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Review

Flavonoids in neuropathic pain management: A new player on an old target

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Neuropathic pain is the consequence of abnormal processing in the peripheral or central nervous system (CNS) elicited by neuronal injury. Due to its heterogeneous nature and important unwanted adverse effects of the commonly prescribed psychoactive drugs like benzodiazepines (BDZ), the treatment of neuropathic pain has remained a challenge for the scientific community. Flavonoids initially isolated from plants and used as tranquilizers in Folkloric medicine, have been reported to possess selective affinity for BDZ binding site. These positive ionotropic modulators of γ-amino butyric acid-A (GABA$_A$) receptors enhance the chloride ion flux and provide a strong inhibitory effect. Therefore, for the treatment of central nervous system-related disorders such as neuropathic pain, these selective GABA$_A$ receptor modulators stand amongst the strongest candidates. This review provides an update on research development that has confirmed the activity of different flavonoids on GABA$_A$ receptors.

Key words: Neuropathic pain, flavonoid, γ-amino butyric acid-A (GABA$_A$) receptors, animal models neuropathy.

INTRODUCTION

Formerly, neuropathic pain was defined by International Association for the Study of Pain (IASP) as "pain initiated or caused by a primary lesion or dysfunction of the nervous system"; but recently it has been revised as "pain arising as a direct consequence of a lesion or disease affecting the somatosensory system" by the IASP neuropathic pain special interest group (NeuPSIG) (Treede et al., 2008). So, this pain has been primarily attributed to be a corollary of a disease upsetting the 'somatosensory system' rather than the 'nervous system' in the revised definition, thus, further clarifying its origin. Under normal conditions, the somatosensory system is involved in the diffusion of noxious information to the central nervous system. Hence lesion of the somatosensory system can not only stop the innervations of nerve cells but can also result in pain with or without sensory hypersensitivity episode in the painful area (Jensen, 2006).

A lesion in the somatosensory system can manifest as positive sensory symptoms or negative sensory symptoms. The negative sensory symptoms arise due to fractional or entire loss of input to the nervous system; whereas, the positive sensory symptoms arise due to regeneration and disinhibition of the nerve cells, as a result of reduced sensory input. The positive symptoms can be either spontaneous or stimulus-evoked.

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Paraesthesias (tingling or ant crawling sensations over the skin), spontaneous ongoing or shooting pain (stimulus independent) and electric shock-like sensations are included in spontaneous positive symptoms; whereas, hyperalgesia and allodynic (further classified according to the static and dynamic nature of the stimulus) pain are stimulus-evoked positive symptoms of neuropathy (Rasmussen et al., 2004). Negative symptoms of neuropathic pain includes hypoesthesia (reduced sensations to non-painful/innocuous stimuli), pallyhypoesthesia (reduced sensations to vibration), hypoalgesia (abridged impression to noxious stimuli) and thermohypoesthesia (abridged impression to cold/warm stimuli) (Jensen, 2006).

Numerous controlled studies have demonstrated potential effectiveness of opioids, tricyclic anti-depressants, gabapentin, pregabalin, phenytoin, lamotrigine, dextromethorphan, tramadol and mexiletine for painful sensory neuropathy (Harden, 1999; Attal, 2001). However, these therapies result in a 30–50% reduction in pain and are often restricted due to significant side effects, with sedation being the most dominant.

PATHOPHYSIOLOGICAL MECHANISMS OF NEUROPATHIC PAIN

Experimental work in rodents has provided a broadened picture of the pathophysiological mechanisms that generate neuropathic pain. It involves both peripheral and central mechanisms. The peripheral sensitization is carried out via unmyelinated C- and thinly myelinated Aδ-primary afferent neurons that normally elicit the pain sensation by responding to noxious stimuli. However, the peripheral nerve lesions sensitize these neurons advancing into spontaneous activity. Moreover, these lesions cause dramatic changes on the cellular and molecular levels triggering the nerve cells (Figure 1) (Baron, 2006).

An increased mRNA expression for voltage gated sodium channels in the primary afferent neurons is considered to be responsible for an ectopic spontaneous activity after a nerve injury. This phenomenon may result in the clustering of these channels that lowers the action potential threshold leading to hypersensitivity. Hence, sodium channel blockers like lidocaine produce pain relief in neuropathic pain via this mechanism (Lai et al., 2003).

Up regulation of a variety of receptor proteins also result from peripheral nerve injury. They are present at the membrane of the primary afferents and are only slightly uttered in physiological circumstances. Vanilloid receptors (TRPV 1) being one of them are involved in the sensing of noxious heat greater than 43ºC (Patapoutian et al., 2003), whereas, TRPM8 receptors have been identified as cold and menthol-sensitive raised by temperature in the range of 8-28ºC. TRPM8 receptor is revealed in small diameter DRG (dorsal root ganglia) neurons (McKemy et al., 2002). A nerve injury may result in either up regulation or gating of this channel leading to peripheral sensitization of C-nociceptors that causes cold hyperalgesia (Wasner et al., 2004).

Acid-sensing ion channels (ASCIs) are believed to be participating in static mechanical hyperalgesia (Price et al., 2001). On the other hand, α_1- and α_2- adrenoceptors located on the cutaneous afferent fibers also play a pronounced function in the hypersensitivity from nerve injury (Baron et al., 1999). Adrenergic sensitivity has been widely expressed in post herpetic neuralgias, complex regional pain syndromes II (CRPS II) and post-traumatic neuralgias; whereas, in polyneuropathies no sensitivity in the primary afferent neurons has been reported (Uphoff and Binder, 2006). Thus, temperature-induced and sympathetically-induced pain could be treated by blocking their respective receptors on nociceptive neurons.

An ectopic activity is also induced by inflammation in both wounded and adjoining normal primary afferent nociceptors triggered by a nerve lesion that release proinflammatory cytokines, especially TNF-α (Sommer, 2003). Deep proximal and paroxysmal pain are pronounced features in the patients diagnosed with peripheral neuropathies e.g., HIV neuropathy. Nerve biopsy specimens of such patient have shown an increased concentration of COX-2 and proinflammatory cytokines (Table 1) (Lindenlaub and Sommer, 2003).

CNS constitutes specific anatomical links with the spinal cord, brain stem, thalamus and the cortex. These connections link sensations generated in the high threshold primary afferents with the cortical regions of the central nervous system, that further process it into ultimate painful sensations (Woolf, 2011). The ongoing hyperactivity generated by injured nerves serves as a trigger for central sensitization and simulates activity-dependent synaptic flexibility occurring within the cortex. Diverse synaptic modulators, changes in ion channel kinetics, excitatory amino acid, increased bulk of ionotropic receptors and pre- and post-synaptic activation of kinases are involved in central sensitization.

Most of the patients presenting with peripheral and all with central neuropathy show predominant synaptic facilitation contributing to allodynia and hypersensitivity (Campbell and Meyer, 2006). Peripheral nerve injury causes "pre-synaptic changes" including modifications in the synthesis of neurotransmitters, neuromodulators and in the density of calcium channels (Hendrich et al., 2008). On the contrary, increased receptor density as a result of enhanced synthesis of ion channels and scaffold proteins and phosphorylation of N-methyl-D-aspartate (NMDA) subunits occur because of "post-synaptic changes" (Cheng et al., 2008). These changes also contribute to an abnormal expression of Nav 1.3 (Hains et al., 2004) and the mitogen-activated protein kinase system (MAPK) (Ji
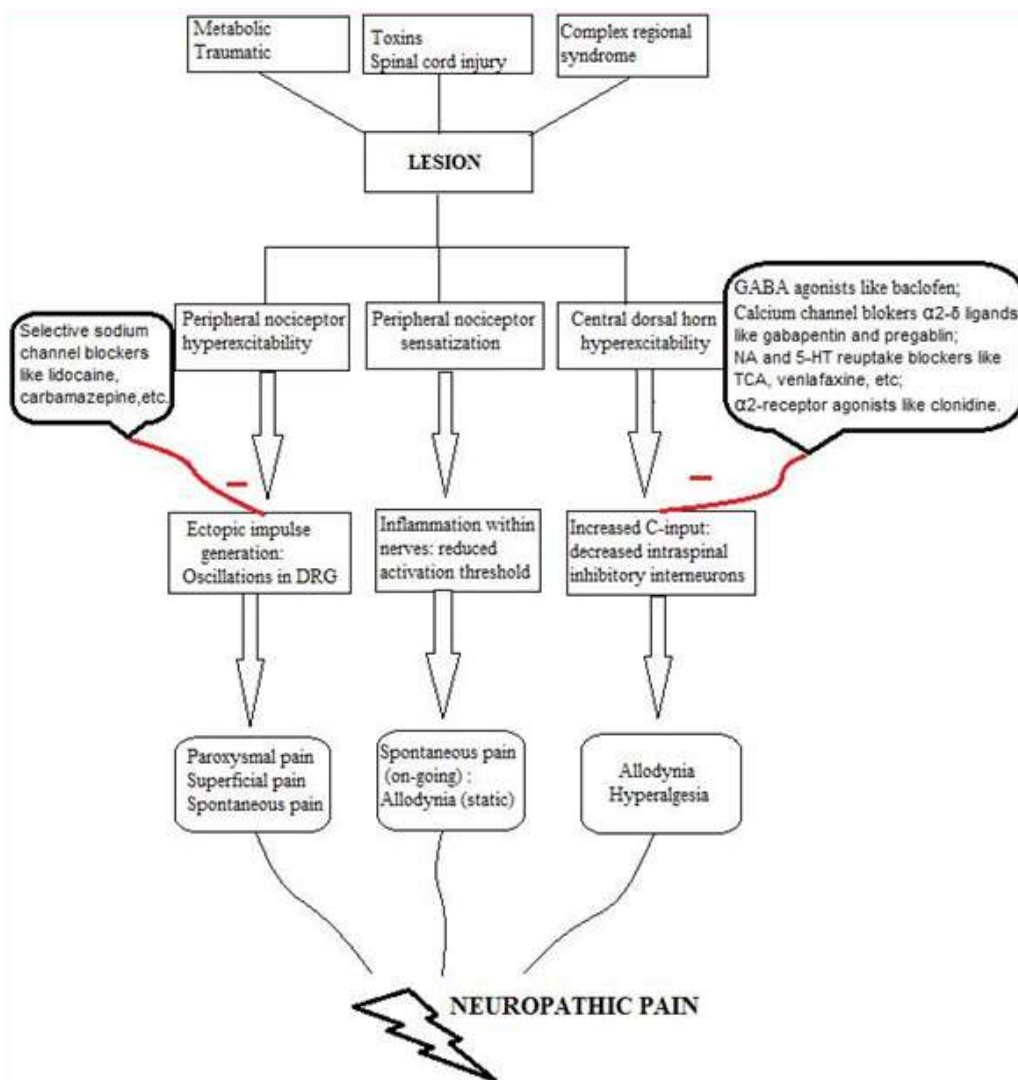
Figure 1. Diagram representing symptoms and mechanism of neuropathic pain with possible medical interventions. NA: nor adrenaline, TCA: tricyclic antidepressants; 5-HT: 5-hydroxy tryptamine; DRG: dorsal root ganglion.

and Woolf, 2001). The role of pathologically sensitized C-fibers which sensitizes neuropeptide substance P and spinal dorsal horn via glutamate release cannot be overlooked. The released glutamate shows an excitatory effect by acting upon post-synaptic NMDA leading to central sensitization (Ultenius et al., 2006). An appreciably abundant evidence reveal the involvement of loss of tonic GABA-conciliated inhibition and increased excitatory neurotransmitters resulted in an initiation of central sensitization, ending in peripheral hypersensitivity, that is, allodynia and hyperalgesia (Knabl, Witschi et al., 2008). Once this sensitivity is developed, the usually innocuous tactile stimuli could activate Aδ and Aβ low threshold mechanoceptors (Tal and Bennett, 1994).

ROLE OF GABA IN NEUROPATHIC PAIN

In the brain, γ-amino-butyric acid (GABA) tends to be the most abundant inhibitory neurotransmitter regulating different physiological characters like sleep, anxiety, memory formation, reward, etc (Zeilhofer et al., 2009) and also control the action of excitatory neurons in the CNS, facilitating a uniform flow of information and therefore, maintaining the homeostasis of neural circuits. Melzack and Wall (1967) previously reported the role of inhibitory neurons in the spinal dorsal horn involved in controlling pain transmission from the periphery to higher levels of the brain (Melzack and Wall, 1967). Later on, GABA was confirmed to be one of the foremost inhibitory

Table 1. Currently available treatment options for neuropathic pain with mechanism, side effects, other benefits, precautions and doses.

Class	Drugs	Mechanism of action	Side effects	Other benefits	Precautions	Doses
Tricyclic antidepressants	Nortriptyline / Desipramine	Norepinephrine and serotonin reuptake inhibition, anticholinergic and sodium channels blockade.	Sedation, anticholinergic effects.	Abolish depression and sleep disturbanc.	Glaucoma, cardiac disease, seizure disorders.	25 mg at bedtime/ 150 mg daily.
Calcium channel $\alpha_2.\delta$ ligands	Gabapentin / Pregabalin	Decrease release of glutamate, NE, etc. via α_2-delta subunit voltage gated calcium channel.	Sedation, peripheral edema, dizziness.	No significant drug interactions.	Renal insufficiency.	Gabapentin: 100-300 mg once or t.i.d/ 1200 mg t.i.d. Pregabalin: 50 mg t.i.d or 75 mg t.i.d.
SSNRIs	Duloxetine / Venlafexine	Norepinephrine and serotonin reuptake inhibition.	Nausea	Improve depression	Hepatic dysfunction, alcohol abuse, tramadol.	Duloxetine: 30 mg once daily/ 60 mg t.i.d. Venlafexine: 37.5 mg once daily.
Topical lidocaine	5% lidocaine patch	Sodium channel blockade.	Erythema, rash.	None	None	1 to 3 patches per day.
Opioid agonists	Morphine Oxycodone Methadone	μ-receptor agonists	Nausea vomiting, dizziness, constipation	Rapid onset of analgesia	Substance abuse, driving impairment, suicide risk.	Morphine:10-15 mg every 4 h.
	Tramadol	μ-receptor agonist, Norepinephrine and serotonin reuptake inhibition.	Same	Same	Same, serotonin syndrome if used in combination with TCA.	50 mg once daily or t.i.d/ 400 mg daily as long-acting drug.

neurotransmitters in the spinal dorsal horn (Yaksh, 1989).

After release from the pre-synaptic neurons, GABA act postsynaptically upon 3 major classes of receptors namely; $GABA_A$ and $GABA_C$ receptors that are ligand-gated ion channels and $GABA_B$ that are G protein-coupled channels (Gavande et al., 2011). Chemically ionotropic $GABA_A$ receptors are formed of transmembrane protein complexes and comprise 5 heteropentameric subunits. In the human brain, $\alpha_1\beta_2\gamma_2$ subunit is considered to be the most dominant one (Wafford, 2005). Upon activation by GABA, the membrane permeability to chloride and carbonate ions increases causing a net inward flow of anions and a consequent hyperpolarization. This hyperpolarizing post-synaptic response is termed as inhibitory post-synaptic potential (Semyanov et al., 2003).

Physiologically, GABA-releasing interneurons, imposes a strong inhibitory control over the dorsal horn neurons. The loss of these neurons could further increase the central sensitization, e.g. as reported in partial peripheral nerve injury models, in which the injury resulted in the reduced spinal GABA release along with decreased GABA-synthesizing enzyme glutamic acid decarboxylase in rodents (Moore et al., 2002), advancing into spontaneous pain and hyperexcitability manifested as allodynia and hyperalgesia.

In diseased states, an increased excitation occurs that is attributed to either a massive loss of GABAergic interneurons or deterioration of interneurons as consequence of depletion of their sensory excitatory inputs or receptors. This imbalance could culminate into many neurological and psychiatric disorders including epilepsy, schizophrenia, neuropathic pain, Alzheimer's (AD) and Parkinson's disease (PD) (Tyson and Anderson 2014).

Hence, the collaborative function of excitatory and inhibitory neurons plays a vital role in controlling various brain activities.

As discussed earlier, central and peripheral sensitization due to nerve injury could result in complex neuropathic pain. Besides other mechanisms, the loss of GABAergic interneurons is now considered to be one of the major contributor to such persistent pain states (Bráz et al., 2012). Recent evidence suggest that the deletion of particular GABA subunits or pharmacological blockade of GABAergic neurotransmission in the spinal cord resulted in hyperalgesia and allodynia (Gwak et al., 2006; Jergova et al., 2012). Similarly, electrophysiological studies have revealed that $GABA_A$ receptor inhibition could induce an embellished behavioral reaction to an
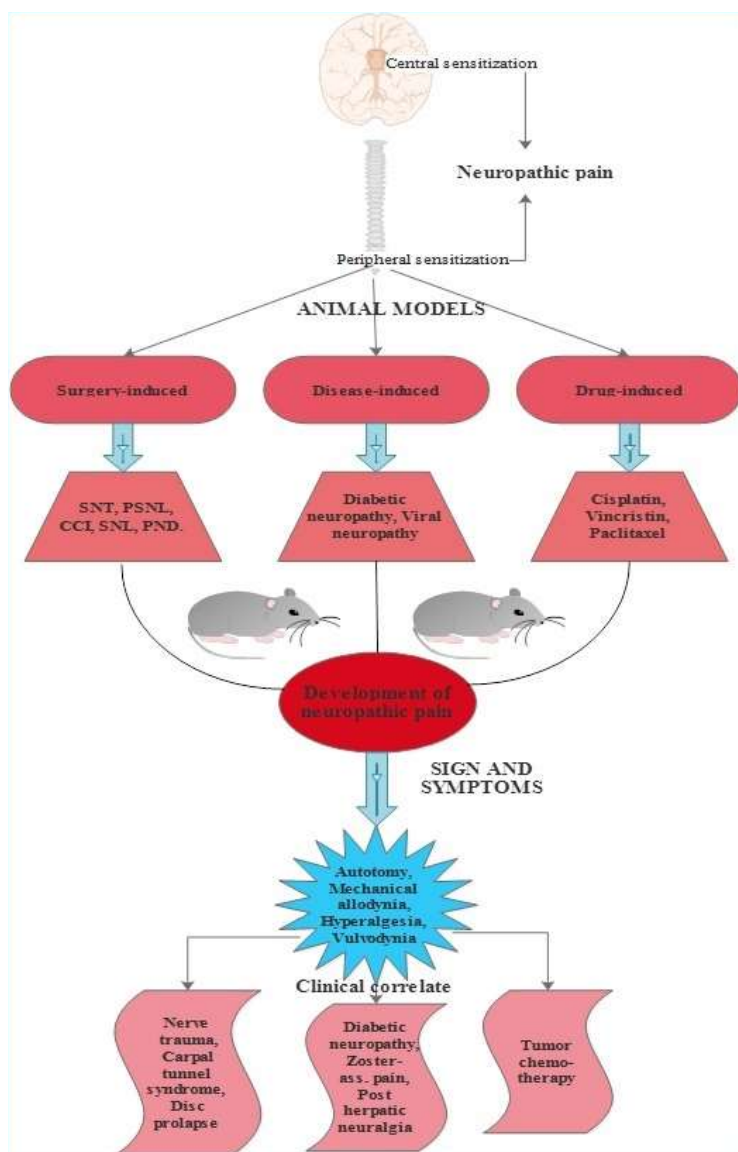
Figure 2. Diagram representing animal models of neuropathic pain. SNT: spinal nerve transaction; PSNL: partial sciatic nerve ligation; CCI: chronic constriction injury; SNL: spinal nerve ligation and PDN: peripheral diabetic neuropathy.

innocuous mechanical stimulus (Hwang and Yaksh, 1997), consistent with the reported high levels of GABAergic receptors in the dorsal horn (Persohn et al., 1991). Moreover, the impaired GABAergic system in the animals presenting with chronic neuropathic pain propose the practical link between such pain and spinal inhibitory neurotransmission (Figure 2) (Zeilhofer, 2008). Therefore, restoration of spinal inhibitory neurotransmission can be a valuable pharmacological advancement in the treatment of neuropathic pain.

GABA$_A$ receptor agonists related antinociceptive effect

has been attributed to the stimulation or blockade of other neurotransmitters as well (McCarson and Enna, 2014). Furthermore, the role of central GABA in opioid-mediated antinociception is well documented (Ossipov et al., 2010). Thus, GABA receptor agonists may play a vital role in treating acute and chronic pain (McCarson and Enna, 2014).

In this regard, GABA$_A$ receptor agonists muscimol and isoguvacine are reported to reverse nerve injury-induced tactile allodynia (Hwang and Yaksh, 1997). These receptors are closely linked to the large diameter

Table 2. Classification of flavonoids.

Class of flavonoids	Example
Flavanol	Kaempferol,Quercetin, Myricetin
Flavanone	Hesperetin, Naringenin
Flavone	Luteolin, Apigenin
Flavans	Catechin, Epicatechin
Isoflavone	Genistein, Daidzein
Anthocyanidin	Cyanidin, Delphinidin
Aurones	Leptosidin, Auresidin
Neoflavonoid	Coutaregenin, Dalbergin
Flavonoid glycosides	Astragalin, Apigenin
Flavonolignan	Silibinin

Adopted from Peterson and Dwyer (1998).

afferents involved in innocuous sensation (Price et al., 1984; Sivilotti and Woolf, 1994; Reeve et al., 1998; Ataka et al., 2000; Riley et al., 2001; Turner, 2003). Great body of evidence suggests that a decreased spinal GABA plays a vital role in inducing and maintaining neuropathic pain. Behavioral and pharmacological studies have shown that a continuous or single intrathecal dose of GABA to the spinal cord or the implantation of GABA releasing cells decrease the signs of neuropathic pain (Eaton et al., 1998, 1999; Stubley et al., 2001; Malan et al., 2002). Moreover, blocking the spinal $GABA_A$ receptors results in aggravating peripheral nerve injury associated hyperalgesia (Yamamoto and Yaksh, 1993).

On the contrary, benzodiazepines, and positive allosteric modulators at $GABA_A$ receptors are widely used drugs not only in anxiety, convulsions and sleep disorders but also as analgesic if administered intrathecally (Tucker et al., 2004). Despite its analgesic potential, its use in pain relief is restricted due to the myriad effects like sedation. Hence, there is an increased need of research in the field of GABAergic modulators that could play a pronounced role in the attenuation of neuropathic pain.

FLAVONOIDS

Flavonoids are widely distributed in vegetables, grains, fruits, barks, roots, flowers, stems, tea, etc. Chemically, they are polyphenolic compounds comprising of a diphenyl propane skeleton (C-6-C-3-C-6), with two aromatic rings and an oxygen-containing heterocyclic benzopyran ring, labeled as fused aromatic ring A, the benzopyran ring adjacent to A is ring C and a phenyl ring B (Middleton, 1998).

Long before the isolation of flavonoids, the natural products bearing them were known for their healthful effects. The decreased mortality rate associated with the use of red wine (constitute flavonoids) was observed in Mediterranean populations that produced an inclination in the flavonoid research, that was further confirmed by epidemiological studies, portraying the cardio protective role of dietary flavonoids in coronary heart disease (Formica and Regelson, 1995; Groot and Rauen, 1998).

Similarly, a new substance isolated from oranges in 1930, was thought to be a vitamin and was called vitamin P, which was later on confirmed to be a flavonoid, that is, rutin which led to an increase in the isolation and study of the mechanisms of various individual flavonoids. A long history of plant species exists that constitute flavonoids and exhibit CNS effects and were used as folk medicines in Europe. Feverfew (*Tanacetum parthenium L. Asteraceae*) was used as prophylactic agent in migraine and Chamomile flowers (*Matricaria recutica L. Asteraceae*) as a tranquillizer for centuries, with both found to be containing apigenin as an active constituent via bioassay-guided fractionation studies (Jäger et al., 2009). Furthermore, Linden flowers (*Tilia sp. Tiliaceae*) were known for their sedative effects and Heather (*Calluna vulgaris [L] Hull. Ericaceae*) as nerve calming remedy, with both shown to have quercetin and kaempferol flavonoids as active components (Aguirre-Hernández et al., 2010).

Flavonoids can be categorized into ten groups (Table 2) (Peterson and Dwyer, 1998). Flavone, isoflavone and flavanol contains a double bond in the ring C, making the fused A-C ring system planar, whereas, the other classes lack this double bond and instead have C_2 and C_3 chiral centers that are located at each end of the plane ring A (Figure 3) (Jager and Saaby, 2011). Besides the isolation of natural flavonoids, various semi-synthetic and synthetic derivatives have been synthesized and screened for their therapeutic potential (Cushnie and Lamb, 2005).

In plants, flavonoids not only provide UV-protection but also aid in the pollination by masking the flowers with attractive colors and patterns. Until now, more than 6000 varieties of flavonoids have been isolated. A variety of interest gasping pharmacological actions for these naturally occurring flavonoid compounds as well as synthetic derivatives have been found (Vidyalakshmi et al., 2010), including both peripheral and central nervous system effects (Figure 4) (Hall et al., 2005). These compounds attribute to a vast range of biological effects, like anticancer (Liu et al., 2010), anti-inflammatory (Wang et al., 2010), antioxidant (Heim et al., 2002), anxiolytic (Ognibene et al., 2008), cardio protective (Yu et al., 2005), antifungal (Ammar et al., 2013), antiviral (Orhan et al., 2010), antimicrobial (Cushnie and Lamb, 2005), neuroprotective (Cho et al., 2013) and antinociceptive activities (Wang et al., 2014).

FLAVONOIDS AS $GABA_A$ RECEPTOR MODULATORS

Flavonoids have been extensively focused for their

Basic structure of flavonoids

Flavone

Flavanone

Flavon-3-ol

Flavan-3-ol
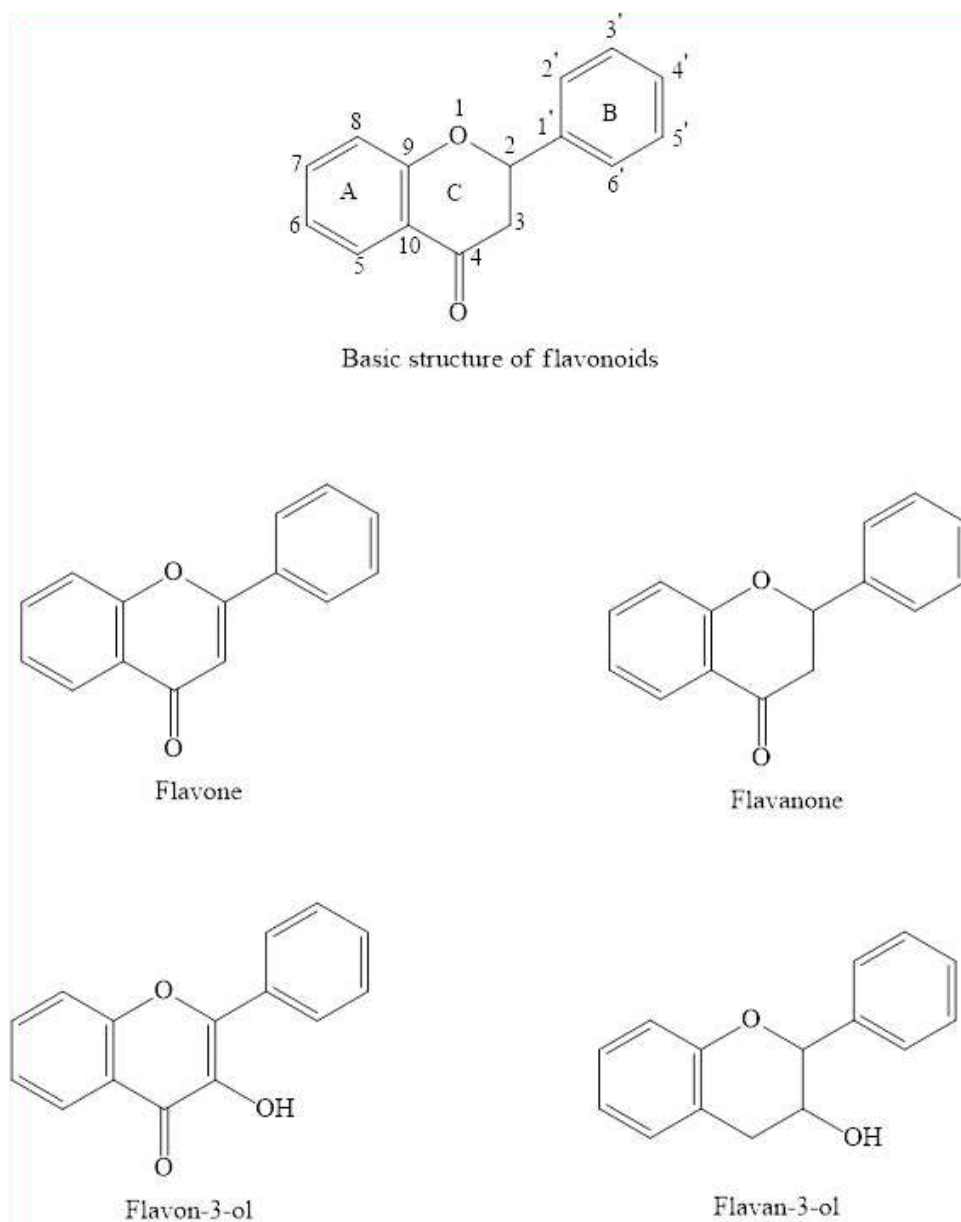
Figure 3. Diagram representing chemical structures of basic flavonoid nucleus and the common subgroups.

peripheral events; however, more recently their selective affinity for GABA$_A$ receptors has been reported in studies using rat and bovine brain membrane binding assays (Hong and Hopfinger, 2003). In conjunction with binding studies, many behavioral studies have also been carried out, which indicate the anxiolytic effects of flavonoids in rodent anxiety models lacking many of the surplus side effects of BDZs (benzodiazepines) (Griebel et al., 1999). Interestingly, the positive, negative and neutralizing allosteric modulatory actions of flavonoids over a wide range of ionotropic GABA receptors has been focused

and strongly supported via a large bulk of evidence.

The isolation of isoflavones from bovine urine that displaced 3[H]-diazepam binding in rat brain laid the foundation of the interaction of flavonoids with BDZ receptors (Luk et al., 1983). In 1990s, flavonoids were defined as a new family of BDZ receptor ligands (Medina et al., 1997; Marder and Paladini, 2002). Classically, they were considered to be acting upon BDZ receptors and a number of synthetic flavonoids having a significant affinity for BDZ binding site were developed accordingly (Yao et al., 2007); until they were reported to be insensitive to
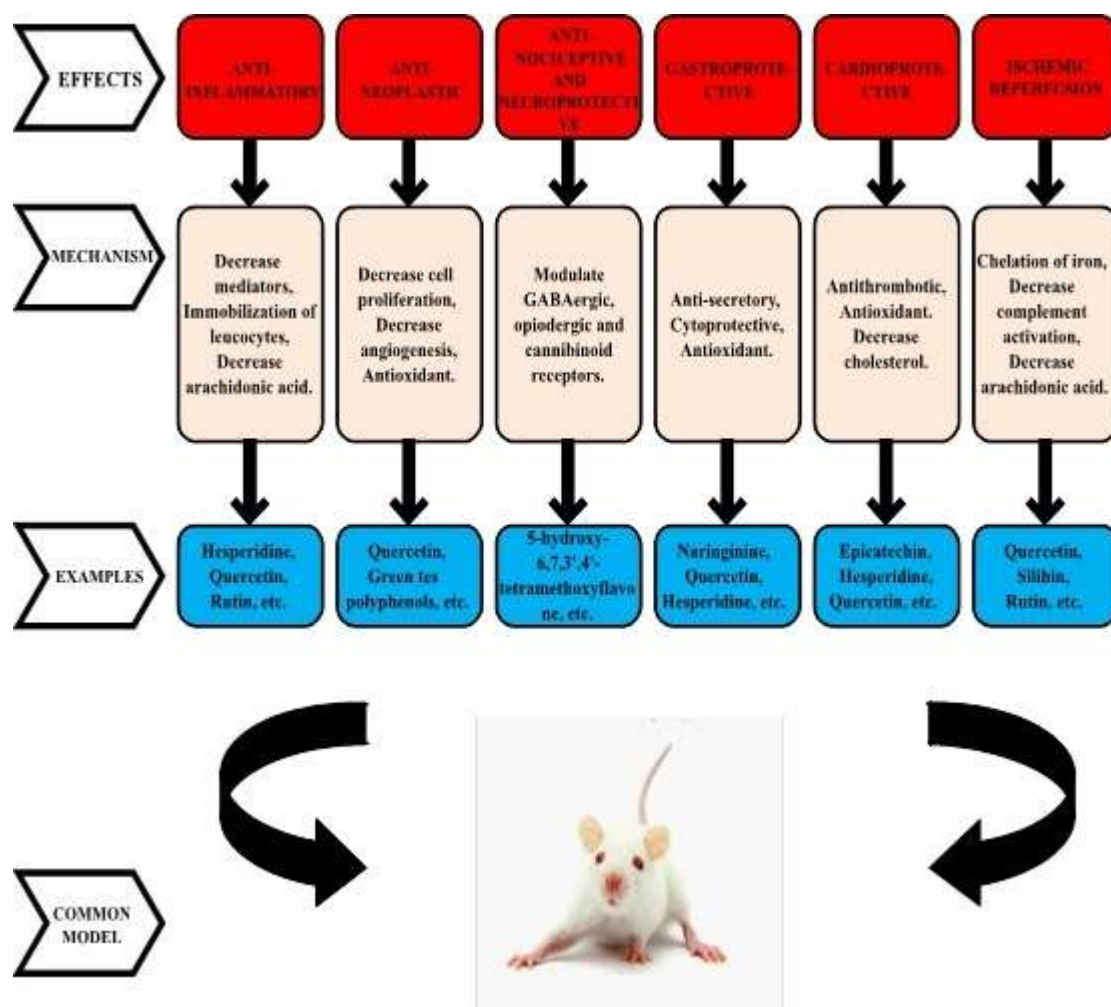
Figure 4. Diagram representing pharmacological effects along with mechanism of action and examples of different natural and synthetic flavonoids.

flumazenil that is BDZ receptor antagonist, thus highlighting a unique site of action for this class (Hanrahan et al., 2011).

The substitution at 6- or 3'-positions of flavones with an electronegative functional group enhanced the affinity towards the BDZ receptors (Paladini et al., 1999). Furthermore, the impact of ligand binding on the GABA binding was used to determine the GABA ratios. These ratios showed that flavones exhibited significant biological activities at BDZ receptors (Hanrahan et al., 2011). 6-Bromoflavone, 6-bromo-3'-nitroflavone and 6-chloro-3'-nitroflavone with a GABA ratio of 1.6-2.0, 1.38 and 2.0 were reported as full agonist (Marder et al., 1996), partial agonist (Wolfman et al., 1998) and an antagonist (Viola et al., 2000) at these receptors.

Positive ionotropic modulators of $GABA_A$ receptors enhance the chloride ion flux and provide a strong inhibitory effect. Therefore, for the management of CNS – allied diseases, including panic disorders, generalized anxiety, seizure disorders, muscle spasm, neuropathic pain and sleep disturbances; these modulators stand amongst the strongest candidates (Rudolph and Möhler, 2006). Moreover, with the discovery of the fact that flavonoids may act upon novel binding sites other than the classical benzodiazepine binding site, opportunities to search for new therapeutic agents with less adverse effects has been provided (Rudolph and Möhler, 2006). In this regard, 6-Methoxyflavonone has been reported to act as positive allosteric modulator at α1β2γ2L and α2β2γ2L subunits of $GABA_A$ receptors (Hall et al., 2014).

The substitution at 6-position on flavones is related to its effects on the recombinant $GABA_A$ receptors. 6-Hydroxyflavone depicted a significant effect at flumazenil-sensitive BDZ site (Ren et al., 2010). Moreover, 6-methoxyflavanone and 6-methoxyflavone have been recently reported to exhibit significant anti-allodynic

effects in streptozotocin- and cisplatin-induced neuropathic pain models (Akbar et al., 2016; Shahid et al., 2017). These protective effects against neuropathic pain were attributed to the positive allosteric modulatory effects of these molecules on opioid and GABA$_A$ receptors respectively (Akbar et al., 2016).

Moreover, myrcitin and baicalin produced significant anti allodynic effects in sciatic nerve ligation models (Meotti et al., 2006; Cherng et al., 2014). Rutin and quercetin has been reported to inhibit oxaliplatin-induced chronic painful peripheral neuropathy (Azevedo et al., 2013). Naringin is also reported as exhibiting anti allodynic potential in the sreptozotocin-induced painful diabetic neuropathy (Kandhare et al., 2012).

OTHER NEUROPATHIC PAIN MODULATING MECHANISMS OF FLAVONOIDS

Besides its action on the GABA$_A$ receptors, flavonoids also exert antioxidant and anti-inflammatory effects. Almost all metabolic diseases are the consequence of oxidative stress. In addition to exogenous damage, the normal oxygen metabolism within the cells and tissues produce free radicals and reactive oxygen species that consistently endanger them. Flavonoids are very well known for their antioxidant potential and are proven to exert positive effects in diseases like atherosclerosis, diabetes, Alzheimer's disease, cancer, etc (De Groot, 1994; Pal and Verma, 2013).

Although the underlying set of events behind the damage caused by the free radicals to the cellular functions is not fully understood, the contribution of lipid per oxidation leading to cellular membrane destruction and activation of inflammatory mediators by the free radicals culminating in ultimate tissue damage, can provide a lot of help in conceiving a pharmacological target. Nature has provided an inborn mechanism against these ROS, comprising of enzymes like superoxidase, glutathione peroxidase and catalase as well as non-enzymatic elements e.g. ascorbic acid, α-tochopherol, etc (Halliwell, 1995). However, these endogenous scavenging compounds are prone to depletion due to an increased oxidative stress caused by many diseased states including conditions that culminate in neuropathic pain e.g. diabetes mellitus (Schreiber et al., 2015).

Epicatechin and rutin are shown to have the ability to be oxidized themselves by the free radicals giving rise to a stable and less reactive species (Hanasaki et al., 1994). Similarly, quercetin inhibits the nitric oxide (NO) induced cell injury. Nitric oxide combines with the free radicals and produces an extremely harmful peroxynitrite that straightly oxidize LDL leading to a permanent destruction of the cell membrane. Hence, quercetin scavenge the free radicals, restraining them from reacting with NO (Shutenko et al., 1999), while silibin react directly with NO (Dehmlow et al., 1996).

Physiologically, the metabolism of xanthine to uric acid is carried out via xanthine dehydrogenase; however, in case of ischemic-reperfusion this enzyme changes into xanthine oxidase that acts as precursor of free radicals. Quercetin, luteolin and silibin flavonoids are known to act as antioxidant via inhibiting xanthine oxidase (Chang et al., 1992; Shoskes, 1998).

Similarly, reperfusion also results in the mobilization of leucocytes causing the consequent release of inflammatory mediators and cytotoxic oxidants provoking the complement system. A number of flavonoids are reportedly involved in the leucocytes immobilization ultimately causing a reduction in the serum complement system and inflammation (Friesenecker and Tsai, 1995; Ferrándiz et al., 1996). A pronounced body of evidence report the involvement of same pathophysiological mechanisms behind both inflammation and neuropathic pain of peripheral origin. Both types of pathologies manifest as allodynia and hyperalgesia (Clatworthy et al., 1995; DeLeo and Yezierski, 2001; Jin et al., 2003). Infiltration of inflammatory cells and their secretary products like arachidonic acid and cytokines (released for nerve regeneration) as a result of peripheral nerve injury is responsible for the generation and maintenance of the ongoing pain (Tracey and Walker, 1995; Cui et al., 2000; Ma and Eisenach, 2003). Cytokines like tumor necrosis factor-α (TNF-α), interleukin-1 (IL-1) and interleukin-6 (IL-6) when injected into the rat paw resulted in the induction of mechanical and thermal hyperalgesia (Cunha et al., 1992; Ferreira et al., 1993). The blockage of TNF–α in the rodent models of painful neuropathy has resulted in the attenuation of hyperalgesia (Sommer et al., 1998). Cytokines once released induce their own production and consequently activate COX–2 dependent prostanoid releases. The role of PGs in inducing inflammation accompanying enhanced sensitivity to pain is well documented. Intrathecal administration of PGs including PGE$_2$ and PGF$_{2α}$ induced allodynia in conscious mice (Minami et al., 1992, 1994), whereas intrathecal injection of PGD$_2$ and PGE$_2$ resulted in the induction of hyperalgesia (Uda et al., 1990). Moreover, synthesis of PG and nitric oxide (NO) via COX-2 and inducible nitric oxide synthase (iNOS) is enhanced in the microglia as a result of peripheral nerve injury leading to hyper sensitization (Hanisch, 2002). Flavonoids exhibit an *in vivo* and *in vitro* anti-inflammatory activity. The *in vivo* anti-inflammatory is attributed to the inhibition of ecosanoid generating enzymes including COX, LOX and phospholipase A$_2$ (Kim et al., 2004).

Flavonoids are known to act upon different enzyme systems, e.g. causes the inhibition of arachidonic acid and blocking the inflammatory response as arachidonic acid tends to start it. By this, antithrombotic and anti-inflammatory features are incorporated into the group (Ferrandiz and Alcaraz, 1991). They also cause decrease

in the release of peroxidase and inhibition of ROS production by neutrophils. This inhibition is carried out via interference with α_1-antitrypsin activation (Middleton and Kandaswami, 1992). Certain flavonoids are involved in iron chelation that causes lipid peroxidation, thereby abolishing an erratic factor for the free radicals development (Nelson et al., 1992; Ferrali et al., 1997).

CONCLUSION

Allosteric modulators at $GABA_A$ receptors modify either the efficacy or affinity of agonists like GABA, consequently regulating their activity. Over the last decade, these modulators have been focused extensively due to the advances in the understanding of the functions of $GABA_A$ receptor subtypes. Flavonoids being potent allosteric modulators may prove to be useful tools in the amelioration of such distressing painful conditions like neuropathic pain. However more studies are required to further elucidate the site of action of these bioactive molecules over the GABAA receptors.

CONFLICT OF INTERESTS

The author has not declared any conflict of interests.

ABBREVIATIONS

AD, Alzheimer's disease; **ASCIs,** acid-sensing ions channel; **BDZs,** Benzodiazepines; **CNS,** central nervous system; **COX-2,** Cycloxygenase-2; **CRPS II,** Complex Regional Pain Syndrome II; **DRG,** dorsal root ganglion; **GABA,** γ-amino butyric acid; **IASP,** International Association for the Study of Pain; **ISSVD,** International Society for the Study of Vaginal Disease; **MAPK,** mitogen-activated protein kinase; **NeuPSIG,** Neuropathic Pain Special Interest Group; **NMDA,** N-methyl-D-aspartate; **PD,** Parkinson's disease; **TNF-α,** tumor necrosis factor- α; **TRPV,** transient receptor potential.

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Full Length Research Paper

Effects of anti-hyperglicemic of yacon leaf (*Smallanthus sonchifolius*) ethanolic extracts using zebrafish as a model

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This study aims to investigate the anti-hyperglicemic effects of yacon leaf (*Smallanthus sonchifolius*) using zebrafish. In this study, zebrafish is used as a model, with alloxan compounds to induce hyperglicemic. The induction stage consists of 3 phases: in the first the zebra was induced at 0.3% for 30 min, in the second stage, it was transferred to a 3% D-glucose solution for 30 min, and in the last, immersion was carried out in a solution containing yacon leaf extract with doses of 20, 30 and 40 mg/ml for 3 h in 3 consecutive days.The results showed that yacon leaf extract in doses of 20, 30 and 40 mg were able to reduce blood glucose levels in zebrafish induced by alloxan.The results indicated that 95% ethanolic extract has an antidiabetic effect.

Key words: Yacon leaf (*Smallanthus sonchifolius*), zebrafish, alloxan.

INTRODUCTION

Diabetes mellitus affects a large percentage of the world's population, significantly altering their quality of life (Lawrence et al., 2008). The definition of diabetes mellitus is a metabolic disease caused by a lack of insulin production or inability of the body to respond to insulin (Stumwoll et al., 2005).

The World Health Organization (WHO) predicts an increase in the number of populations living with diabetes mellitus, considering it a global health threat (WHO, 2016). In Indonesia, it is estimated to rise from 8.4 million in 2000 to 21.3 million by 2030, with a staggering increase of 2-3 times that by 2035 (Wild et al., 2004). Meanwhile, the International Diabetes Federation (IDF, 2015) predicts an increase of 9.1 million in 2014 with an expected 14.1 million by 2035 (IDF, 2015).A natural ingredient found in Indonesia and capable of reducing blood glucose levels is the yacon (*Smallanthus sonchifolius*) plant. In a study conducted by Genta et al. (2010), it was found that a decrease in blood glucose levels from the extract was caused by caffeonylquinic, caffeic and chlorogenic compounds. In addition, enhydrin and sesquiterpenic lactone in the leaves are effective for reducing post-prandial glucose and is useful in the treatment of animal diabetes (minimum dose: 0.8 mg / kg body weight). According to research conducted by Dou et al. regarding chemical compounds from *Smallanthus sonchifolius*, a structure of new compounds were identified that are as efficacious as diabetes agents,
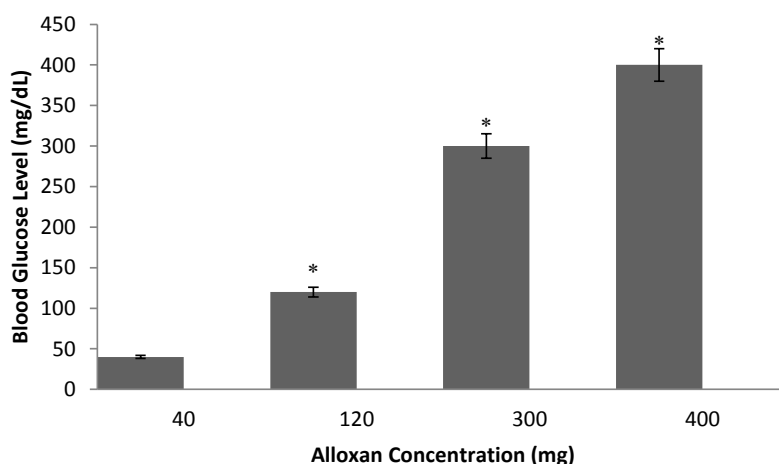
Figure 1. Blood glucose levels on alloxan solution concentration. The zebrafish were exposed to various alloxan concentrations in half saline solution; 40 mg/100 ml (40 mg), 120 mg/100 ml (120mg), 300 mg/100 ml (300 mg), or 400 mg/100 ml (400 mg) for 30 min, moved to a 1% water glucose solution in water for 30 min, and then exposed to water for 1 h. After induction, blood glucose levels were measured; *$p < 0.05$.

namely ent-Kaurane-3β,16β, 17,19-tertol, ent-Kaurane-16 β, 17,18,19-tertol, ent Kaurane-3β, 16β, 17-triol, ent-Kaurane-16β, 17-diol-19-oic acid, ent-Kauran-6β, 17,18-triol and 1-Pentacosanol (Dou et al., 2010).

The yacon leaves contain fructooligosaccharide, carbohydrates, and flavonoids which can reduce glucose levels in blood and effectively transport components of phenol and caffeonylquinic which inhibit glucosidase (Djamil et al., 2014). Baroni et al. (2008) found that the crude extract of yacon leaves significantly reduced blood glucose levels of diabetic rats in the experimental model induced by STZ. Most of the available models are rodent-based, which have drawbacks in that they are labour intensive and because of ethical issues, only small groups of animals can be used. To overcome these limitations, the zebrafish (Danio rerio) has been increasingly used to study diabetes and its related diseases, chosen because of the high similarities in organ physiology and metabolism between zebrafish and mammals (Zhang et al., 2017).

Zebrafish is an excellent model when compared to rodents in understanding the conditions and pathology of vertebrates (Zhang et al., 2018). Used in previous studies and induced by alloxan monohydrate at a dose of 300 mg and glucose 1%, considerable damage to the pancreatic Langerhans cells, causing diabetes was observed (Shin, 2012). This study aims to determine the effect of 95% ethanolic extracts of yacon leaves (*Smallanthus sonchifolius*) on the production of insulin using the zebrafish model. The development of zebrafish-based antidiabetes compound screening is founded on the discovery of marked similarities, which justify the use of

the fish to screen antidiabetic compounds.

MATERIALS AND METHODS

Animal experimentals

The experimental animals used in this study were black striped zebrafish weighing180-220 mg, obtained from Bogor Agricultural University, Bogor, West Java and were used after ethical approval. 100 zebrafish were divided into 10 tails per aquarium used during acclimatization and were fed at 4% per tail daily (Kinkel and Prince, 2009). Fish were fed twice daily, at 10 am with gelatin containing shrimp as described below and at 4 pm with Zeigler Adult Diet (Aquatic Habitats). Aerators were installed, to provide oxygen, and to prevent stress and death. Specific conditions were necessary, including approximately 10 h of darkness for rest periods, made by covering a tank with a trash bag. However, zebrafish activities required approximately 14 h of bright lighting, with an optimum temperature of 28.5 (Zhang et al., 2017). After acclimatization, they were chosen on the basis of activity levels and weighed (220-260 mg). The zebrafish were divided into 6 groups, with each consisting of 10 randomly picked tails, and given treatment for 3 days. Group 1 is a normal control, without any treatment, Group 2 a negative control with 3% alloxan monohydrate, hyperglycemic, D-glucose, and treated water induced. Group 3 is a positive control and was given liquid containing metformin. Positive control was comparison; Group 4 with the administration of 20 mg dosage of yacon leaves ethanolic extracts; Group 5, with the administration of 30 mg dosage of ethanolic extracts; Group 6, with the administration of 40 mg dosage of ethanolic extracts.

The zebrafish were exposed to various alloxan concentration in half saline solution; 40 mg/100 ml (40 mg), 120 mg/100 ml (120 mg), 300 mg/100 ml (300 mg) and 400 mg/100 ml (400 mg) for 3 days. After induction, blood glucose levels were measured as shown in Figure 1. To optimize alloxan levels on the zebrafish, alloxan doses of 40, 120, 300 and 400 mg were used. 300 mg / 100 ml dosage was the most optimal as blood glucose levels had
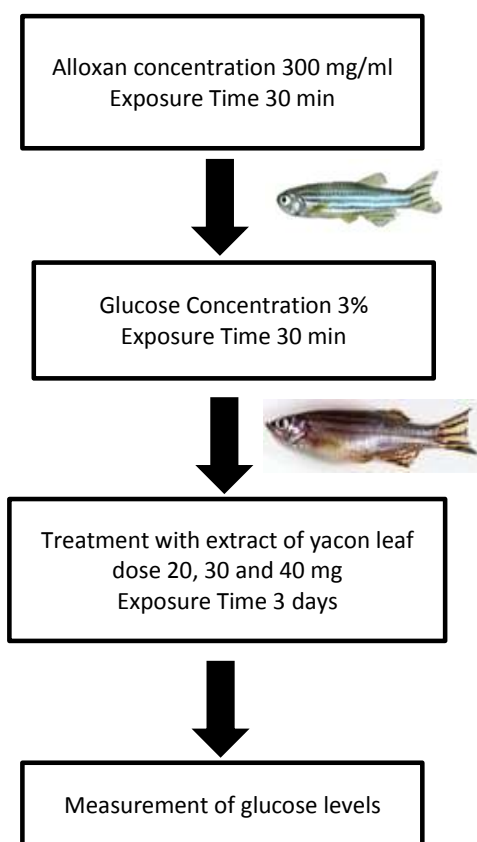
Figure 2. Process for antidiabetic assay with Zebrafishmodel.

reached 300.45-378.34 mg / dl and all exposed zebrafishes did not die.The dosage of 400 mg / 100 ml blood glucose levels could reach levels of 400.34-456.67 mg / dl, and therefore, more than 50% of the Zebrafish died. The diabetic zebrafish model induced with only water glucose is required for a longer time compared to the model induced with alloxan.

Changes in the body weight before and after diabetogenic induction is shown in Figure 3. The zebrafish were also weighed to determine the amount of feed to be offered daily. This is used to maintain control variables and identify the differences between the administration of metformin and 95% ethanol extract of yacon leaves.

Antidiabetic activation test

The selection of the dosage of yacon leaf extract was carried out after optimization; a dose of 20, 30 and 40 mg was given for 3 days after the hyperglycemic induction, and the zebrafish blood glucose levels were measured (Figure 2).

Statistic analysis

Glucose level data obtained from each treatment group was tested for normality and homogeneity. If the data are normally distributed and homogeneous, parametric statistical tests were carried out

using one-way analysis of variance (ANOVA). If the results showed a significant difference, the analysis was then followed by the Tukey Post Hoc test to see the variations in each group at a significance level of 0.05%.

RESULTS AND DISCUSSION

The data before the acclimatization initially showed non-uniform results; however, afterwards they gained weight and were broadly uniform. This may be a result of food factors, given everyday for 7 days. After the induced hyperglycemic, the zebrafish lost weight. This is because weight loss is one of the typical symptoms of metabolic disorders, experienced by patients with diabetes mellitus, despite having very good appetite (Zhang et al., 2017). The zebrafish lost weight because their energy needs were not met from glucose metabolism because of the fat and protein overhaul. This condition is caused by pancreatic β cell damage after being induced by alloxan, leading to a decrease in the production of in the body, causing glucose to not binding to insulin to produce energy (Zhang et al., 2018; Capiotti et al., 2014).

Figure 4 highlights the blood glucose levels of the normal, diabetic group, positive controls, and administration of yacon leaf extract in 20, 30 and 40 mg dosages. It can be seen that there is a decrease in blood glucose levels by to day 3 in the positive control with yacon leaf extract dosages of 20, 30, and 40 mg. The measurement of blood glucose level of the yacon leaf ethanol extract shows that the higher the dose of ethanol extract, the higher the rate at which blood glucose levels are decreasing in zebrafish.

In the positive control group, metformin had a better decrease in activity because it can reduce more blood glucose levels of zebrafish than the 3 groups of dosages of 95% ethanolic extracts. In another study, the antidiabetic effects of yacon leaf extract were investigated using rat animals induced with streptozotocin. Animal models type 1 diabetes mellitus was caused by the loss of pancreatic β- cells resulting in reduced insulin production (Kinkel and Prince, 2009). An alloxan-induced acute hyperglycemia zebrafish model of type 1 diabetes lead to the generation of a destroyed pancreas. This model is a mild acute hyperglycemia model for short-term experimentation; therefore, further research is needed to develop a permanent model (Kinkel and Prince, 2009).

Based on the results, the hyperglycemic induction with 0.3% alloxan monohydrate using the immersion method with 3% D-glucose and aquadest, damaged the pancreatic β cells of the zebrafish, resulting in reduced insulin production in the body, and high glucose in the blood. Glucose in zebrafish bodies could not bind to insulin and glucose levels in normal controls do not change the condition of hyperglycemia from day 1 to 3. This is due to an increase in blood glucose levels by zebrafish treated with alloxan induction and glucose,
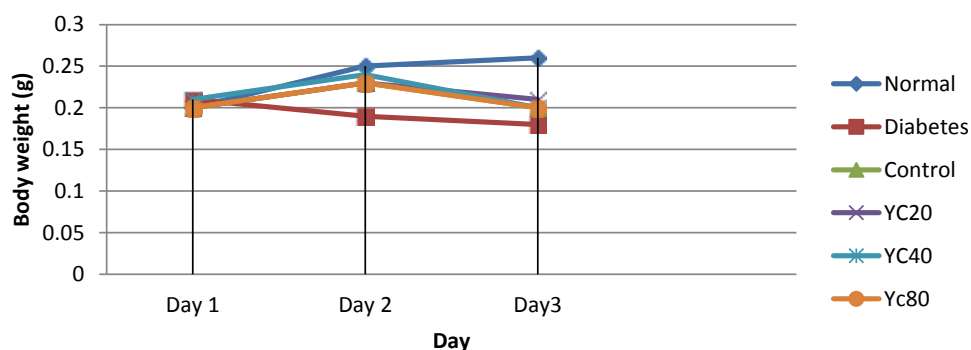
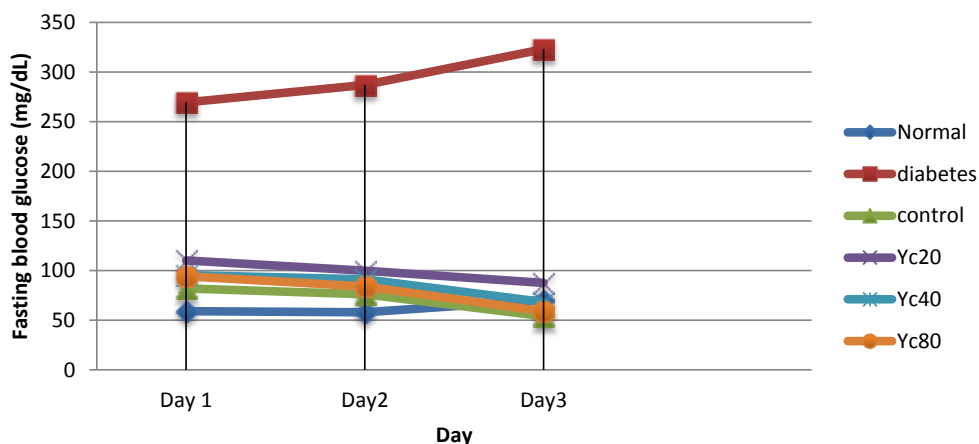**Figure 3.** Body weight of the zebrafish before and after induction.

Figure 4. Average of blood glucose of treated zebrafish (Day 1, 2 and 3 treatment time with yacon dose 20 mg/100 ml (Yc20), yacon dose 40 mg/100 ml (Yc40), yacon dosen 80 mg/100 ml (Yc80) and with metformine (control), diabetes (negative) and normal).

resulting in an inability to regulate insulin. This led to the destruction of pancreatic β cells which could be seen by increased blood glucose levels of zebrafish. Overall, these data indicated that zebrafish could be induced to the develop mild and stable hyperglycemia and this model was appopriate for these experiments (WHO, 2016). A significant advantage of using zebrafish for diabetes research is that hyperglycemic can be induced by simply adding glucose to the fish water. In contrast, rodent models of diabetes typically require the injection of the toxic glucose analogue, streptozotocin or alloxan, which preferiantally kill pancreatic β cells (Dou et al., 2010).

Conclusion

The results of this study indicated that yacon leaf ethanol extract could reduce blood glucose levels with a dosage of 40 mg. The research showed that an active compound isolated from yacon leaf extract had an antidiabetic effect.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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