Inhibition of Soluble Epoxide Hydrolase by Astaxanthin for Anti-depressant Effects

Precious Agboinghale

A thesis submitted to the University of Ottawa in partial fulfillment of the requirements for the Master's degree in Chemistry and Biomolecular Sciences.

Department of Chemistry and Biomolecular Science

Faculty of Science

University of Ottawa

ACKNOWLEDGEMENT

I would like to thank **God** for showing me kindness and giving me strength during this period of my life. Everything that I have and will accomplish is through the grace of **God**.

Next, I would like to thank my supervisor, **Dr. Chibuike Udenigwe**, for his mentorship and guidance. He took a chance on me, and I remain grateful; he encouraged me through my projects and most of my decisions here in Canada. I would like to thank everyone in the lab, from the lab coordinator, Ph.D. candidates, current master's students, visiting colleagues and honours students, for their help and for the fun memories over the last few years.

I especially want to thank **Dr. Paul Mayer** for all his help as my co-supervisor and TAC committee member and for providing me with relevant information through my master's program. To **Dr. Appollinaire Tsompo and Dr. Adam Shuhendler**, thank you for your guidance and expertise sharing with me, thank you for listening to my TAC meeting presentation and questions, as well as providing feedback.

I would like to thank the **Dr. Yu Scholarship in Chemistry** and **Nutrition and Mental Health Scholarship** for funding my research and studies. These scholarships gave me the opportunity to learn without financial burdens.

My family has been of great help and influence in my life. To my parents, **Mr. Frederick Agboinghale** and **Mrs. Violet Agboinghale**, thank you for your support, unconditional love, and constant prayers. To my brother, **Justice Agboinghale**, thank you for always believing in me and encouraging me. To my second mum, **Bridget Odufuwa**, you have shown me never-ending kindness and support, I am so grateful. I want to say a big thank you to **Dr. Ogadimma Okagu** for his help, mentorship, advice, and constant encouragement.

ABSTRACT

The enzyme soluble epoxide hydrolase (sEH) plays a major role in the pathogenesis and pathophysiology of neurodegenerative diseases like depression by catalyzing the hydrolysis of epoxyeicosatrienoic acids (EETs) into dihydroxyicosatrienoicacids (DHETs), its less biologically active form, influencing the anti-inflammatory system and promoting inflammation. Therefore, inhibiting sEH leads to increased levels of EETs, reducing inflammation, especially in the brain and can help mitigate neurodegenerative diseases. This study investigated sEH inhibition by a phenolic carotenoid compound, astaxanthin and its inhibitory mechanism of action. Enzyme inhibitory activity and kinetics demonstrated that astaxanthin had a half-maximal inhibitory concentration (IC_{50}) of 26 ± 0.92 μM and is a mixed-non-competitive inhibitor of sEH. *In silico* ADME/tox analysis showed that astaxanthin is bioavailable, biostable, and non-toxic when taken orally. Molecular docking study demonstrated that astaxanthin binds to an allosteric site of sEH and formed a contact and clashing-only interaction with the ASP333 residue of the hydrolase pocket of sEH. In this study, we highlight the potential therapeutic application of astaxanthin as a natural sEH inhibitor in the treatment of inflammation-related diseases, particularly neurodegenerative diseases.

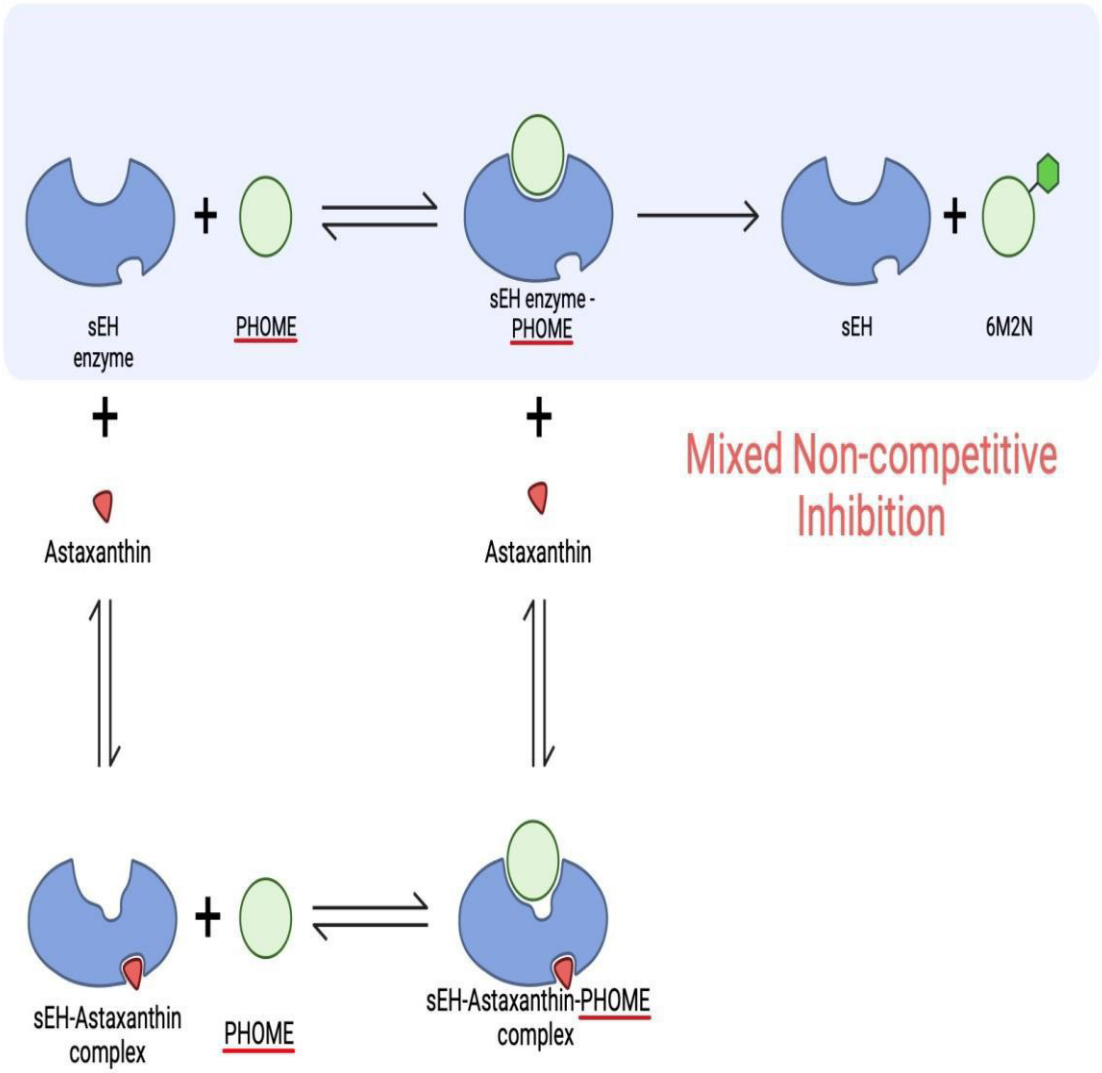


Figure 1: Graphical illustration of abstract created with Bio render.

Table of Contents

LIST OF ABBREVIATIONS

6M2N: 6-methoxy-2-napthaldehyde

AEPU: 1-adamantyl-3-(5-(2-(2-ethoxy ethoxy) ethoxy) pentyl)) urea

AUDA-BE: 12-(3-adamantan-1-yl-ureido)-dodecanoic acid butyl ester

AUDA: 12-(3-adamantan-1-yl-ureido)-dodecanoic Acid

BKca: Big Potassium and Calcium-activated Channels

BSA: Bovine Serum Albumin

CDU: 1-cyclohexyl-3-dodecyl urea

COX: Cyclooxygenase

CRP: C-Reactive Protein

DHA: Dihydroxy Fatty Acids

DiHDPAs: Dihydroxydocosapentaenoic Acids

DiHETEs: Dihydroxy eicosatetraenoic Acids

DMSO: Dimethyl sulfoxide

EETs: Epoxyeicosatrienoic acids

ENOS: Endothelial Nitric Oxide Synthase

EpDAs: Epoxydocosapentaenoic Acids
EpETEs: Epoxyeicosatrienoic Acids

EPFAs: Epoxy Fatty Acids
HDHAs: Hydroxy docosahexaenoic Acids

HEPEs: Hydroxy eicosapentaenoic Acids kDa:

KiloDaltons

LOX: Lipoxygenase
MDD: Major Depressive Disorder

NO: Nitric Oxide

PFC: Prefrontal Cortex

PHOME: 3-phenyl-cyano(6-methoxy-2-naphthalenyl) methyl ester-2-oxirane acetic Acid

PUFAs: Polyunsaturated Fatty Acids sEH: Soluble epoxide Hydrolase t-AUCB: trans-4-

(4-(3-adamantan-1-yl-ureido)-cyclohexyl)-benzoic acid

TNF: Tumor Necrosis Factor

TUPS:1-(1-methanesulfonyl-piperidine-4-yl)-3-(4-trifluoromethoxy-phenyl)-urea

DW: Dry weight WW:

Wet weight

ICAM-1: Intracellular adhesion molecule-1 iNOS:

Inducible nitric oxide

LIST OF TABLES

LIST OF FIGURES

CHAPTER ONE

GENERAL INTRODUCTION

Major depressive disorder (MDD) is one of the most widespread and incapacitating forms of psychopathology; people in all communities worldwide are impacted by depression, which contributes significantly to the global burden of disease. Depression is a common psychological condition that manifests as low mood, lack of interest or pleasure, diminished energy, guilt or feelings of low self-worth, interrupted sleep or food, and difficulty concentrating (Marcus et al., 2012). Due to its high incidence, high recurrence rates, chronic nature, and comorbidity with physical illness, effective and early treatment is essential. (Kessler et al., 2005; WHO 2012; WHO 2017). According to epidemiological surveys, the lifetime prevalence of MDD is 16.6%, with estimates for women exceeding 21.3% (Kessler et al., 2005; Kessler & Bromet, 2013). These numbers underscore the urgent need to understand the pathophysiology of this disease and discover new therapies that can attenuate the global burden of this disease.

An appreciable amount of evidence suggests that inflammation has a role in the pathophysiology of depression, with much evidence mainly from the assessment of cytokine messengers in peripheral blood, particularly interleukin-6 (IL-6), tumour necrosis factor-α (TNF-α) and others, of untreated depressed patients when compared to controls (Miller & Raison, 2016; Strawbridge et al., 2015; Kohler et al., 2017). Certainly, elevated amounts of cytokines circulating in the periphery can pass through the cortex, striatum, and hippocampus which are the permeable regions of the blood-brain barrier (BBB) and affect brain activity associated with depressive symptoms (Borsini et al., 2015; Miller & Raison, 2016).

Additionally, findings from post-mortem research have demonstrated that people with a history of depression had higher levels of proinflammatory cytokine TNF-α gene expression in their prefrontal cortex (PFC) and hippocampi (Dean et al., 2010). Ultimately, these studies suggest that

inflammation is an underlying factor contributing to the onset of depression and that antiinflammatory medication treatment can significantly lessen its symptoms (Borsini, 2021). Considering the crucial part inflammation plays in depression, increasing evidence indicates that inflammation, which is linked to the development of major psychiatric diseases, is influenced by alterations in the enzyme soluble epoxide hydrolase (sEH) in the metabolism of polyunsaturated fatty acids (PUFAs) (Hashimoto, 2019). Ren et al. discovered that the expression of the sEH protein increased more in the parietal cortex, striatum, and hippocampus of mice that exhibited depressionlike behaviours and in the brains of people with major depressive illness (Ren et al., 2016). The sEH enzyme transforms the anti-inflammatory ω-3 PUFAs CYP-derived epoxy epoxyeicosatetraenoic acid (EpETEs) and epoxydocosapentaenoic acids (EpDPAs) into their less active diols, dihydroxyeicosatetraenoic acid (DiHETEs) and dihydoxydocosapentaenoic acid (DiHDPA). DiHETEs and DiHDPAs have been shown to have less anti-inflammatory effects than their progenitors, EpETEs and EpDPAs (Ishihara et al., 2019). This indicates that decreasing the bioavailability of EpETEs and EpDPAs by activating the sEH enzyme can impair their biological activity, eventually resulting in depressive symptoms. These findings imply that sEH plays a significant role in the pathophysiology of depression and that sEH inhibitors may be an alternative treatment option for people who experience this illness (Ren et al., 2016; Swardfager et al., 2018). To date, most sEH inhibitors are synthetic compounds (Sun et al., 2021). In a recent study, it was observed that 1-[1-propionylpiperidin 4-yl]-3-[4-(trifluoromethoxy) phenyl] (TTPU), an sEH inhibitor significantly reduced both spontaneous seizures and depression-like behaviours (Shen et al., 2019). However, the metabolism, appropriate dosing and overall safety and effectiveness of TPPU remain unknown (Debin et al., 2019).

Current sEH inhibitors have high lipophilicity, which indicates poor absorption, high metabolic rate, and poor solubility, making these compounds potentially harmful as they can be stored in the body for a long period and become toxic (Liu et al., 2011). Many known sEH inhibitors function

by binding to the active site, but due to their poor water solubility, they are not effective in treating sEH-related disorders and inflammatory diseases (Sun et al., 2021).

Antidepressants and antipsychotics are employed as a treatment for depression and have been effective to a large degree. However, about one-third of individuals suffering from depression do not respond fully to medication **(**Ren, 2019). This was thought to be partly due to the large size of these compounds, making them susceptible to biotransformation in the gastrointestinal tract reducing their bioavailability and biostability (Sun et al., 2021). Therefore, the need for new therapies, including functional foods and nutraceuticals that are bioavailable and biostable, cannot be overstated.

Astaxanthin is a deep, red-coloured phenolic compound, a prominent member of xanthophylls that can be synthesized by *Haematococcus pluvialis* microalgae (Reigner et al., 2015). Astaxanthin has previously been reported to possess antioxidative, anti-inflammatory, anti-apoptotic and several other health-promoting properties (Reigner et al., 2015). There are no known adverse effects from consuming astaxanthin (6 mg/day) with meals (Rao et al., 2010; Stewart et al., 2008). Using acute, mutagenicity, teratogenicity, embryotoxicity, and reproductive toxicity testing, the biotechnological company Hoffman-La Roche validated the safety of astaxanthin (Kidd, 2011). Astaxanthin possesses both lipophilic and hydrophilic properties; it can be transported by fat molecules directly to tissues and organs that require it the most, such as the brain, retina, and skeletal muscle, owing to its fat-solubility (Higuera-Caiapara et al., 2006). Astaxanthin can easily cross the BBB, protecting the brain against acute injury and long-term neurodegeneration (Shen et al., 2009; Ying et al., 2015). Anti-oxidative, anti-inflammatory, and anti-apoptosis activities are some of the neuroprotective properties of astaxanthin (Zhang et al., 2014a; Zhang et al., 2014b). To the best of our knowledge, there are no studies focusing on the effects of astaxanthin on the inhibition of soluble epoxide hydrolase.

This study aimed to evaluate the effects of astaxanthin on the enzymatic activity of soluble epoxide hydrolase. Using enzyme kinetics, the mechanism of inhibition was determined, and the biomolecular interactions between astaxanthin and sEH were further characterized towards providing a potential food-based treatment for depression.

CHAPTER TWO

LITERATURE REVIEW

2.1 Depression

Major depressive disorder (MDD) is one of the most prevalent and debilitating psychiatric diseases; its widespread occurrence, high recurrence rates, chronic nature, and comorbidity with physical illness make it a significant disease burden worldwide (Troubat et al., 2021; WHO, 2017). Roughly 280 million individuals worldwide suffer from depression, including 5.0% of adults and 5.7% of adults above 60 years; suicide can result from depression at its worst. Every year, approximately 700,000 people die by suicide (GHDx, 2021). For people aged 15 to 29, suicide is the fourth most common cause of death (GHDx, 2021). It is not unexpected that much research is being carried out to improve the understanding of the genesis, maintenance, and treatment of depression, given the high prevalence and significant burden on the healthcare system. Numerous studies have demonstrated that inflammation is linked to the pathogenesis of major psychiatric diseases like depression and is primarily influenced by alterations in the soluble epoxide hydrolase (sEH) in the metabolism of polyunsaturated fatty acids (PUFAs) (Hashimoto et al., 2019a; Hashimoto et al., 2019b; Swardfarger et al., 2018).

2.2 Physiological roles of sEH

The sEH (EC 3.3.2.10) is a 62-kDa enzyme constituting the N-terminal domain (involved in lipid phosphate hydrolysis) and C-terminal domain (involved in the hydrolysis of epoxides to diols (Anita et al., 2021; Hammock *et al.,* 2021). The *EPHX2* gene codes for the human sEH enzyme that is expressed in a myriad of tissues throughout the body, some of which include the smooth muscle, brain, heart, kidney, liver, lungs, and vascular endothelium (Borsini, 2021).

The cyclooxygenase (COX) and lipoxygenase (LOX) pathways convert polyunsaturated fatty acids into eicosanoids, active lipid signalling molecules that possess diverse physiological roles (Wagner *et al.,* 2017). Unlike the roles stated above two ways, which have been extensively studied (Wagner *et al.,* 2017), the physiological roles of cytochrome P450 enzymes in the metabolism of epoxy fatty

acids (EpFAs) are beginning to gain significant attention. Epoxy and hydroxy fatty acids are antiinflammatory compounds produced by the CYP system (Gabbs et al., 2015). Epoxy fatty acids include epoxydocosapentaenoic (EpDPAs) and epoxyeicosatrienoic (EpETEs) from EPA and DHA, respectively. The eicosapentaenoic acids (18, 19, and 20-HEPEs) from EPA and the hydroxy docosahexaenoic acids (20, 21, and 22-HDHAs) from DHA are examples of hydroxy fatty acids. The sEH enzyme converts epoxy fatty acids (EpETEs and EpDPAs) into their corresponding diols, dihydroxy eicosatetraenoic acids (DiHETEs) and dihydroxy docosapentaenoic acids (DiHDPAs). The sEH enzyme catalyzes the conversion of bioactive epoxides to diols by the addition of a water molecule (Anita et al*.,* 2021). The physiological functions of diols generated by sEH are not clearly defined; however, they are considered less beneficial than epoxides (Hildreth *et al.,* 2020). These EpDPAs and EpETEs possess antiinflammatory, antihypertensive, neuroprotective, and analgesic activities and are known as proresolving lipid mediators (Chang *et al.,* 2018; Anita et al*.,* 2021). Conversely, their corresponding diols have been known to elicit cytotoxic (Anita et al*.,* 2021), leukotoxic (Hu et al., 2017), and neurotoxic effects (Hu *et al.,* 2017).

Overall, these previous studies indicate that inflammation is an underlying mechanism and a factor in the onset of depression. This evidence also suggests that sEH overexpression plays a significant role in the pathophysiology of depression and that inhibitors of this enzyme may serve as an alternative therapeutic approach for people with depression.

sEH is expressed in every brain region (Domingues et al*.,* 2020); however, it is abundant in the neurons, astrocytes, and microglia (Borsini, 2021). The role sEH plays in neurotransmission and general brain signal transduction has been described. Big potassium (BK) channels are voltagegated potassium channels that conduct large amounts of potassium ions (K^+) across the cell membrane (BKCa). The release of neuropeptides from the amygdala has been thought to recruit BKCa channels for calcium signalling; furthermore, dihydroxyeicosatrienoic acids (DHETs) and epoxyeicosatrienoic acids (EETs) could function in the release of neuropeptide because of their

actions on BKCa channels (Domingues *et al.,* 2020). Inhibition of sEH activity has been shown to improve neuronal synaptic neurotransmission via elevated levels of postsynaptic glutamatergic receptors and persistent strengthening of the synapses (Domingues *et al.,* 2020). Intriguingly, sEH, however, plays a significant role in the modulation of nitric oxide (NO)-mediated endothelial cell function and endothelial nitric oxide synthase (eNOS) activity (Hou *et al.*, 2015). NO is known to be involved in long-term potentiation (LTP), contributing to learning and memory processes (Domingues *et al.,* 2020). NO has also been reported to mediate the signalling of glutamateNMDA receptor (NMDAr) in neurons (Wu & Tymianski, 2018). Therefore, the sEH significantly influences brain signaling, including synaptic plasticity.

The role of the phosphatase activity of sEH in the central nervous system is yet to be thoroughly explored. However, overexpression of sEH phosphatase activity has been shown to increase cholesterol levels, leading to increased production of amyloid precursor proteins (APP), which contributes to the development of Alzheimer's disease (AD) and other neurodegenerative diseases (Domingues *et al.,* 2020). A thorough exploration of the sEH phosphatase activities in future studies is recommended as it could provide critical information on the pathophysiology of neurodegenerative diseases. Furthermore, overexpression of sEH in the anterior cingulate cortex, and parietal cortex have been associated with bipolar disorder and schizophrenia, as observed in a post-mortem study of patients with major depressive disorder (Zhang *et al.,* 2020), indicating the potential involvement of sEH in emotional disorders.

There is substantial evidence that sEH contributes to the etiology of hypertension due to the hydrolytic activity of its C-terminal domain on EpFA (Ma et al., 2020). EETs, the substrates of sEH in the C-terminal domain, play crucial roles in regulating blood pressure by activating the smooth muscle large-conductance Ca^{2+}-activated K^{+}-channels in diverse vascular beds (Papiashvili et al., 2021). EETs have been reported to elicit several cardioprotective mechanisms.

Some short-term effects include promoting pro-survival signalling, attenuating apoptosis, and preserving mitochondrial structure and function (Jamieson, 2020). In the long term, adverse remodelling can be prevented by EETs and maintain systolic function (Jamieson, 2020). Inhibition of sEH activity has been suggested to alleviate renal tubular mitochondria dysfunction in diabetic animal models (Jiang *et al.,* 2020). EETs are also produced in the pancreas and may be involved in modulating β-cell function (Shah *et al.,* 2020). In addition, inhibition of sEH was demonstrated to rescue insulin resistance by preventing glucose reabsorption via down-regulation of SGLT2 gene expression in the kidney (Luo *et al.,* 2021). Due to the analgesic effects of EpFAs, inhibition of sEH may be a target for managing pains associated with diabetic neuropathies (Hammock et al., 2021). Therefore, sEH plays a crucial role in various physiological processes. Its overexpression has been linked to the pathogenesis of many diseases, making the enzyme a critical therapeutic target in many conditions.

2.3 Roles of sEH in Depression

Several lines of evidence have recently suggested that sEH distribution is critical in developing inflammatory and brain disorders. Although there are no known specific biomarkers of depression, inflammation has been implicated in its physiopathology (Hennebelle et al., 2017). The levels of various biomarkers of inflammation, such as tumour necrosis factor-α (TNF-α), interleukin-1 (IL1), interleukin-6 (IL-6), and acute-phase protein C-reactive protein (CRP) have been established to be increased in the blood of patients with major depressive disorders (Lotrich et al., 2011). A school of thought believes that inflammatory biomarkers associated with depressive symptoms may be brought about by pre-existing medical conditions such as cancer, cardiovascular diseases, and neurodegenerative diseases (Swardfager et al., 2018).

Winter depression was associated with changes in plasma levels of CYP- and sEH-derived epoxy acids, according to an observational study examining individuals with seasonal depression. In

particular, when compared to summer, CYP-derived 14,15-EpETE decreased, while sEH-derived 16,17-DiHDPA and 19,20-DiHDPA increased. (Hennebelle et al., 2017). Due to the lower bioavailability of the anti-inflammatory ω-3 PUFAs-derived epoxy fatty acids in patients with seasonal depression, these studies suggest that an increase in sEH-dependent metabolism underlies a more inflammatory state in these individuals. The serum levels of 10,11-EpDPE and 17,18DiHETE were also found to have a negative and positive link with depressive symptoms in different investigations of people with type 2 diabetes and severe depression, respectively (Anita et al., 2021), demonstrating once more how important these epoxy acids are in the treatment of depression.

2.4 Pharmacological inhibitors of sEH

Inflammatory pathways were linked to the etiology of depression and the anti-depressant activity of certain substances in several studies (Hashimoto, 2016; Miller & Raison, 2016; Zhang et al., 2016a). **Figure 2.1** below shows the mechanism of action of sEH inhibitors in depression.

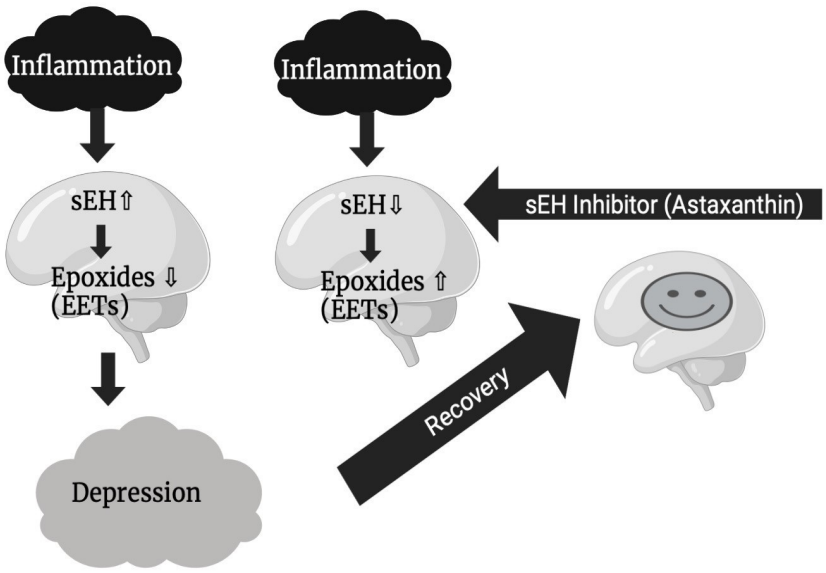


Figure 2.1: Possible mechanism of inhibiting depression by sEH inhibitors

The initial inhibitors of soluble epoxide hydrolase discovered were compounds that contain epoxide. Enhancing absorption, distribution, metabolism, and excretion could lead to the development of efficient in vivo inhibitors.

Initial compounds, for instance, (AUDA)12-(3-adamantan1-yl-ureido)-dodecanoic acid, are generally utilized to examine enzyme biological functions, although their increased melting point, low solubility and poor metabolic stability in water or several organic solvents hinder their pharmacological use (Morisseau et al., 2002; Kim et al. 2004; Kim et al. 2007). Several compounds

have been discovered after AUDA, which integrated functional groups expected to bind to the sites by hydrogen bonding within the site of catalysis. Some of the new compounds, such as (TPAU)1trifluoromethoxyphenyl-3-(1-acetylpiperidin-4-yl) urea and (t-AUCB) trans-4(4(3adamantan1-yl-ureido)-cyclohexyl)-benzoic acid, have better metabolic stability than earlier sEH inhibitors, possibly due to their higher resistance to metabolism and water solubility. (Hwang et al. 2007). sEH inhibition results in high EETs levels and potentially has favourable therapeutic effects on inflammation, lipid, carbohydrate metabolism and blood pressure (Yu et al. 2019).

2.5 Astaxanthin

Astaxanthin is a red xanthophyll carotenoid primarily found in marine organisms and animals (Galasso et al., 2017; Ambati et al., 2014). Astaxanthin differs from other carotenoids and xanthophylls because it contains two hydroxyl groups (Ambati et al., 2014).

Astaxanthin (3,3′-dihydroxy-β, β-carotene-4,4′-dione; Molecular weight 596.8g/mol molecular formula $C_{40}H_{52}O_4$) consists of a polyene chain connecting two terminal β-ionone-type rings. It contains a hydroxyl group (-OH) on each end and two asymmetric carbons at the 3,3′ position of the β-ionone ring. The ring system contains oxygen in the form of hydroxyl and keto (C=O) groups (**Figure 2.2)** (Tatas et al., 2020). Astaxanthin is found naturally in a wide range of organisms, such as complex plants, microalgae, and shellfish (Hussien et al., 2005). The yeast *Phaffia rhodozyma* and the algae *Haematococcus pluvialis* are the primary sources of astaxanthin used in commercial products. Astaxanthin is closely related to other carotenoids like β-carotene, lutein, and zeaxanthin because it is a member of the xanthophyll group (Stahl & Sies, 2005). The different biological activities of astaxanthin and its natural sources are highlighted below (**Tables 2.2 and 2.3**).

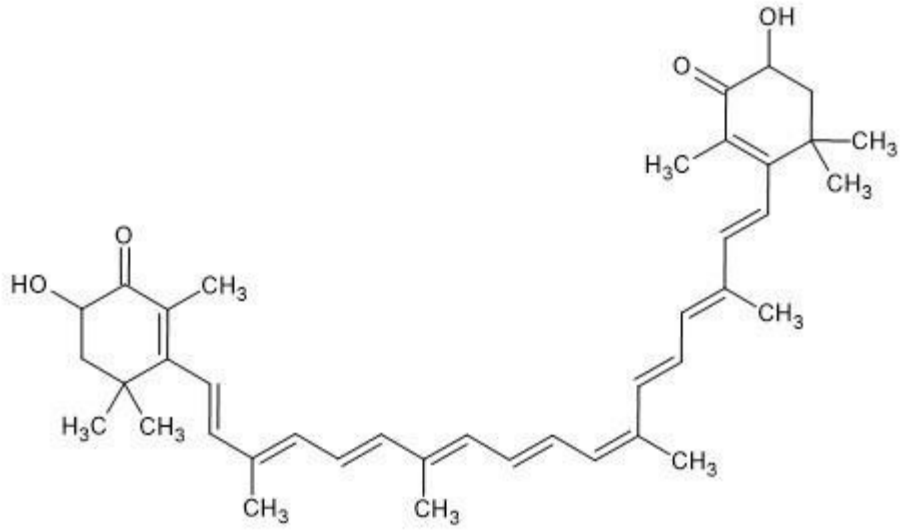


Figure 2.2: Chemical structure of Astaxanthin

Table 2. 1: Biological activities of astaxanthin

Study Model	**Effect**	**Dosage**	**Target**	**Disease**	**Reference**
Wistar Rat	Anti-aging	10 mg/kg/d	IL-1β, IL-10	Brain aging	Balietti et al., 2016
ETS Mouse	Antiinflammation	40 and 80 mg/kg	IL-6, TNF-α, MDA, SOD, CAT	Cognitive Impairment	Yang et al., 2019
Wistar Rat	Neuroprotective	10 mg/kg	GPx, MDA	Alzheimer's disease	Taksima et al., 2019
HT 22 Cells	Neuroprotective	1.25-5 uM	HO-1, Cyto-C	Alzheimer's disease	Wen et al., 2015

Abbreviations: **CAT**- Catalase, **GPx**- Glutathione peroxidase, **MDA**- Malondialdehyde, **SOD**- Superoxide dismutase, **HO-1**- Heme oxygenase -1, **TNF**α- Tumor necrosis factor, **IL-6**- Interleukin-6, **IL-10**- Interleukin-10, **IL-1β**- Interleukin 1 beta, **Cyto-C**- Cytochrome C, **pAKT**- Protein Kinase B.

Table 2. 2: Natural sources of astaxanthin

Source	**Species**	**Astaxanthin Concentration (mg/kg)**	**Reference**
Crustaceans	*Litopenaeus Vanamei*	31.8 w.w	Parisenti et al., 2011
	Marsupenaeus Japonicus		Chien & Shiau, 2011
Shrimp	*Chionoecetes Opilio*	418 d.w	Higueria-Ciapara et al., 2011
Crabs		119.6 d.w	
	Procambarus Clarkii		Charset et al., 2001
	Atlantic Salmon (salmo salar)		Ambati et al., 2014
Crawfish		197.9 d.w	
	Sockeye Salmon (Oncorhynchus nerka)		Lim et al., 2018
Fish		6.8 w.w	
		26-38 w.w	

Bacteria	*Agrobacterium aurantiacum* *Sphingomonas astaxanthinifaciens* *Brevundimonas sp. M7*	140 d.w 690 d.w 1300 d.w	Yokoyama et al., 2014 Barredo, 2012 Barredo, 2012
Yeast	*Candida utilis* *Phaffia rhodozyma PR 190*	400 d.w 970 d.w	Miura et al., 1998 Kusdiyantini et al., 1998
Zooplankton	*Amphiascoides atopus* *Acartia bifilosa* *Pseudocalanus acuspes*	619 d.w 487 d.w 305 d.w	Caramujo et al., 2012 Lotocka et al., 2004 Lotocka et al., 2004

Phytoplankton	*Chlorella zofingiensis* *SAG-211/14* *Euglena sanguinea* *Chlorococcum sp.*	6800 dw - 	Orosa et al., 2001 Chen & Lim, 2020 Zhang & Lee, 1997
		4230 d.w	
Archea	*Halobacterium salinarium NRC-1*	265 d.w	Calo et al, 1995
Microorganism	*Haematococcus* *pluvialis NIES-144* *Haematococcus* *pluvialis CCAP-34/7* *Haematococcus* *lacustris*	98,000 d.w 22,700 d.w 43,100 d.w	Domínguez- Bocanegra et al., 2004 Orosa et al., 2001 Lee & Soh, 1991

D.W – dry weight and **W.W** -wet weight.

2.6 Astaxanthin as a potential inhibitor of sEH

The positive effects of astaxanthin have been the subject of numerous investigations. In experimental models of neurological diseases, astaxanthin mediates neuroprotection through antioxidant, anti-inflammatory, and anti-apoptotic pathways (Shen et al., 2009; Yamagishi & Aihara, 2014). Administration with astaxanthin attenuated lipid peroxidation and oxidative DNA

damage in an *in vivo* mouse model and inhibited N-methyl-D-aspartate (NMDA)-triggered retinal damage (Nakajima et al., 2008). Another study showed that astaxanthin exhibited neuroprotective effects against oxidative stress induced by oxygen-glucose deprivation in SH-SY5Y cells (a thricesubcloned cell line derived from the SK-N-SH neuroblastoma cell line) and 10-min global cerebral ischemia in rats (Lee et al., 2010). Uncontrolled or severe inflammation is destructive to the body and can harm the body's cells and tissues (Brown & Neher, 2010); inflammation plays a critical role in acute and chronic neurodegenerative diseases (Lucas et al., 2006).

Remarkably, astaxanthin directly inhibited the activity of inducible nitric oxide synthase (NOS) to elicit anti-inflammatory effects in lipopolysaccharide-induced uveitis animal models (Ohgami et al., 2003). **Figure 2.3** shows the anti-inflammatory properties of astaxanthin in neurological diseases. Furthermore, astaxanthin reduced the gene expression of inflammatory mediators (such as TNF-α and IL-1β), and by blocking the NF-β-dependent signalling pathway, it decreased the severity of endotoxin-induced uveitis animal model (Suzuki et al., 2006). Astaxanthin has also proved to be a potent inflammation inhibitor by restoring the normal levels of protein tyrosine phosphatase-1, inhibiting ROS-induced nuclear expression of

NF-κB (p65) and decreasing the downstream production of pro-inflammatory cytokines (such as IL-1, IL-6, and TNF-α) in U937 mononuclear cells (SHP-1) (Speranza et al., 2012). Similarly, astaxanthin inhibited cyclooxygenase and down-regulated prostaglandin E2 (PGE2) and TNF-α in mice by inhibiting nitric oxide production and inducible nitric oxide synthase (iNOS) activity in macrophages (Ohgami et al., 2003). Astaxanthin provided neuroprotection against EBI in a prechiasmatic cistern subarachnoid hemorrhage (SAH) model by modulating cerebral inflammation (Zhang et al., 2014). Zhang et al. reported that after SAH, astaxanthin post-treatment significantly reduced neutrophil infiltration inhibited NF-κB activity, decreased the protein and mRNA levels of inflammatory mediators IL-1β, TNF-α, and intracellular adhesion molecule-1 (ICAM-1), and reversed brain inflammation (Zhang et al., 2014). Following astaxanthin

administration, secondary brain damage cascades, neuronal degeneration, BBB disruption, cerebral edema, and neurological dysfunction were all mitigated (Zhang et al., 2014). In young, healthy adult human participants, dietary astaxanthin supplementation promoted T-cell and B-cell mitogeninduced lymphocyte proliferation, improved the cytotoxic activity of natural killer cells, and augmented interferons IFN- and IL-6 production in the blood (Park et al., 2010). Astaxanthin has been regarded as a safe (8mg/day) and effective natural substance against neurological disorders because of its positive effects in the treatment of neurological conditions (Wu et al., 2014). Given all these studies, astaxanthin is a promising candidate for treating neurological disorders and can play a critical role in inhibiting the soluble epoxide hydrolase, therefore lowering sEH-induced inflammation, and alleviating neurological disorders and mental health diseases like depression. Astaxanthin has anti-inflammatory, antioxidant, anti-diabetic, anti-apoptotic, antiproliferative, neuroprotective, and hepato-protective properties (Zeynab et al., 2022). All of these convey the multifunctional and potential therapeutic applications of astaxanthin.

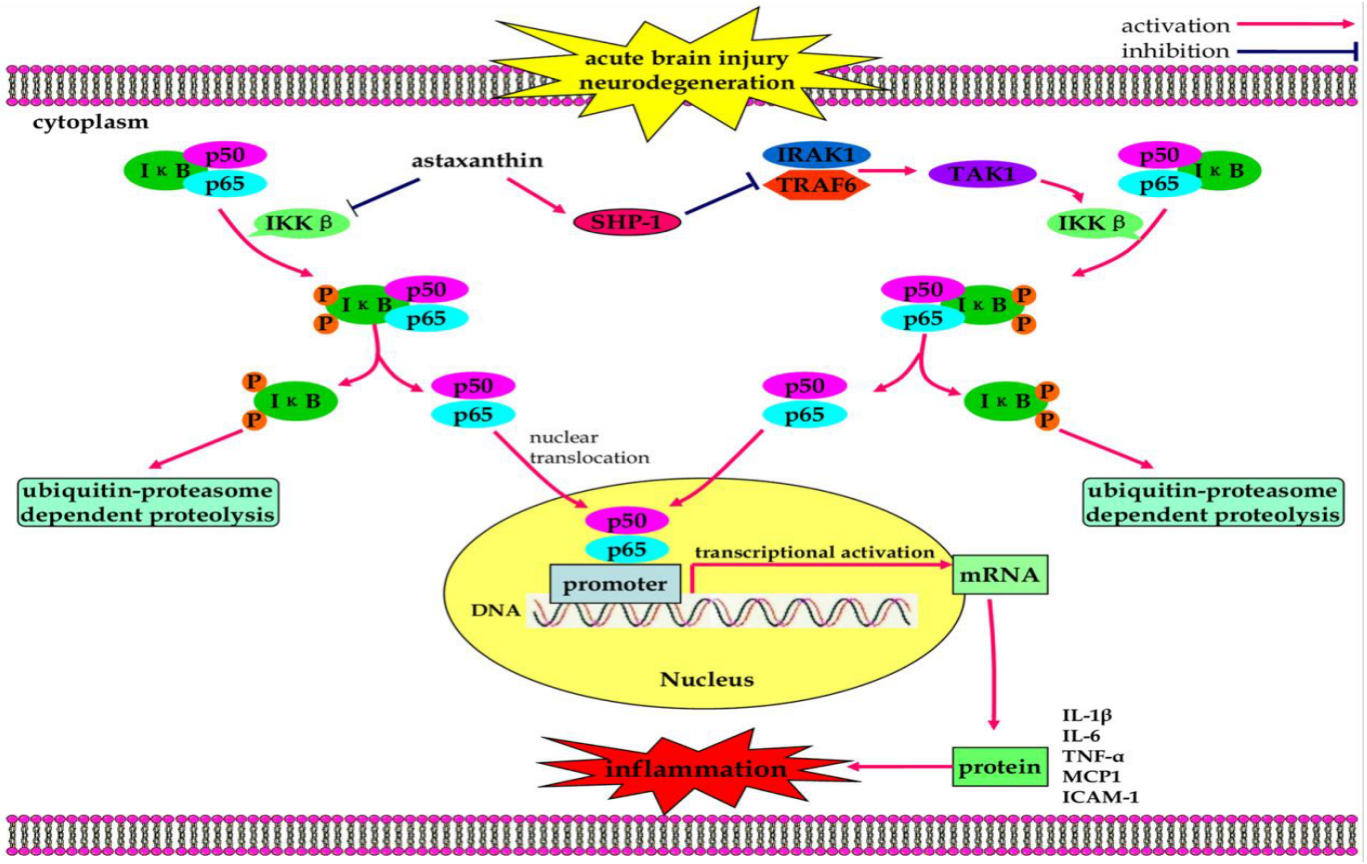


Figure 2.3: The anti-inflammatory properties of astaxanthin in neurological diseases.

The inflammatory molecules IL-6, ICAM-1, and MCP1 are not expressed, while astaxanthin is present because of the inhibition of IκB-α breakdown and NF-κB nuclear translocation. By restoring physiological levels of SHP-1, astaxanthin also inhibits NF-B nuclear expression and lowers downstream production of proinflammatory cytokines (Wu et al., 2015).

2.7 Hypothesis, objective, and study impact

It is hypothesized that astaxanthin will inhibit sEH activity, which is relevant in reducing inflammation in the brain. The aim of this study was to determine the effect of the natural compound astaxanthin on sEH enzymatic activity, the mode of its inhibition, and its kinetics mechanism.

This study investigated the direct neurophysiological benefits of naturally occurring carotenoids and, thus, will provide information on the possibilities of replacing conventional synthetic sEH inhibitor candidates with safer natural compounds like astaxanthin.

CHAPTER THREE

MATERIALS AND METHODS

3.1 MATERIALS

Dimethyl sulfoxide (DMSO), 3-phenyl-cyano(6-methoxy-2-naphthalenyl) methyl ester-2-oxirane acetic acid (PHOME), 6-methoxy-2-napthaldehyde (6M2N), recombinant human soluble epoxide hydrolase and AUDA were purchased from Cayman Chemicals (Ann, Arbor MI, USA).

Astaxanthin, bovine serum albumin (BSA, 96% purity) and Bis-Tris HCl were purchased from Millipore Sigma, Ottawa, Canada. Milli-Q water (18.2 cm resistivity at 25, total organic carbon level 5 ppb) was purified using a commercial water-purification system (Advantage A10 Q-POD Milli-Q Water System).

3.2 METHODS

3.2.1a Sample Preparation

Astaxanthin (inhibitor) solution was prepared by dissolving it in DMSO, creating a stock at a final concentration of 1 mM. Before performing the inhibition and kinetics studies, the stock solution was added to 25 mM Bis-Tris HCl, pH 7.0 buffer containing 0.1 mg/mL of BSA. DMSO <6% for all concentrations.

3.2.1b AUDA

In a small microtube, 1 mM stock of 12-(3-adamantan-1-yl-ureido)-dodecanoic acid (AUDA; Cayman Chemicals) was prepared DMSO and diluted in phosphate buffer and stored at −20◦ C.

3.2.1c Buffer

5.23 g of Bis-Tris was dissolved in 400 mL of water. The pH was adjusted to 7.0 at room temperature with 1 M HCl and adjusted with Milli-Q water to a final volume of 1 litre and stored at room temperature. Before performing enzyme assays, a 5 mg fraction of bovine serum albumin was dissolved in 50 mL of the buffer. This buffer is 25 mM Bis-Tris HCl, pH 7.0, containing 0.1 mg/mL of BSA. BSA was included to stabilize pure sEH and help retain its lipophilic compounds in the solution.

3.3 In Silico Study; ADME/Tox Analysis

Astaxanthin and AUDA absorption, distribution, metabolism, and excretion (ADME) properties were analyzed *in silico* and compared. SMILES strings of AUDA were obtained from the NCBI PubChem Compound database (http://www.pubchem.ncbi.nlm.nih.gov/compound), and the SMILES strings of astaxanthin were obtained from the National Library of Medicine (PubChem). SwissADME was used to obtain the physiochemical properties, pharmacokinetics and druglikeness (Daina et al., 2017). The potential toxicity of astaxanthin was assessed via AdmetSAR 2 prediction tool (Yang et al., 2019).

3.4 sEH Inhibition assay

sEH inhibition was measured using 3-phenyl-cyano(6-methoxy-2-naphthalenyl) methyl ester2oxirane-acetic acid (PHOME) as the fluorescent substrate as previously described (Bumistrov,

2021), with some modifications. Astaxanthin was assayed for inhibitory activity against sEH. In a 96-well plate, 50 µL of 16 ng/mL recombinant human sEH was incubated with varying concentrations (2.5 µM -100 µM) of astaxanthin (inhibitor) dissolved in DMSO for 5 minutes in 25 mM Bis-Tris HCl buffer, pH 7.0 containing 0.1% of bovine serum albumin BSA at a temperature of 30 °C before the addition of substrate 5 µM. The activity was determined by monitoring the appearance of fluorometric product 6M2N over six hours with an excitation wavelength of 330 nm and emission wavelength of 465 nm at 30ºC temperature by relative fluorescence unit (RFU) using a Microplate reader (Tecan, USA). AUDA was used as a positive control. The reaction rate (RFU/min) was calculated as:

$$\text{Rate} = \frac{!"\#\$"}{\%\&}$$

Tf and Cf are the fluorescence intensities of the assay mixture in the presence and absence of inhibitor, respectively, over 1 h of the enzyme assay. The inhibitory activity of sEH was calculated using: Percentage % inhibitory activity = $1 - \frac{\%'()*+,-}{\%/012\&314} \times 100$

$\Delta Fsample$ and $\Delta Fcontrol$ are changes in the fluorescence intensity in the presence and absence of inhibitor, respectively, over 1 h of enzyme assay.

The half-maximal **inhibitory concentration** (IC_{50}) is a measure of the potency of a substance in inhibiting a specific biological or biochemical function. The reported IC_{50} value is the average of three replicates obtained from the experiment, determined using linear regression obtained from

the curve of the percent inhibition vs inhibitor concentrations. Values were reported as average ± standard deviation.

3.5 sEH inhibition kinetics analysis

Kinetics analysis of the sEH enzyme was conducted to determine astaxanthin's mode of inhibition. This analysis was conducted at different PHOME concentrations (1.25-20 μM) (Jo et al., 2016) in the presence and absence of varying astaxanthin concentrations (5-35 μM). The data was analyzed using Michaelis-Menten plots. Lineweaver-Burk analyses were performed to determine their mode of inhibition (competitive, non-competitive, uncompetitive). Maximum rate (V_{max}), Michaelis constant (K_m) were also calculated. The enzyme kinetics parameters (V_{max} and K_m) were determined using GraphPad Prism version 9.1.2 for Windows (GraphPad Software, La Jolla, CA, USA). Kinetics assay was carried out for 6 h to determine the kinetic inactivation parameters ($[P]_\infty$, apparent inactivation rate constant *A*) and to test for the effect of astaxanthin on sEH inactivation in the presence and absence of PHOME.

3.6 Molecular Docking

The molecular docking study was carried out as previously described (Fatoki et al., 2022). Briefly, human soluble epoxide hydrolase (PDB ID: 3OTQ) was obtained from NCBI Pubchem. The structures of the ligands (astaxanthin and AUDA) were obtained NCBI PubChem Compound database (http://www.pubchem.ncbi.nlm.nih.gov/compound) in SMILES (Simplified Molecular Input Line Entry Specification) format, and their 3D structure was optimized using ACDLab/Chemsketch software, and saved in .mol format. PyMol software was used for ligand file conversion from .mol to .pdb and for the preparation of protein by removal of water and existing ligands. Ligand and protein were prepared for docking using AutoDock Tools (ADT) v1.5.6 (Morris et al., 2009) at default settings, and the output file was saved in pdbqt format. Active site amino acid residues of the enzyme were noted from the literature (Schiøtt and Bruice, 2002) and used to

define the docking parameters: center grid box (78.369 × -7.222 × 65.775 points), size (70 × 70 × 70 points), and spacing (0.375 Å). The molecular docking program AutoDock Vina v1.2.3 (Trott and Olson, 2010; Eberhardt *et al*., 2021) was employed for the docking experiment. The blind docking parameters used were center grid box (54.319 × 3.400 × 75.079 points), size (116 × 100 × 100 points), and spacing (0.675 Å). After docking, close binding interactions of the target with the ligands were analyzed and visualized using PyMol and ezLigPlot (Tao et al., 2019).

3.8 Statistical Analysis

GraphPad Prism version 9.1.2 (GraphPad Software, La Jolla, CA, USA)was used using Tukey's or Dunnett's multiple comparisons tests with a significance level set at $p < 0.05$. All analyses were conducted in experimental triplicates. Data were expressed as mean ± standard deviation.

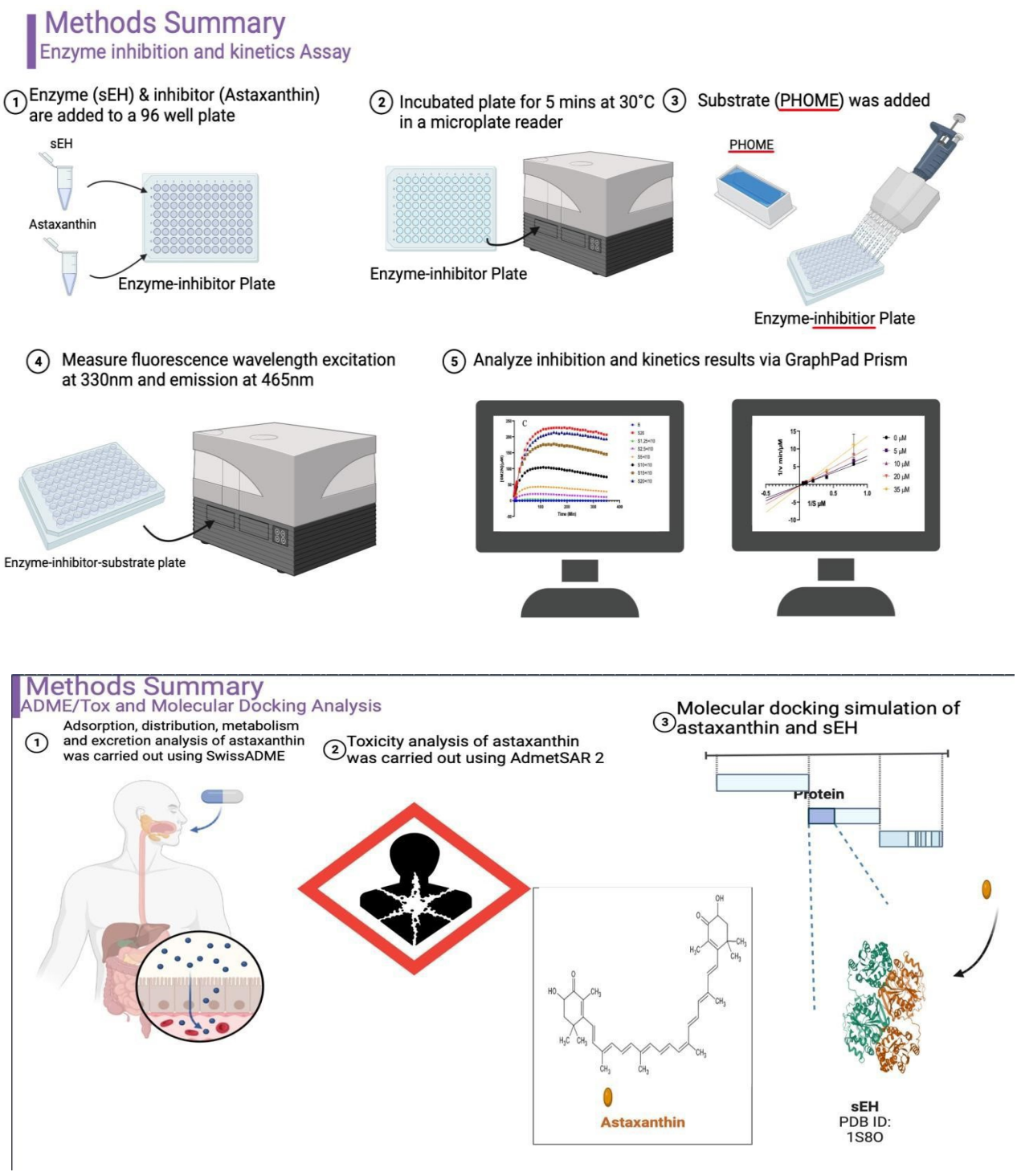


Figure 3.1: Graphical illustration of the methods section. Created with Bio render.

CHAPTER FOUR

RESULTS & DISCUSSION

4.1 *In silico* ADME analysis of AUDA and astaxanthin

Non-invasive computational technologies can predict the behaviour of molecules in biological systems (Diana et al., 2017). The adsorption, distribution, metabolism, and excretion (ADME) of astaxanthin, lipophilicity, solubility parameters, physiochemical properties, pharmacokinetics, and drug-likeness were predicted using SwissADME software (Diana et al., 2017). In (**Table 4.1**), astaxanthin's ADME parameters were compared with those of a notable sEH inhibitor, AUDA. The interaction of molecules in lipid and aqueous environments can be assessed using solubility and lipophilicity in pharmacokinetics. The oral bioavailability of a compound can be predicted by its ROTB (number of rotatable bonds), and TPSA (topological polar surface area) properties (Ji et al., 2020). Compounds with rotatable bonds >10 have a higher molecular weight and characteristics of poor oral bioavailability, whereas compounds with low TPSA values (between 20 and 130) may have high oral bioavailability (Mbarik et al., 2019). As shown in (**Table 4.1)** the TPSA and ROTB values of astaxanthin indicate that it has good oral bioavailability. Nonetheless, the bioavailability score of astaxanthin was low and much lower than that of AUDA, according to predicted oral bioavailability (**Table 4.1**).

The CLogP value of astaxanthin is considerably higher than that of AUDA; this indicates a more hydrophobic compound with lesser affinity for the aqueous phase. Although most sEH inhibitors bind to the sEH active site, they remain insoluble in water, hindering their effective clinical use for the treatment of sEH-related diseases (Sun et al., 2021). Another shortcoming of these sEH inhibitors may be their large molecular size, making it impossible to escape biotransformation in the gastrointestinal tract, thus reducing their bioavailability (Sun et al., 2021). Previous studies on astaxanthin have shown its ability to resist biotransformation in the gastrointestinal tract (Tatas et al. 2020). Astaxanthin is predicted to be a P-glycoprotein substrate and not an inhibitor of

cytochrome P450 3A4 (**Table 4.1**); therefore, it can be removed quickly from cells and undergo speedy metabolism while still exerting its beneficial actions, making it desirable for human use. Bioavailability radar images of astaxanthin and soluble epoxide inhibitors AUDA and TPPU are shown below in **Figure 4.1**. Bioavailability radar illustrates the drug-likeness of a compound through its physiochemical properties: size, polarity, lipophilicity, solubility, saturation, and flexibility (Diana et al., 2017). The proximity of each parameter to the centre of the radar gives a smaller value, and the pink area represents the optimal range for each property. Astaxanthin, AUDA and TPPU each generated a distinct bioavailability radar image, but AUDA and TPPU had similarities in size, polarity, and solubility. Astaxanthin and TPPU showed similarities in size, polarity, and solubility. The similarities in solubility demonstrate that astaxanthin is a soluble molecule which will greatly facilitate many drug development activities, especially the formulation and handling process. Astaxanthin had good polarity (TPSA) properties (not too polar); this predicts that astaxanthin may be orally bioavailable. From the bioavailability radar, astaxanthin was predicted to be too flexible, having more than 9 rotatable bonds, violating the criteria for flexibility.

Astaxanthin was analyzed using AdmetSAR 2 for specific toxicity criteria: mutagenicity, acute oral toxicity, carcinogenicity, hepatotoxicity and reprotoxicity (**Table 4.2**). Astaxanthin belongs to the third-class toxicity group corresponding to LD_{50} between 500 and 5000 $mg.kg^{-1}$, which is considered safe based on the Hodge and Sterner toxicity scale. Astaxanthin was considered safe in every toxicity criterion, except that it was predicted to be a potential reprotoxic compound. It will therefore be necessary to pay attention to this parameter during further development of astaxanthin for therapeutic applications.

Table 4.1: In Silico ADME Parameters of Astaxanthin and AUDA using SwissADME

		AUDA	ASTAXANTHIN
LIPHOPHILICITY	TPSA (Å²)	78.43 Å²	74.60 Å²
	CLogP (o/w)	4.69	8.24
Pharmacokinetics and Drug likeness	Bioavailability Score	0.56	0.17
	GIA	High	Low
	BBB Permeation	Low	High
	Lipinski Filter	High	High
	CYP 3A4 Inhibitor	High	Low
	P-glycoprotein Substrate	Low	High/Yes
Physiochemical Properties	Molecular weight (g/mol)	392.58	596.84
	ESOL (Log S)	-4.98	-9.35
	ROTB (n)	15	10
	HBA (n)	3	4
	HBD (n)	3	2
	Formula	$C_{23}H_{40}N_2O_3$	$C_{40}H_{52}O_4$

Abbreviations: Gastrointestinal Absorption (GIA), Number of Rotatable Bonds (ROTB), Hydrogen Bond Acceptors (HBA), Hydrogen Bond Donors (HBD), Estimated Solubility (ESOL), Topological Polar Surface Area (TPSA), Logarithm of Compound Partition Coefficient Between

N-Octanol and Water (ClogP), Lipinski Filter (Lipinski's Rule-Of-5), Cytochrome P450 3A4 (CYP3A4), and Blood Brain Barrier (BBB).

Table 4.2: Toxicity parameters of astaxanthin obtained with AdmetSAR 2 ASTAXANTHIN

Toxicity Risks	
Acute Oral Toxicity	Class III
Mutagenicity	LOW
Carcinogenicity	LOW
Hepatotoxicity	LOW
Reprotoxicity	HIGH

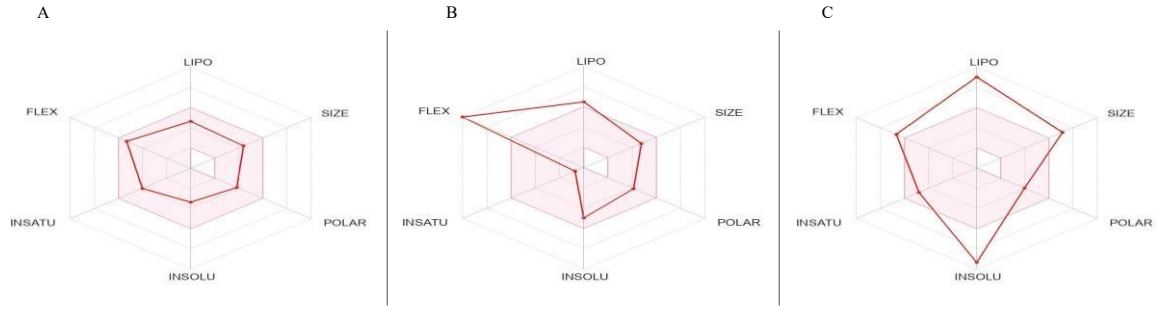
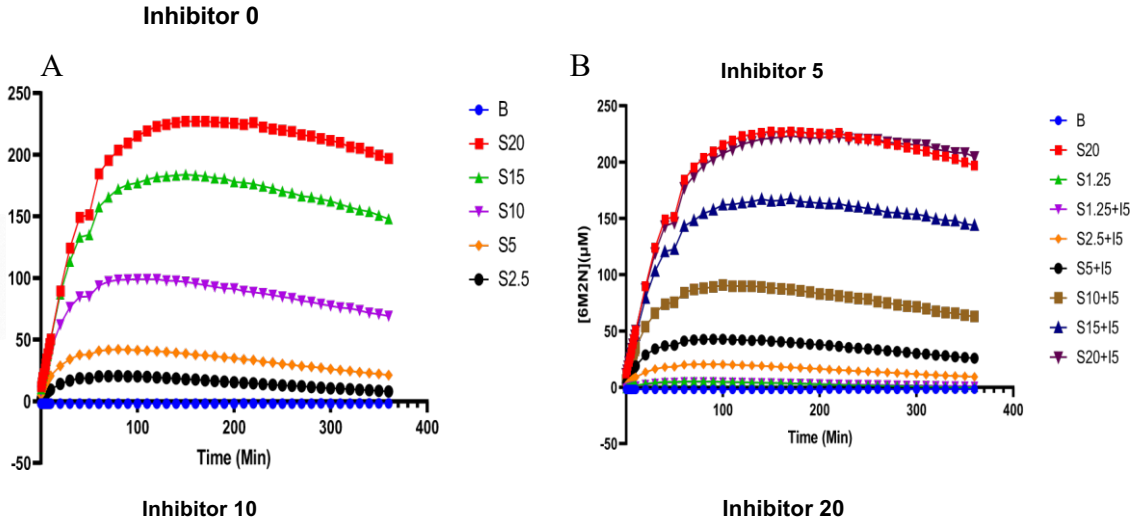


Figure 4.1: Bioavailability radar images of (A) TPPU, (B) Astaxanthin and (C) AUDA generated using SwissADME. Bioavailability radar displayed six physiochemical properties: Lipophilicity, flexibility, size, polarity, solubility, and saturation.

4.2 sEH inhibition: Product formation dependence on substrate concentration

Time curves (plots of $[P]_t$ vs. time) for 0, 5, 10, 20 and 35 µM astaxanthin showed that the concentration of product formed [P] (PNP) formed at any time interval, t, was directly related to the substrate concentration (**Figure 4.2**). With an increase in the time of reaction, $[P]_t$ the point at which the concentration of product remains constant $[P]_\infty$ changed, depending on the substrate concentration. With a lower substrate concentration, $[P]_\infty$ was reached at a lower concentration. From time 100-120 min, there was optimum product formation, after which product formation decreased. This may be due to the inhibitory actions of astaxanthin, stopping the catalysis of the reaction. It was observed that the $[P]_\infty$ for each substrate concentration [S] decreased with a corresponding increase in astaxanthin (inhibitor) concentration. The higher the inhibitor concentration, the lower the product concentration at which $[P]_\infty$ was reached. Also, with increasing inhibitor concentrations, the activity of sEH can be observed to decrease consistently. This implies that astaxanthin inhibits soluble epoxide hydrolase activity. Once $[P]_\infty$ was attained, a decrease over time was observed, i.e., with increasing astaxanthin concentration, a gradual decline in $[P]_\infty$ was observed. This suggests that sEH inhibition occurred by partly decreasing its affinity for the substrate PHOME (Chilaka & Nwamba, 2008).



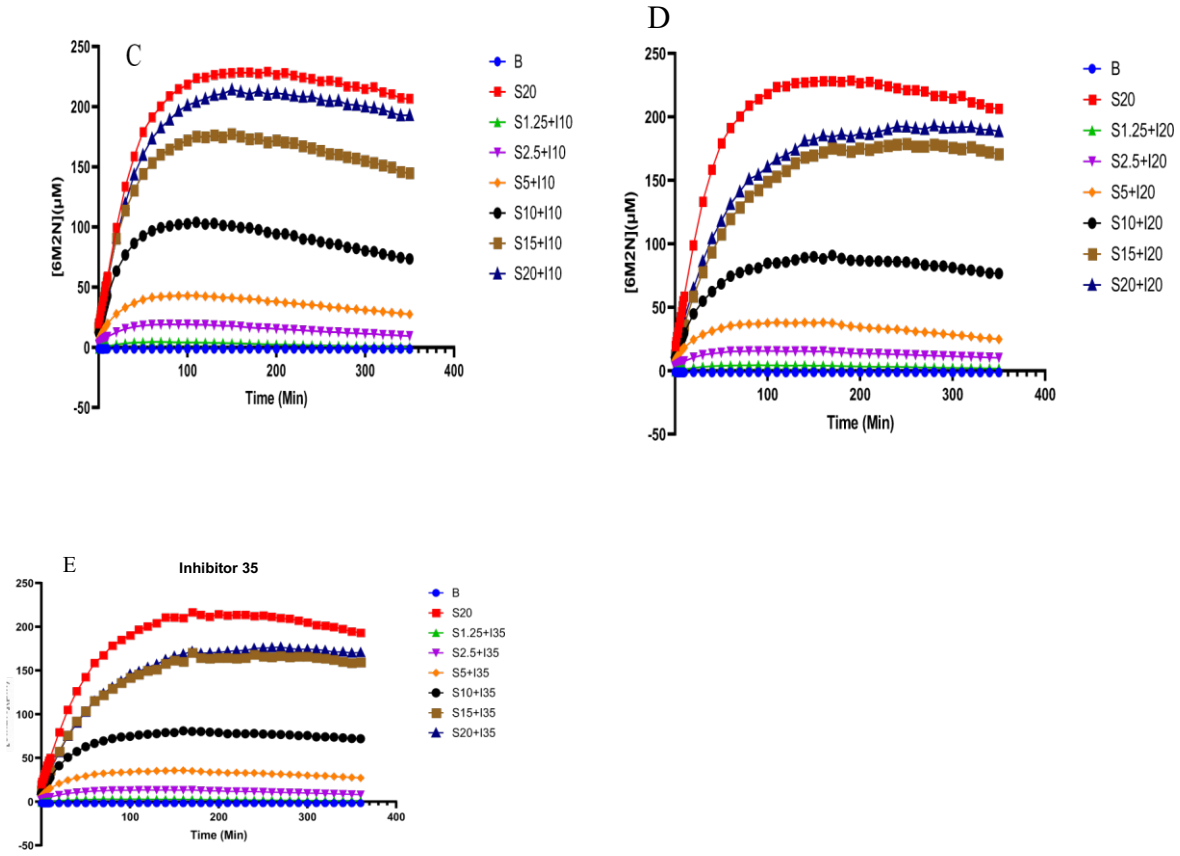


Figure 4.2: Plot of time curves of the product (6M2N) concentrations formed during the inhibition of sEH by Astaxanthin under varying substrate (PHOME) concentrations (1.2520 uM) and Inhibitor concentrations (5-35uM). The inhibitor concentration consisted of (A) Absence of Astaxanthin, (B) 5 uM Astaxanthin, (C) 10 uM Astaxanthin, (D) 20 uM Astaxanthin, and 35 uM Astaxanthin.

4.3 Inhibitory mode of astaxanthin on sEH

The inhibitory effect of astaxanthin on soluble epoxide hydrolase was analyzed by dose-effects graphs. Astaxanthin showed an inhibitory effect on soluble epoxide hydrolase, which increased with increasing concentrations of astaxanthin (5 - 100 μM). Astaxanthin inhibited sEH in a dosedependent manner, with an IC_{50} value of 26 ± 0.45 μM (**Figure 4.3**). **Figure 4.3B** shows the IC_{50} value of astaxanthin plotted with a linear regression graph, the IC_{50} value is derived where the line meets the negative x-axis. AUDA was used as a positive control (IC_{50} value of 2.0 ± 0.2 nM). The half maximal inhibitory concentration (IC_{50}) value for astaxanthin, is estimated to be 26 ± 0.45 μM, although, an IC_{50} value not >10 μM is considered therapeutically more significant, astaxanthin

had an IC_{50} value comparably lower than other food or bioactive peptide inhibitors, for example, the compound extracts of *Lycopus lucidius* (compounds 1 and 3 (triterpenes), 6 (phenylpropanoids), and 10 (9 methyl lithospermate) with IC_{50} values 67.8 ± 3.2 µM, 42.1 ± 4.0 µM, 52.0 ± 1.9 µM and 35.7 ± 2.1 µM respectively) (Han et al., 2021). Compound extracts costunolide and alantolactone from *Inula helenium* had IC50 values of 31.56 ± 0.76 µM and 32.46

± A 1.42 µM (He et al., 2020); this B demonstrates that astaxanthin showed promising inhibitory activity against sEH. (**Figure 4.3**). This indicates the potential of carotenoid compounds like astaxanthin as natural inhibitors of sEH.

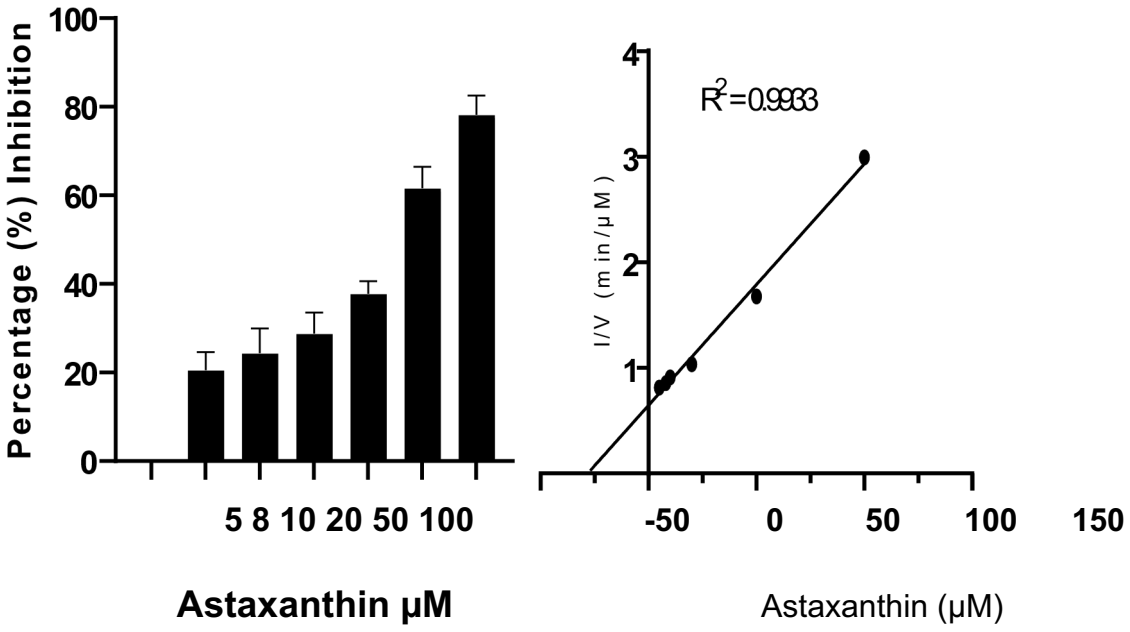


Figure 4.3: Inhibitory activities of astaxanthin against soluble epoxide hydrolase (sEH) at a range of concentrations

4.4 Kinetics Study of Soluble Epoxide Hydrolase (sEH) by Astaxanthin

The Michaelis-Menten and Lineweaver-Burk plots (double reciprocal graphs) were used to determine the inhibition kinetics parameters (Michaelis constant (K_m) and the maximum rate (V_{max})) and mode of astaxanthin inhibition of soluble epoxide hydrolase. With increasing

concentrations of astaxanthin, both K_m and V_{max} decreased, which indicates that astaxanthin reduced the activity of sEH and affected the binding affinity of the substrate PHOME to the enzyme sEH via a mixed-type (non-competitive & uncompetitive) mechanism of inhibition. The K_m value of sEH in the absence of astaxanthin was 71.09 ± 0.10 (**Table 4.2**). The double reciprocal Lineweaver-Burk plots passed through the x-axis and intercepted at the centre above the 1/s axis and both the x and y-axis (**Figure 4.4a**), indicating that astaxanthin behaved as a mixed inhibitor of sEH (non-competitive and uncompetitive). In this type of inhibition, the inhibitor binds to both the free enzyme and the enzyme-substrate complex (Zheng et al., 2020). With an increase in the concentration of astaxanthin, the V_{max} decreased significantly, and K_m decreased steadily (**Table 4.3)**, indicating that astaxanthin was predominantly a non-competitive inhibitor of sEH. This mixed inhibition mode is closely similar to that reported of dimethyl lithospermate isolated from the roots of *Lycopus lucidius* (Han et al., 2021). However, the significant noticeable change in the K_m values of the enzyme (**Table 4.2**) implies that it is a mixed inhibitor.

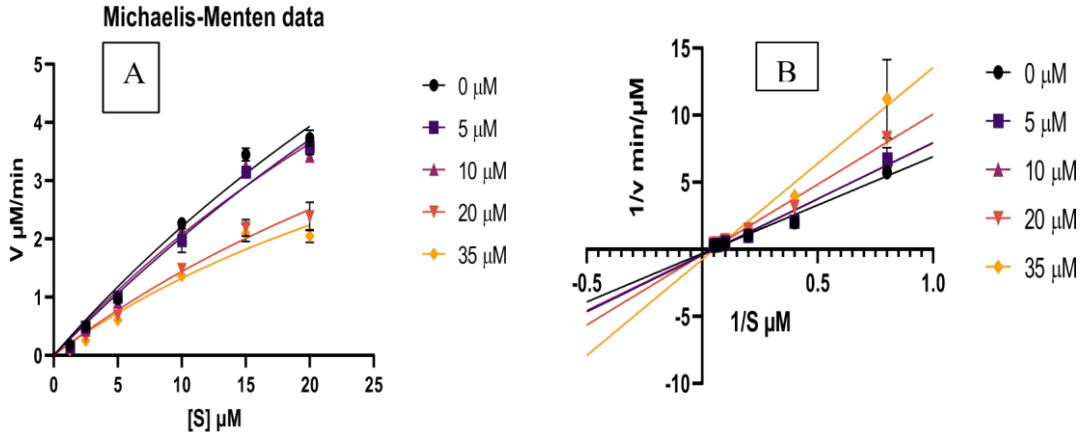


Figure 4.4a: Michaelis-Menten and Lineweaver-Burk double reciprocal Plot of sEH at different astaxanthin concentration

Figure 4.4a: (A) Michaelis-Menten and (B) Lineweaver-Burk double reciprocal Plot of sEH at different astaxanthin concentration

Table 4.3: Enzyme kinetics parameter of sEH in the absence and presence of astaxanthin

Astaxanthin (µM)	V_{max} (µM/min)	K_m (µM)
0	17.91 ± 0.28	71.09 ± 0.10
5	16.23 ± 0.49**	67.05 ± 0.18**
10	14.8 ± 0.33**	61.42 ± 0.12**
20	9.663 ± 0.33***	57.2 ± 0.12**
35	4.22 ± 1.05***	44.59 ± 0.39***

Km, Michaelis constant in the absence and presence of the inhibitor, Vmax, maximum reaction velocity in the absence and presence of the inhibitor, **, significant at $0.01 > p > 0.001$, ***, significant at $0.001 > p > 0.0001$ ****, significant at $p < 0.0001$ compared to control sEH (at 0 µM astaxanthin).

The plots of $[P]_\infty$ vs. astaxanthin showed an increase in $[P]_\infty$ as astaxanthin concentration increased, after which a decrease in $[P]_\infty$ was observed as more astaxanthin at higher concentrations of PHOME (15 & 20 µM) was added to the assay. This was significantly opposite at lower substrate concentrations (PHOME 2.5 – 10 µM), where no significant change was observed. This observation might be because most of the substrates formed have been converted to products resulting in similar $[P]_\infty$ across inhibitor concentrations. The lowest substrate concentration, 2.5 µM, had the highest slope in the presence and absence of an inhibitor, suggesting that the two ligands have different binding sites on soluble epoxide hydrolase, where substrates binding affects astaxanthin binding and vice versa, leading to a decrease in $[P]_\infty$ With an increase in astaxanthin concentration, a higher inhibition and progressive decrease in $[P]_\infty$ was observed. This confirms that astaxanthin may have inhibited soluble epoxide hydrolase affinity for the substrate, hence inhibiting its activity.

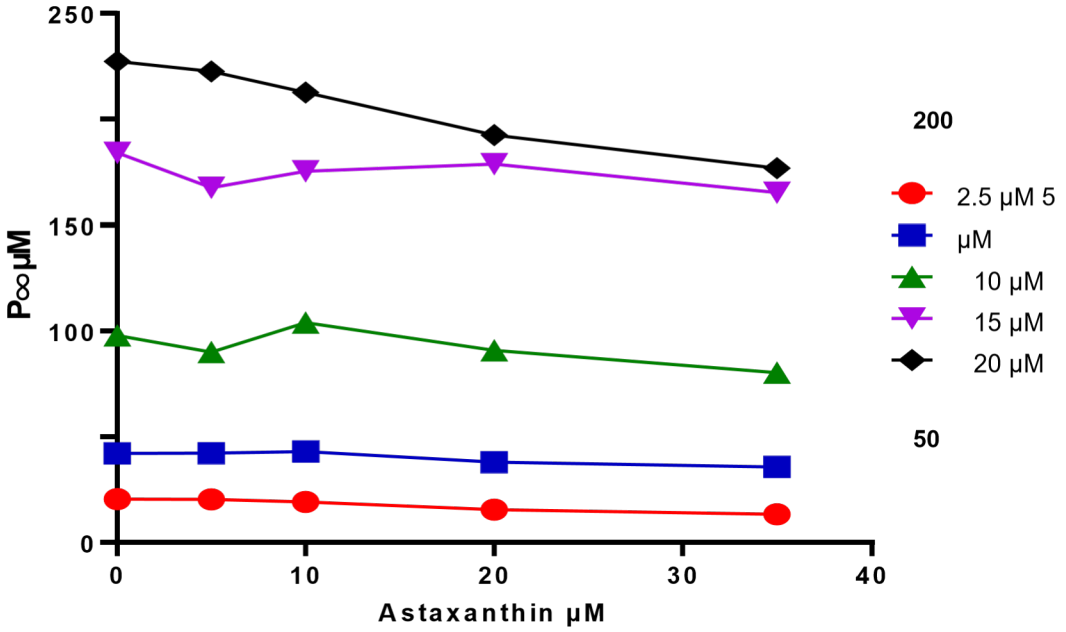


Figure 4.4b: Plots of $[P]_\infty$ at varying concentrations of astaxanthin with changing substrate concentration (PHOME 2.5 – 20µM).

4.5 Apparent Inactivation Rate Constant A and the Protective Effect of Substrate on Inactivation Constant

Plots of $\ln([P]_5 - [Pt])$ vs. time gave first-order kinetics with slopes that were dependent on inhibitor concentration and corresponding to *A* (**Figure 4.5**), the apparent inactivation rate constant. At the lowest concentration of astaxanthin (5µM), first-order kinetics with a negative slope was observed at a lower substrate concentration (< 10µM), while zero-order kinetics was observed at high (> 10µM) substrate concentration.

The protective effects a substrate may have on the enzyme are described by *A* (Chilaka & Nwamba, 2008). The plot derived from 1/*A* vs. [S] showed no protective effect by the substrate in the presence of astaxanthin, which is characterized by the non-linear pattern of the 1/A vs [S] graph (**Figure 4.6**). It also showed no change in *A* at substrate concentration below 10 µM followed by a gradual increase in A as substrate increased in the presence of astaxanthin at a concentration of 10 µM on the other hand, at a high concentration of astaxanthin (35 µM), an increase in *A* was observed below substrate 10 µM followed by a decrease in A (**Figure 4.6**). The absence of effect on *A* at low

substrate can be attributed to the lack of protective effect by the substrate making A independent of [S] at a low concentration of astaxanthin and not as much at 35 µM of astaxanthin. No protective effects of the substrate were also observed at the highest concentration of astaxanthin 35 µM. All of these findings indicate the absence of concentration-dependent effects of the inhibitor on the protective effects of the substrate.

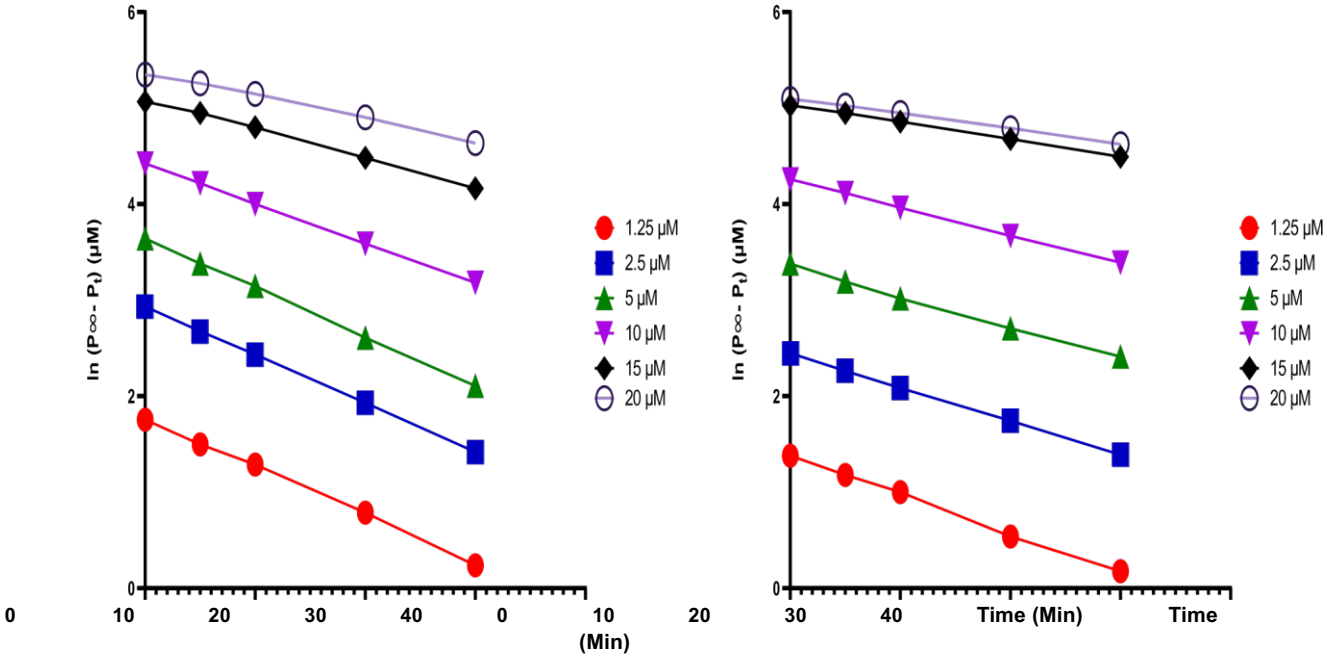


Figure 4.5: Plots of ln($[P]_5 - [Pt]$) vs. time to determine the apparent inactivation rate constant of soluble epoxide hydrolase in the presence of inhibitor (A) 5 µM Astaxanthin, (B) 35 µM Astaxanthin.

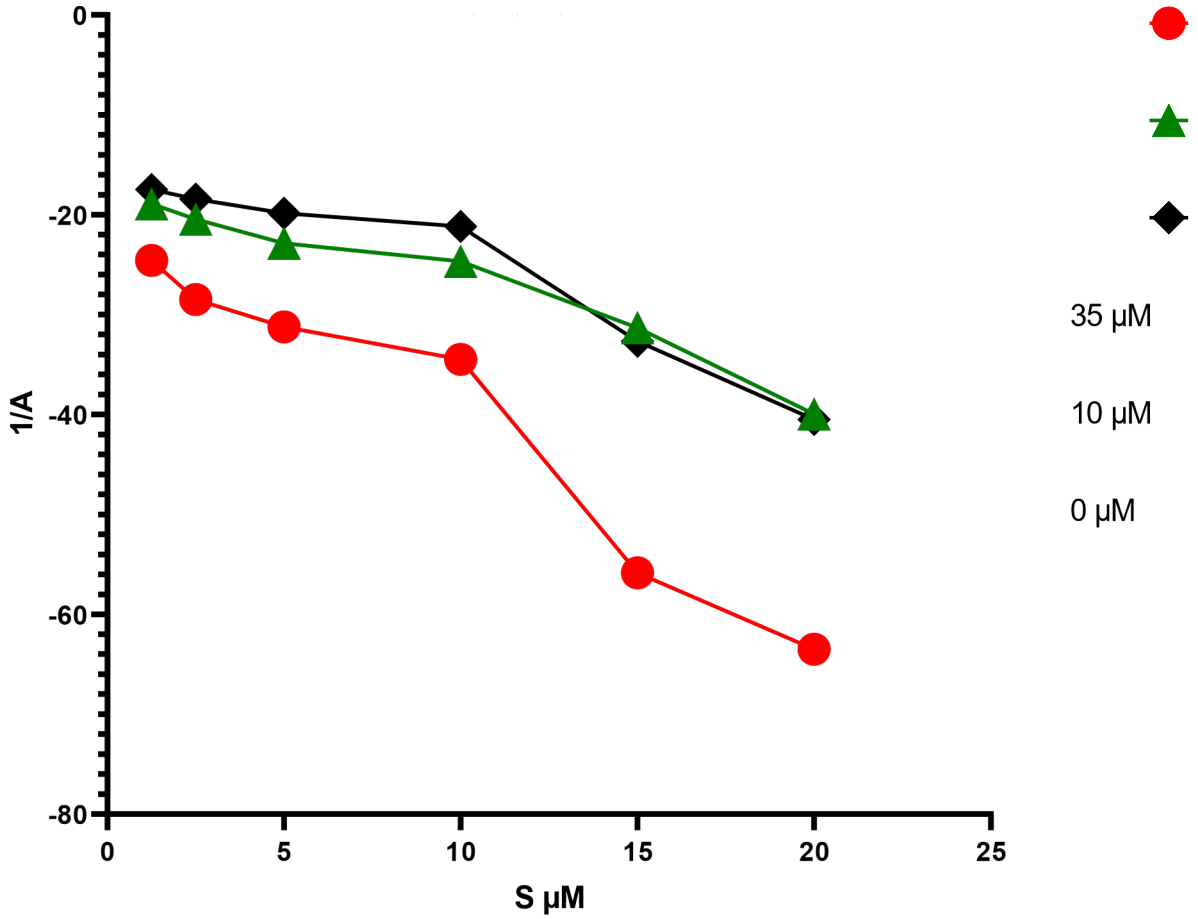


Figure 4.6: Plot of the reciprocal of apparent inactivation rate constant A vs. substrate concentration in the absence and presence of varying concentrations of astaxanthin (10 and 35 µM).

4.6 Analysis of Molecular Docking Studies

To further determine the inhibition mechanism of astaxanthin on soluble epoxide hydrolase, molecular docking studies were conducted. Active site and blind docking analysis were used to determine this inhibition parameter. The C-terminal hydrolase pocket in the sEH active site contains two tyrosine residues, Tyr381 and Tyr465, and a catalytic triad, Asp333-Asp495-His523, which is located directly opposite the hydrolase pocket. Epoxide hydrolysis requires the formation of an ester bond with the carboxylic acid of Asp333, which is a critical step. Since Asp333 is nucleophilic, it can accept a hydrogen atom from the inhibitor peptide's amino group (-NH) to form a hydrogen bond with its oxygen atom. Astaxanthin docking at the active site showed a clash and contact-only hydrophobic interaction with sEH at TRP525A, PHE497A, TYR466A and SER415A (**Figure 4.8b**). Other interactions between astaxanthin and side chains (HIS524A, MET503A, LEU499A, TYR383A, VAL498A, and MET419A), backbone amino acids (SER412A, LEU417A and LEU408A) and backbone side chain residues (ASP335A and TRP336A) were also observed with no presence of hydrogen bond. From the results, it was observed that astaxanthin in the presence of substrate PHOME could bind to an allosteric site of soluble epoxide hydrolase with binding affinity energy of -8.270 kcal/mol and the binding of astaxanthin to the enzyme had no effect on the substrate binding to the enzyme. (**Table 4.3 and Figure 4.8a & b**). Another observation from this analysis is that astaxanthin in the presence (ES, ESI complex) and absence (EI complex) of the substrate (PHOME) binds to an allosteric site of soluble epoxide hydrolase, thereby eliminating the possibility of a competitive inhibition model. The higher binding affinity of PHOME to sEH (-9.722 kcal/mol) indicates that PHOME competitively outweighs both astaxanthin and AUDA binding to sEH active site. Hence, the allosteric site binding of astaxanthin to sEH strengthens the predicted astaxanthin model of mixedtype (non-competitive and uncompetitive) inhibition. The blind docking of astaxanthin to sEH showed side-chain interactions at PRO83A, LEU30A, ALA29A, ARG25A, GLU90A and

VAL22A, and backbone sidechain residues (ASN85A, THR26A, ILE91A, LYS94A and ALA95A) with a binding affinity of -7.898 kcal/mol. Due to the presence of another binding site on sEH, it can be deduced that the substrate PHOME was bound more easily to the active site of soluble epoxide hydrolase, and astaxanthin was bound to another site of soluble epoxide hydrolase (allosteric site), corroborating the earlier observation of a mixed-type (non-competitive and uncompetitive) inhibition. **Figure 4.7** illustrates the chemical structure of AUDA.

Table 4.4: Molecular docking properties and binding energy score

SN	Ligands (Compounds)	sEH Active site docking binding affinity ($kcal.mol^{-1}$) and amino acid residues	sEH-PHOME complex Active site docking binding affinity ($kcal.mol^{-1}$) and amino acid residues	sEH Blind docking binding affinity ($kcal.mol^{-1}$) and amino acid residues	sEH-PHOME complex Blind docking binding affinity ($kcal.mol^{-1}$) and amino acid residues
1	Astaxanthin (Pubchem CID: 5281224)	**-9.035** H-Bond: None MET330, ASP335, TRP336, MET339, LEU408, SER415, MET419, TYR466, VAL498, PHE497, MET503, TRP525	-8.270 H-BOND: HIS146 (1.874 Å) HIS146, ARG103, PRO279, LEU110, LYS114, GLN107, ALA280	-7.898 H-Bond: None ALA29, THR26, VAL22, ILE91, LYS94	-6.515 H-BOND: None ARG133, LEU136, GLN155, GLU152, ILE151, PHE168, THR172, PHE149, PHE147.
2	AUDA (Pubchem CID: 10069117)	**-7.591** H-Bond: TYR343 (2.24 Å) TRP336, TYR343,	-6.595 H-BOND: TYR230 (1.975 Å) ARG103, GLN107,	-6.355 H-Bond: HIS146 (2.07 Å) ARG103, LEU106,	-6.027 H-Bond: ASN535 (1.927 Å) LEU106, ARG103,

		ILE363, PRO364, PRO371, SER374, TYR466, MET469, ASN472	LEU106, LEU110, THR230, PRO229, PRO227,	HIS146, THR230, PRO227, ASN535	LEU110, GLN107, THR230, PRO229, PRO227, ALA280, ASN535
3	PHOME (Pubchem CID: 53394628)	**-9.722** H-Bond: ASP335 (2.989 Å) MET339 (3.123 Å) TRP336, MET339, VAL380, PHE381, GLN384, TYR466, LEU499, HIS524	ND	-8.657 H-Bond: None ILE363, ASN366, PHE381, PRO371, GLN384, ILE375	ND

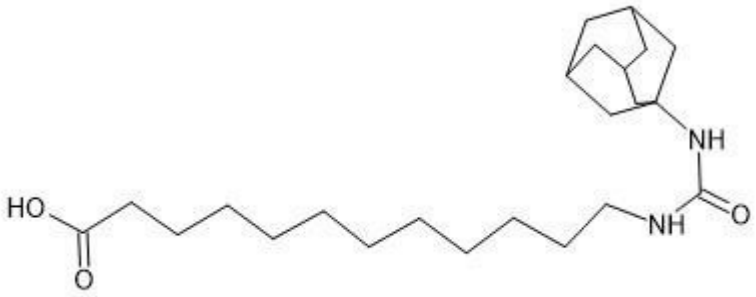


Figure 4.7: Chemical structure of 12-(3-(Adamantan-1-yl)ureido)dodecanoic acid (AUDA).

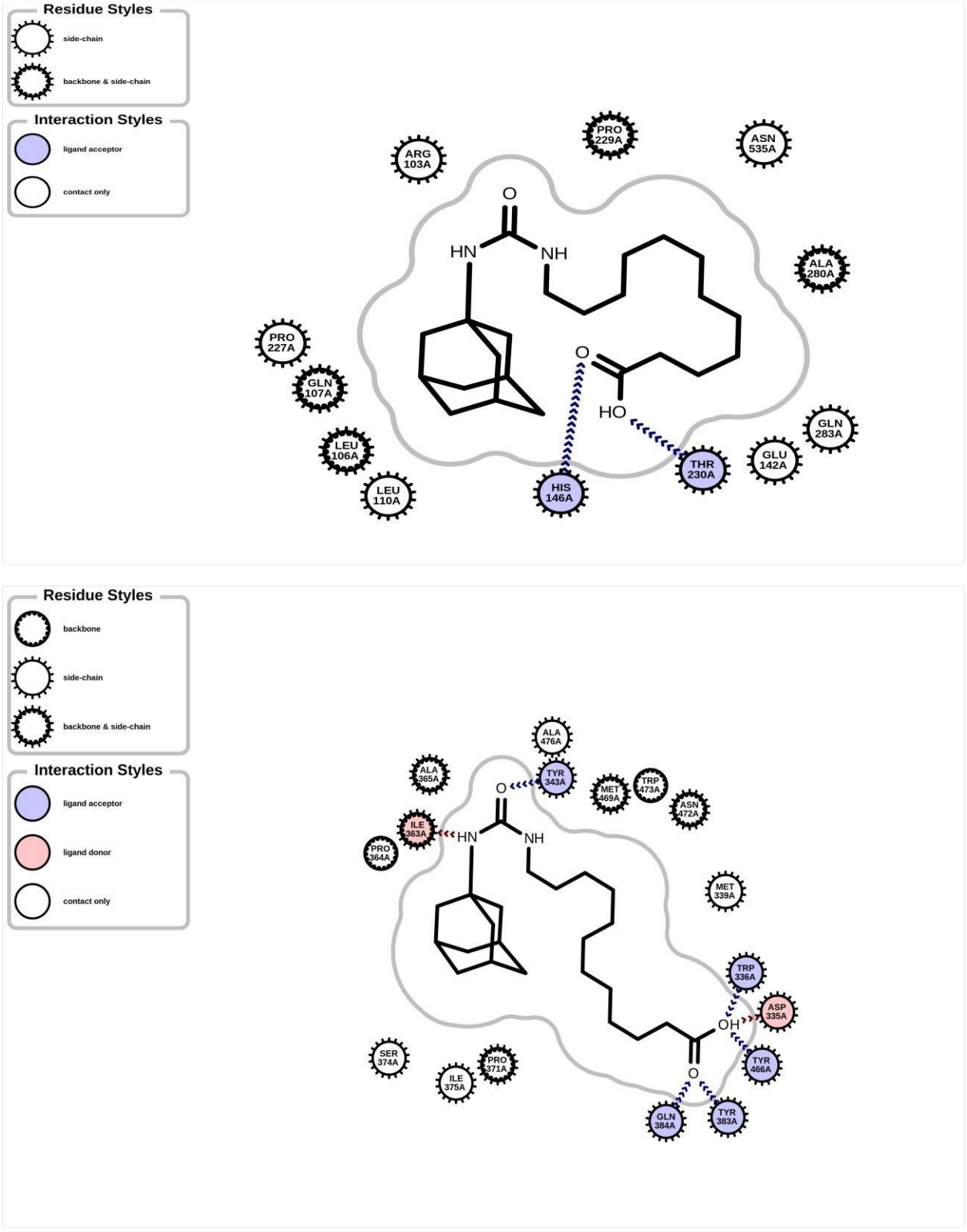
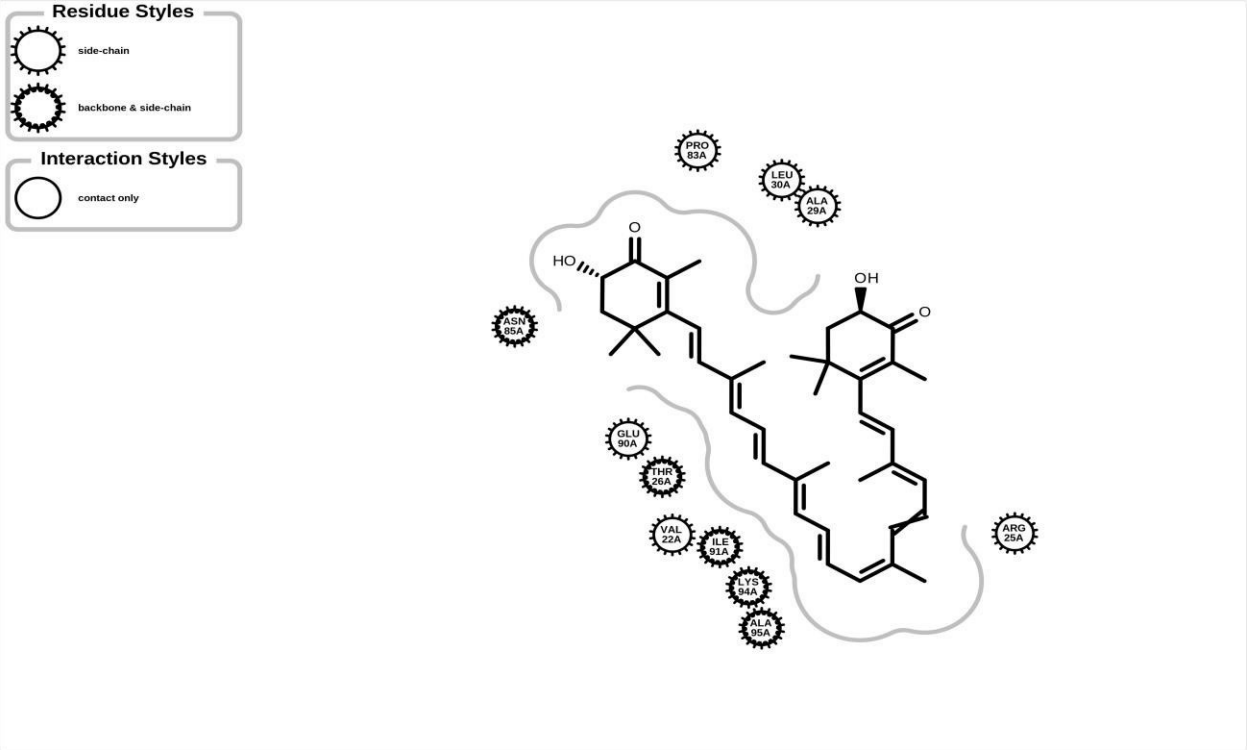


Figure 4.8a: Blind and Active docking results of AUDA to soluble epoxide hydrolase (sEH)



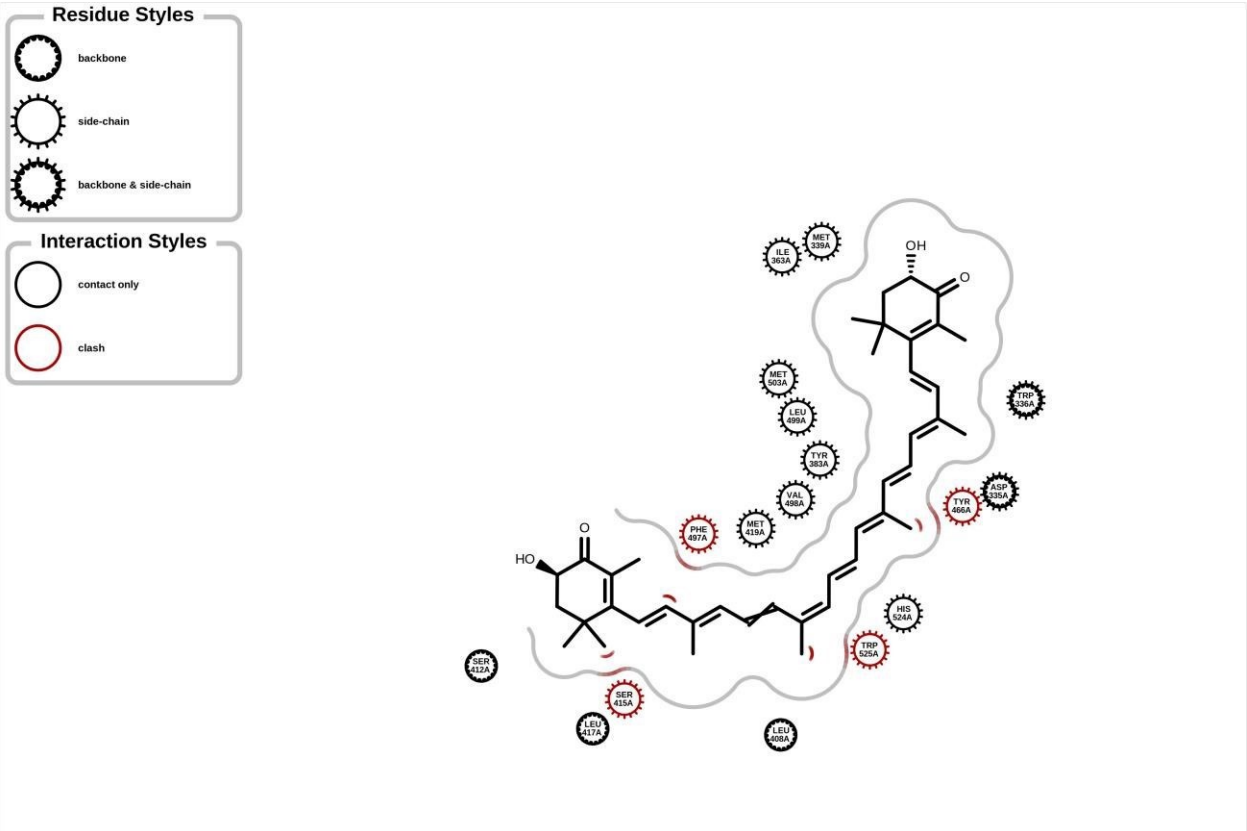
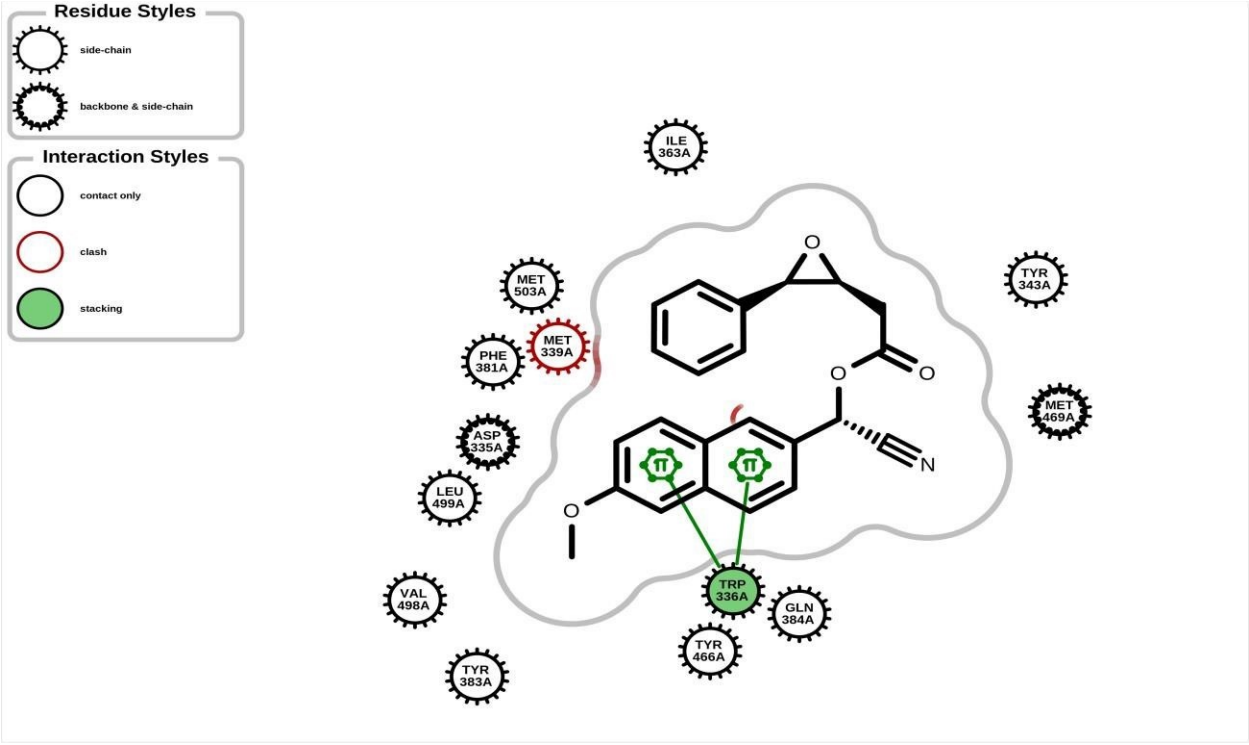


Figure 4.8b: Blind and active docking results of astaxanthin soluble epoxide hydrolase (sEH)



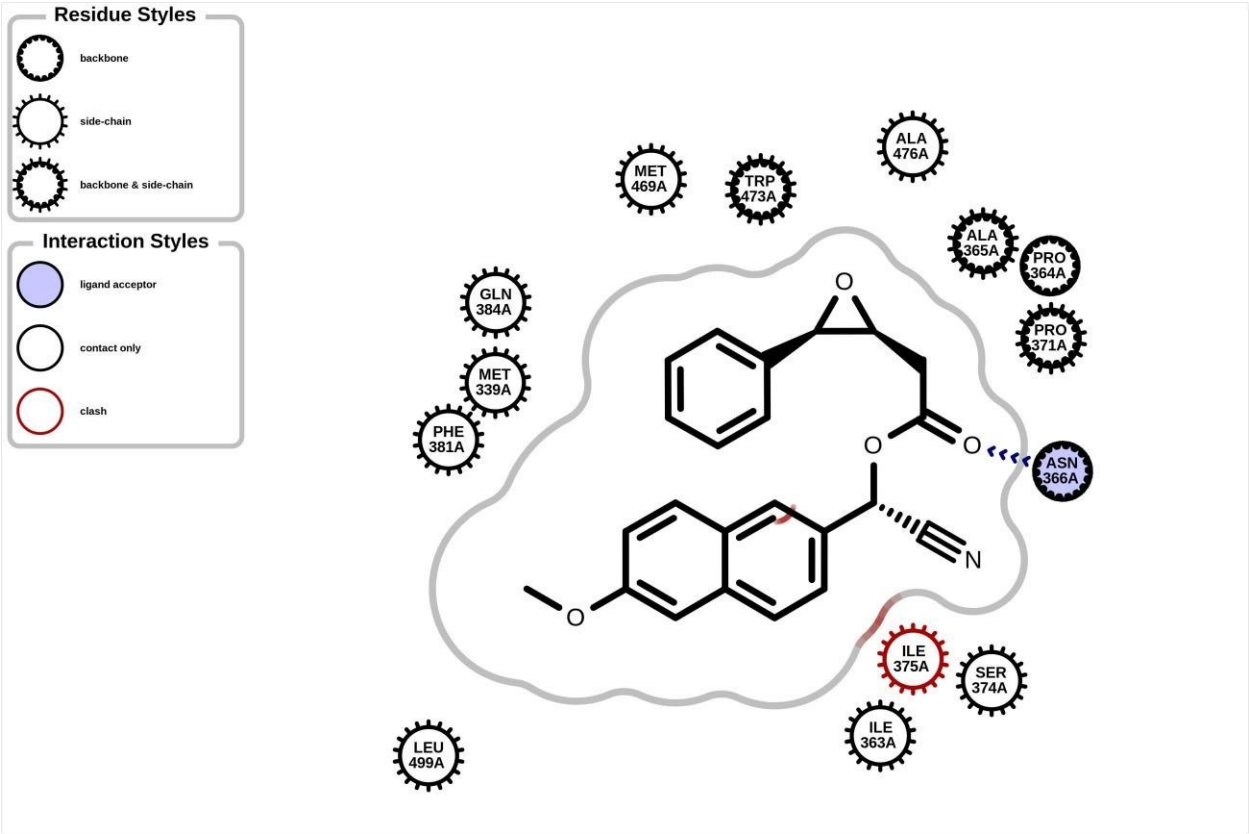


Figure 4.8c: Blind active docking of PHOME and astaxanthin to soluble epoxide hydrolase (sEH)

CHAPTER FIVE

CONCLUSION

CHAPTER FIVE

CONCLUSION & FUTURE DIRECTION

The sEH enzyme transforms the anti-inflammatory ω-3 PUFAs CYP-derived epoxy EpETEs and EpDPAs into their less active diols, DiHETEs and DiHDPAs. This indicates that decreasing the bioavailability of EpETEs and EpDPAs by activating the sEH enzyme can impair their biological activity, eventually resulting in depressive symptoms. These findings imply that sEH plays a significant role in the pathophysiology of depression and that naturally occurring compound inhibitors of sEH may be an alternative treatment option for people who experience this illness. In conclusion, this study investigated the potential of astaxanthin, a naturally occurring carotenoid, in inhibiting soluble epoxide hydrolase (sEH). Astaxanthin was identified as a mixed-type (noncompetitive and uncompetitive) inhibitor of sEH with an IC_{50} value of 26 ± 0.45 μM. Astaxanthin demonstrated no potential adverse side effects or acute toxicity when investigated computationally for its adsorption, distribution, metabolism, excretion, and toxicity profile. The findings revealed that astaxanthin bonded to the allosteric site of soluble epoxide hydrolase both in the presence and absence of the substrate PHOME, where the substrate binding does not affect the binding of the inhibitor, and vice versa and inhibited the activity of the enzyme as well as the affinity of the enzyme for the substrate PHOME. Enzyme kinetics study showed noticeable decreases in V_{max} and $K_{m,}$ which indicate that astaxanthin affected the binding of the enzyme sEH to the substrate PHOME via a mixed-type (non-competitive & uncompetitive) mechanism of inhibition, a high K_m value suggested that the enzyme sEH needs a high concentration of PHOME to be saturated. The findings from the protective effect of substrate on the enzyme (*A) indicate* that *A* was independent of the substrate [S] and the absence of concentration-dependent effects of the inhibitor on the protective effects of the substrate. An increase in astaxanthin concentration led to

a higher inhibition and a progressive decrease in $[P]_{\infty}$, confirming that astaxanthin may have inhibited soluble epoxide hydrolase activity as well as the enzyme's affinity for the substrate. The increase in the apparent rate of inactivation constant also suggests that astaxanthin inhibited the enzyme non-competitively and stopped the reaction from being catalyzed/moving forward. The predictions from ADME indicate that some pharmacokinetics and drug-likeness properties of astaxanthin are considerably better than those of AUDA. Although astaxanthin is a large molecule, which prevented its interaction with the ASP333A or other relevant residues in the hydrolytic pocket of sEH. Previous research has shown that astaxanthin is easily absorbable by the GIT and can cross the blood-brain barrier (BBB), a major requirement/consideration for my study.

Thus, the data from this study provide important information on the inhibition activity and mechanism of astaxanthin, a naturally occurring compound on sEH activity towards future application as a nutraceutical for managing and treating inflammatory diseases, particularly major depressive disorder.

FUTURE DIRECTION

Further research is required to determine the effect of astaxanthin on the secondary structure of sEH to better understand the structural mechanism of inhibition. One of this initial study objectives was to investigate astaxanthin's neurophysiological effects in suppressing depressive-like inflammatory responses in cultured brain cells. This future study will provide evidence of the ability of astaxanthin to repress depressive-like inflammatory responses in brain cells prior to in vivo studies. It is expected that the physiological effects of astaxanthin or other sEH-inhibiting bioavailable and biostable nutraceuticals will facilitate their use as a therapy for depression, thereby reducing the complications associated with the treatment/management of depression by synthetic inhibitors.

REFERENCES

Ambati, R, Phang, S, M, Ravi, S & Aswathanarayana, R, G **2014**, Astaxanthin: Sources, extraction, stability, biological activities, and its commercial applications—A review. *Marine Drugs*, *12*, 128–152.

Anita, N, Z, Forkan, N, Kamal, R, Nguyen, M, Yu, D, Major-Orfao, C, Wong, S, K, Lanctôt, K, L, Herrmann, N, Oh, P, I, Shah, B, R, Gilbert, J, Assal, A, Halperin, I J, Pedersen, T, L, Taha, A, Y & Swardfager, W **2021**, Serum soluble epoxide hydrolase related oxylipins and major depression in patients with type 2 diabetes. *Psychoneuroendocrinology*, *126*, 105149.

Balietti, M, Giannubilo, S, R & Giorgetti, B **2016**, "The effect of astaxanthin on the aging rat brain: gender-related differences in modulating inflammation," *Journal of the Science of Food and Agriculture*, vol. 96, no. 2, pp. 615–618

Barredo J.-L **2012**, "Microbial carotenoids from Bacteria and microalgae," Methods and Protocols, *Methods in Molecular Biology*, vol. 892.

Borsini, A **2021**, The role of soluble epoxide hydrolase and its inhibitors in depression, *Brain, behaviour, & immunity - health*, *16*, 100325.

Borsini, A, Zunszain, P, A, Thuret, S & Pariante, C, M **2015**, The role of inflammatory cytokines as key modulators of neurogenesis, *Trends Neuroscience*, 38, 145–157.

Bourassa, P, Bariyanga, J & Tajmir-Riahi, H, A **2013**, Binding sites of resveratrol, genistein, and curcumin with milk and caseins, *Journal of Physical Chemistry B*, *117*(5), 1287– 1295, doi.org/10.1021/jp3114557.

Brown, G, C & Neher, J, J **2010**, Inflammatory neurodegeneration, and mechanisms of microglial killing of neurons, *Molecular Neurobiology*, 41, 242–247.

Burmistrov, V, V, Karlov, D, S, Butov, G, M & Novakov, I, A **2021**, Prospects for the inhibition of the phosphatase domain of human soluble epoxide hydrolase (sEHP). *Russian Chemical Bulletin*, *70*(6), 1067-1074.

Galasso, C, Corinaldesi, C & Sansone, C **2017**, "Carotenoids from Marine Organisms: Biological

Functions and Industrial Applications," *Antioxidants*, vol. 6, no. 4, p. 96.

Calo, P, T, Miguelde, C, Sieiro, J, B, Velasquez & Vilia, T, G **1995**, "Ketocarotenoids in halobacteria: 3-hydroxy- echinenone and trans-astaxanthin," *Journal of Applied Bacteriology*, vol. 79, no. 3, pp. 282–285.

Caramujo, M, J, Carla, C, C, R. De Carvalho, Soraya, J, S, & Kevin, R, C **2012**, "Dietary carotenoids regulate astaxanthin content of copepods and modulate their susceptibility to UV light and copper toxicity," *Marine Drugs*, vol. 10, no. 12, pp. 998–1018.

Chang, L, H, Lin, H, C, Huang, S, S, Chen, I, C, Chu, K, W, Chih, C, L, Liang, Y, W, Lee, Y, C, Chen, Y, Y, Lee, Y, H & Lee, I, H **2018**, Blockade of soluble epoxide hydrolase attenuates post-ischemic neuronal hyperexcitation and confers resilience against stroke with TrkB activation. *Scientific reports*, *8*(1), 118.

Charest, D, J, Balaban, M, O, Marshall, M, R & Cornell, J, A **2001**, "Astaxanthin extraction from crawfish shells by super-critical CO2 with ethanol as Cosolvent," *Journal of Aquatic Food Product Technology*, vol. 10, no. 3, pp. 81–96.

Chilaka, F, C & Nwamba, C, O **2008**, Kinetic analysis of urea-inactivation of beta-galactosidase in the presence of galactose, *Journal of Enzyme Inhibition Medicinal Chemistry*, 23(1):7-15. doi: 10.1080/14756360701450244. PMID: 18341246.

Chien, Y, H & Shiau, W, C **2005**, "The effects of dietary supplementation of algae and synthetic astaxanthin on body astaxanthin, survival, growth, and low dissolved oxygen stress resistance of kuruma prawn, Marsupenaeus japonicus Bate," *Journal of Experimental Marine Biology and Ecology*, vol. 318, no. 2, pp. 201–211.

Daina, A, Michielin, O & Zoete, V **2017**, SwissADME: A free web tool to evaluate pharmacokinetics, drug-likeness and medicinal chemistry friendliness of small molecules, *Scientific Report*, 7, 42717.

Dean, B, Tawadros, N, Scarr, E & Gibbons, A, S, **2010**, Regionally specific changes in levels of tumour necrosis factor in the dorsolateral prefrontal cortex obtained post-mortem from subjects with major depressive disorder, *Journal of Affective Disorder*, 120, 245–248.

Debin, W, Yang J, McReynolds, C, B, Barnych, B, W, Karen M, Morisseau, C, Hwang, S, H, Sun Jia, Blöcher, R & Hammock, B, D **2019**, In vitro and in vivo Metabolism of a Potent Inhibitor of Soluble Epoxide Hydrolase, 1-(1- Propionylpiperidin-4yl)-3-4(trifluoromethoxy)phenyl) urea *Frontiers in Pharmacology*, 10:1663-9812.

Domingues, M, F, Callai-Silva, N, Piovesan, A, R & Carlini, C, R **2020**, Soluble Epoxide Hydrolase and Brain Cholesterol Metabolism, *Frontiers in molecular neuroscience*, *12*, 325.

Domínguez-Bocanegra, A, R, Guerrero, L, I, Martinez, J, F & Tomasini, A, C **2004**, "Influence of environmental and nutritional factors in the production of astaxanthin from Haematococcus pluvialis," *Bioresource Technology*, vol. 92, pp. 209–214.

Eberhardt, J, Santos-Martins, D, Tillack, A, F & Forli, S **2021**, AutoDock Vina 1.2.0: New Docking Methods, Expanded Force Field, and Python Bindings, *Journal of Chemical Information and Modelling*, doi.org/10.1021/acs.jcim.1c00203.

Fatoki, T, Chukwuejim, S, Ibraheem, O, Oke, C, Ejimadu, B, Olaoye I, Oyegbenro, O, Salami, T, Basorun, R, Oluwadare, O & Salawudeen, Y **2022**, Harmine and 7,8-dihydroxyflavone synergistically suitable for amyotrophic lateral sclerosis management: An *in silico* study, *Research Results in Pharmacology*, 8(3):49-61, DOI 10.3897/rrpharmacology.8.83332.

Gabbs, M, Leng, S, Devassy, J, G, Monirujjaman, M & Aukema, H, M **2015**, Advances in our understanding of oxylipins derived from dietary PUFAs, *Advanced Nutrition* 6, 513–540.

Hammock, B, D **2007**, Design of bioavailable derivatives of 12-(3-adamantan-1-yl-ureido)dodecanoic acid, a potent inhibitor of the soluble epoxide hydrolase, *Bioorganic and Medicinal Chemistry*, 15:312–23.

Hammock, B, D, McReynolds, C, B, Wagner, K, Buckpitt, A, Cortes-Puch, I, Croston, G, Lee, K, Yang, J, Schmidt, W, K & Hwang, S, H **2021**, Movement to the Clinic of Soluble Epoxide Hydrolase Inhibitor EC5026 as an Analgesic for Neuropathic Pain and for Use as a Nonaddictive Opioid Alternative, *Journal of Medicinal Chemistry*, *64*(4), 1856–1872.

Han, Y, K, Lee, J, S, Yang, S, Y, Lee, K, Y & Kim, Y, H **2021**, In Vitro and In Silico Studies of Soluble Epoxide Hydrolase Inhibitors from the Roots of *Lycopus lucidus*. *Plants 10*, 356.

Hashimoto, K **2016**, Soluble epoxide hydrolase: A new therapeutic target for depression. *Expert Opinion on Therapeutic Targets*, 20(10), 1149-1151.

Hashimoto, K **2019a**, Role of soluble epoxide hydrolase in the metabolism of PUFAs in psychiatric and neurological disorders *Frontiers in Pharmacology*, 10, 36.

Hashimoto, K **2019b**, Recent advances in the early intervention in schizophrenia: future direction from preclinical findings, *Current Psychiatry*, Rep. 21, 75. https://doi.org/10. 1007/s11920-019-1063-7.

He, X, Zhao, W, Y, Shao, B, Zhang, B, J, Liu, T, T, Sun, C, P, Huang, H, L, Wu, J, R, Liang, J, H & Ma, X, C **2020**, Natural soluble epoxide hydrolase inhibitors from Inula helenium and their interactions with soluble epoxide hydrolase, *International Journal of Biological Macromolecules*, S0141-8130(20)33090-7.

Hennebelle, M, Otoki, Y, Yang, J, Hammock, B, D, Levitt, A, J, Taha, A, Y & Swardfager, W **2017**, Altered soluble epoxide hydrolase-derived oxylipins in patients with seasonal major depression: An exploratory study. *Psychiatry Research*, pp. *252*, 94–101.

Higuera-Ciapara, I, Félix-Valenzuela, L & Goycoolea, F, M **2006**, "Astaxanthin: a review of its \ chemistry and applications," *Critical Reviews in Food Science and Nutrition*, vol. 46, no. 2, pp. 185–196, 2006.

Hildreth, K, Kodani, S, D, Hammock, B, D & Zhao, L **2020**, Cytochrome P450-derived linoleic acid metabolites EpOMEs and DiHOMEs: a review of recent studies. *The Journal of Nutritional Biochemistry*, p. *86*, 108484.

Hou, H, H, Liao, Y, J, Hsiao, S, H, Shyue, S, K & Lee, T, S **2015**, Role of phosphatase activity of soluble epoxide hydrolase in regulating simvastatin-activated endothelial nitric oxide synthase, *Scientific Reports*, p. *5*, 13524.

Hu, J, Dziumbla, S, Lin, J, Bibli, S, I, Zukunft, S, de Mos, J, Awwad, K, Frömel, T, Jungmann, A, Devraj, K & Cheng, Z, W **2017**, Inhibition of soluble epoxide hydrolase prevents diabetic retinopathy. *Nature* 552, 248–252, https://doi.org/10.1038/nature25013.

Hussein, G, Nakamura, M, Zhao, Q, Iguchi, T, Goto, H, Sankawa, U & Watanabe, H **2005**, Antihypertensive and neuroprotective effects of astaxanthin in experimental animals. *Biological and Pharmaceutical Bulletin*, *28*, 47–52.

Hwang, S, H, Tsai, H, J, Liu, J, Y, Morisseau, C & Hammock, B, D **2007**, Orally bioavailable, potent soluble epoxide hydrolase inhibitors, *Journal of medicinal chemistry* 50(16), 3825.

Imig, J, D, Zhao, X, Capdevila, J, H, Morisseau, C & Hammock, B, D **2002**, Soluble epoxide hydrolase inhibition lowers arterial blood pressure in angiotensin II hypertension. *Hypertension*, 39(2), 690–694.

Institute of Health Metrics and Evaluation. Global Health Data Exchange (GHDx), **2021.**

Ishihara, T, Yoshida, M & Arita, M, **2019**, Omega-3 fatty acid-derived mediators that control inflammation and tissue homeostasis, *International Immunology*,31, 559–567.

Jamieson, K, L **2020**, Cardioprotective Effects of Soluble Epoxide Hydrolase Inhibition Following Myocardial Infarction, *Faculty of Pharmacy and Pharmaceutical Sciences University of Alberta.*

Ji, D, Xu, M, Udenigwe C, C & Agyei, D **2020**, Physicochemical characterization, molecular docking, and drug-likeness evaluation of hypotensive peptides encrypted in flaxseed proteome, *Current Research in Food Science*, 3: 41-50.

Jiang, X, S, Xiang, X, Y, Chen, X, M, He, J, L, Liu, T, Gan, H & Du, X, G **2020**, Inhibition of soluble epoxide hydrolase attenuates renal tubular mitochondrial dysfunction and ER stress by restoring autophagic flux in diabetic nephropathy, *Cell death & disease*, *11*(5), 385.

Jo, A, R, Kim, J, H, Yan, X, T, Yang, S, Y & Kim, Y, H **2016**, Soluble epoxide hydrolase inhibitory components from Rheum undulatum and in silico approach, *Journal of Enzyme Inhibition Medicinal Chemistry*, 31(2), 70-78.

Jung, O, Brandes, R, P, Kim, I, H, Schweda, F, Schmidt, R, Hammock, B, D & Fleming, I **2005**, Soluble epoxide hydrolase is a main effector of angiotensin II–induced hypertension, *Hypertension*, 45(4), 759–765.

Kanakis, C, D, Tarantilis, P, A, Polissiou, M, G & Tajmir-Riahi, H, A **2013**, Probing the binding sites of resveratrol, genistein, and curcumin with milk lactoglobulin, *Journal of Biomolecular Structure and Dynamics*, *31*(12), 1455–1466.

Kessler, R, C & Bromet, E, J **2013**, The epidemiology of depression across cultures, *Annual Review of Public Health*, 34, 119–138.

Kessler, R, C, Berglund, P, Demler, O, Jin, R, Merikangas, K, R & Ellen, E, W **2005**, Lifetime prevalence and age-of-onset distributions of DSM-IV disorders in the national comorbidity survey replication, *Archives of General Psychiatry*, 62(6), 593.

Kessler, R, C, Chiu, W, T, Demler, O & Ellen, E, W **2005**, Prevalence, severity, and comorbidity of 12-month DSM-IV disorders in the national comorbidity survey replication, *Archives of General Psychiatry*, 62(6), 617.

Kidd, P **2011**, Astaxanthin, cell membrane nutrient with diverse clinical benefits and anti-aging Potential, *Alternative Medicine Review*, *16*, 355–364.

Kim, I, H, Morisseau, C, Watanabe, T & Hammock, B, D **2004**, Design, synthesis, and biological activity of 1,3-disubstituted urea as potent inhibitors of the soluble epoxide hydrolase of increased water solubility, *Journal of Medicinal Chemistry*, 47:2110–22

Kohler, C, A, Freitas, T, H, Maes, M, de Andrade, N, Q, Liu, C, S, Fernandes, B, S, Stubbs, B,

Solmi, M, Veronese, N, Herrmann, N, Raison, C, L, Miller, B, J, Lanctot, K, L & Carvalho, A, F **2017**, Peripheral cytokine and chemokine alterations in depression: a meta-analysis of 82 studies, *Acta Psychiatrica Scandinavica*, 135, 373–387.

Kusdiyantini, E, P, Gaudin, G, G & Blanc, P, J **1998**, "Growth kinetics and astaxanthin production of Phaffia rhodozyma on glycerol as a carbon source during batch fermentation," *Biotechnology Letters*, vol. 20, no. 10, pp. 929–934.

Lee, Y, K & Soh, C, W **1991**, "Accumulation of Astaxanthin in Haematococcus lacustris (Chlorophyta)," *Journal of Phycology*, vol. 27, no. 5, pp. 575–577.

Lee, D, H, Lee, Y, J & Kwon, K, H **2010**, Neuroprotective Effects of Astaxanthin in Oxygen-Glucose Deprivation in SH-SY5Y Cells and Global Cerebral Ischemia in Rat, *Journal of Clinical Biochemistry Nutrition*, 47, 121–129.

Lim, K, C, Yusoff, F, M, Shariff, M & Kamarudin, M, S **2018**, "Astaxanthin as feed supplement in aquatic animals," *Reviews in Aquaculture*, vol. 10, pp. 738–773.

Liu, X, Testa, B &Fahr, A **2011**, Lipophilicity and its Relationship with Passive Drug Permeation, *Pharmaceutical Research*, 28,962-977.

ŁOtocka, M, E, Styczyńska-Jurewicz, & BŁędzki, L, A **2004**, "Changes in carotenoid composition in different developmental stages of copepods: Pseudocalanus acuspes Giesbrecht and Acartia spp," *Journal of Plankton Research*, vol. 26, no. 2, pp. 159–166.

Lotrich, F, E, El-Gabalawy, H, Guenther, L, C, & Ware, C, F **2011**, The role of inflammation in the pathophysiology of depression: different treatments and their effects, *The Journal of Rheumatology*, 88, 48–54.

Lucas, S, M, Rothwell, N, J & Gibson, R, M **2006**, The role of inflammation in CNS injury and disease, *British Journal of Pharmacology*, *147*, S232–S240.

Luo, J, Hu, S, Fu, M, Luo, L, Li, Y, Li, W & Xu, X **2021**, Inhibition of soluble epoxide hydrolase alleviates insulin resistance and hypertension via downregulation of SGLT2 in the mouse kidney, *Journal of Biological Chemistry*, *296*.

Ma, L, Zhao, H, Yu, M, Wen, Y, Zhao, T, Yan, M, Liu, Q, Jiang, Y, Cao, Y, Li, P & Niu, W **2020**, Association of Epoxide Hydrolase 2 Gene Arg287Gln with the Risk for Primary Hypertension in Chinese, *International Journal of Hypertension*, 2351547.

Marcus, M, Yasamy, M, T, van Ommeren, M, V, Chisholm, D & Saxena, S **2012**, Depression: A global public health concern.

Mbarik, M, Poirier, S, J, Doiron, J, Selka, A, Barnett, D, A, Cormier, M, Touaibia, M & Surette, M, E **2019**, Phenolic acid phenethylesters and their corresponding ketones: inhibition of 5lipoxygenase and stability in human blood and HepaRG cells, *Pharmacology Research & Perspective,* 7: 1-12.

Miller, A, H & Raison, C, L **2016**, The role of inflammation in depression: from evolutionary imperative to modern treatment target, *Nature Reviews Immunology*, *16*(1), 22–34.

Miura, Y, K, Kondo, T, Saito, H, Shimada, P, Fraser, D & Misawa, N **1998**, "Production of the carotenoids lycopene, β-caro- tene, and astaxanthin in the food yeast Candida utilis," *Applied and Environmental Microbiology*, vol. 64, no. 4, pp. 1226–1229.

Morisseau, C, Goodrow, M, H, Newman, J, W, Wheelock, C, E, Dowdy, D, L & Hammock, B, D **2002**, Structural refinement of inhibitors of urea-based soluble epoxide hydrolases, *Biochemical Pharmacology* 63:1599–608.

Morisseau, C, Du, G, Newman, J, W & Hammock, B, D **1998**, Mechanism of mammalian soluble epoxide hydrolase inhibition by chalcone oxide derivatives, *Archives of Biochemistry and Biophysics*, 356: 214–228.

Morisseau, C & Hammock, B, D **2007**, Measurement of Soluble Epoxide Hydrolase (SEH) Activity, *Current Protocols in Toxicology*, 33, 4.23.1−4.23.18.

Morris, G, M, Huey, R, Lindstrom, W, Sanner, M, F, Belew, R, K, Goodsell, D, S & Olson, A, J **2009**, AutoDock4 and AutoDockTools4: automated docking with selective receptor flexibility, *Journal of Computational Chemistry* 30(16):2785–2791.

Nakajima, Y, Inokuchi, Y, Shimazawa, M, Otsubo, K, Ishibashi, T & Hara, H **2008**, Astaxanthin, a dietary carotenoid, protects retinal cells against oxidative stress *in vitro* and in mice *in vivo*, *Journal of Pharmacy and Pharmacology, 60*, 1365–1374.

Ohgami, K, Shiratori, K, Kotake, S, Nishida, T, Mizuki, N, Yazawa, K & Ohno, S **2003**, Effects of astaxanthin on lipopolysaccharide-induced inflammation *in vitro* and *in vivo*, *Investigative Ophthalmology and Visual Science*, 44, 2694–2701.

Okagu, O, D, Jin, J & Udenigwe, C, C **2021**, Impact of succinylation on pea protein-curcumin interaction, polyelectrolyte complexation with chitosan, and gastrointestinal release of curcumin in loaded-biopolymer nano-complexes, *Journal of Molecular Liquids*, *325*.

Okagu, O. D, Verma, O, McClements, D, J & Udenigwe, C, C **2020**, Utilization of insect proteins to formulate nutraceutical delivery systems: Encapsulation and release of curcumin using mealworm protein-chitosan nano-complexes, *International Journal of Biological Macromolecules*, *151*, 333–343.

Orosa, M, Valero, J, F, Herrero, C & Abalde, J **2001**, "Comparison of the accumulation of astaxanthin in Haematococcus pluvialis and other green microalgae under N-starvation and high light conditions," *Biotechnology Letters*, vol. 23, no. 13, pp. 1079–1085.

Papiashvili, N, Gongadze, N, Bakuridze, A & Bakuridze, K **2021**, Antihypertensive And Cardioprotective Effects of Epoxyeicosatrienoic Acid Analogs and Soluble Epoxide Hydrolase Inhibitors, *Georgian Medical News*, (312), 125-132.

Parisenti, J, Beirão, L, H & Maraschin, M **2011**, "Pigmentation and carotenoid content of shrimp fed with Haematococcus pluvialis and soy lecithin," *Aquaculture Nutrition*, vol. 17, no. 2, pp. e530–e535.

Park, J, S, Chyun, J, H, Kim, Y, K, Line, L, L & Chew, B, P **2010**, Astaxanthin decreased oxidative stress and inflammation and enhanced immune response in humans, *Nutrition and Metabolism, 7,*18.

Rao, A, R, Reddy, R, L, R, Baskaran, V, Sarada, R & Ravishankar, G, A **2010**, Characterization of microalgal carotenoids by mass spectrometry and their bioavailability and antioxidant properties elucidated in a rat model, *Journal of Agriculture and Food Chemistry*, *58*, 8553–8559.

Regnier, P, Bastias, J, Rodriguez-Ruiz, V, Caballero-Casero, N, Caballo, C, Sicilia, D, Fuentes, A, Maire, M, Crepin, M & Letourneur, D **2015**, Astaxanthin from Haematococcus Pluvialis Prevents Oxidative Stress on Human Endothelial Cells without Toxicity, *Marine Drug*, *13*, 2857–2874.

Ren, Q **2019**, Soluble Epoxide hydrolase inhibitor: A Novel potential therapeutic or prophylactic drug for psychiatric disorders, *Frontiers in pharmacology*, 10:420.

Ren, Q, Ma, M, Ishima, T, Morisseau, C, Yang, J, Wagner, K, M, Zhang, J, C, Yang, C, Yao, W, Dong, C, Han, M, Hammock, B, D & Hashimoto, K **2016**, Gene deficiency and pharmacological inhibition of soluble epoxide hydrolase confers resilience to repeated social defeat stress, *Proceedings of the National Academy of Sciences of the United States of America*, *113*(13), E1944–E1952.

Schiøtt, B, & Bruice, T, C., **2002**, Reaction mechanism of soluble epoxide hydrolase: insights from molecular dynamics simulations, *Journal of the American Chemical Society*, 124, 49, 14558-14570.

Shah, S, A, Mehmood, M, H, Khan, M, Bukhari, I, A, Alorainey, B, I & Vohra, F **2020**, Inhibition of soluble epoxide hydrolase offers protection against fructose-induced diabetes and related metabolic complications in rats, *Journal of Physiology and Pharmacology*, *71*(5), 615.

Shen, H, C & Hammock, B, D **2012**, Discovery of inhibitors of soluble epoxide hydrolase: a target with multiple potential therapeutic indications, *Journal of Medicinal Chemistry*, 8;55(5):1789-808.

Shen, H, Kuo, C, C, Chou, J, Delvolve, A, Jackson, S, N, Post, J, Woods, A, S, Hoffer, B, J, Wang, Y & Harvey, B, K **2009**, Astaxanthin reduces ischemic brain injury in adult rats, *The FASEB Journal, 23*, 1958–1968.

Si, P & Zhu S **2022**, Biological and neurological activities of astaxanthin (Review), *Molecular Medicine Reports*, 26, 300.

Speranza, L, Pesce, M, Patruno, A, Franceschelli, S, de Lutiis, M, A, Grilli, A & Felaco, M **2012**, Astaxanthin treatment reduced oxidative-induced pro-inflammatory cytokines secretion in U937: SHP-1 as a novel biological target, *Marine Drugs, 10*, 890–899.

Stewart, J, S, Lignell, A, Pettersson, A, Elfving, E & Soni, M, G **2008**, Safety assessment of astaxanthin-rich microalgae biomass: Acute and subchronic toxicity studies in rats, *Food and Chemical Toxicology*, *46*, 3030–3036

Strawbridge, R, Arnone, D, Danese, A, Papadopoulos, A, Herane Vives, A & Cleare, A, J, **2015**, Inflammation and clinical response to treatment in depression: a meta-analysis, *The journal of the European College of Neuropsychopharmacology*, 25, 1532–1543.

Sun, C, P, Zhang, X, Y, Morisseau, C, Hwang, S, H, Zhang, S, H, Hammock, B, D & Ma, X, C **2021**, Discovery of soluble epoxide hydrolase inhibitors from chemical synthesis and natural products, *Journal of Medicinal Chemistry*, 64.

Suzuki, Y, Ohgami, K, Shiratori, K, Jin, X, H, Ilieva, I, Koyama, Y, Yazawa, K, Yoshida, K, Kase, S & Ohno, S **2006**, Suppressive effects of astaxanthin against rat endotoxin-induced uveitis by inhibiting the NF-κB signaling pathway, *Experimental Eye Research, 82*, 275– 281.

Swardfager, W, Hennebelle, M, Yu, D, Hammock, B, D, Levitt, A, J, Hashimoto, K & Taha, A, Y **2018**, Metabolic/inflammatory/vascular comorbidity in psychiatric disorders; soluble epoxide hydrolase (I) as a possible new target, *Neuroscience and Biobehavioral Reviews*, 87, 56–66.

Taksima, T, Chonpathompikunlert, P, Sroyraya, M, Hutamekalin, P, Limpawattana, M & Klaypradit, W **2019**, "Effects of astaxanthin from shrimp shell on oxidative stress and behaviour in animal model of Alzheimer's disease," *Marine Drugs*, vol. 17, no. 11, p. 628.

Tatas, H, P, B, Leenawaty, L, Edi, S & Heriyanto, **2020**, "Structures of Astaxanthin and Their Consequences for Therapeutic Application," *International Journal of Food Science*, vol. 2020, 2156582, 16 PP.

Tao, A, Huang, Y, Shinohara, Y, Caylor, M, L, Pashikanti, S & Xu, D **2019**, EzCADD: A Rapid 2D/3D Visualization-Enabled Web Modeling Environment for Democratizing Computer-Aided Drug Design, *Journal of Chemical Information and Modelling*, 59(1):18-24

Trott, O & Olson, A, J **2010**, AutoDock Vina: improving the speed and accuracy of docking with a new scoring function, efficient optimization, and multithreading, *Journal of Computational Chemistry*, 31(2):455–461.

Troubat, R, Barone, P, Leman, S, Desmidt, T, Cressant, A, Atanasova, B, Brizard, B, El-Hage, W, Surget, A, Belzung, C & Camus, V **2021**, Neuroinflammation and depression: A review, *European Journal of Neuroscience,* 53(1):151-17.

Wagner, K, M, McReynolds, C, B, Schmidt, W, K & Hammock, B, D **2017**, Soluble epoxide hydrolase as a therapeutic target for pain, inflammatory and neurodegenerative diseases, *Pharmacology & therapeutics*, pp. *180*, 62–76.

Wagner, K, Yang, J, Inceoglu, B & Hammock, B, D **2014**, Soluble epoxide hydrolase inhibition is antinociceptive in a mouse model of diabetic neuropathy, *The Journal of Pain*, 15(9), 907–914.

World Health Organization, **2012**, World suicide prevention day.

World Health Organization, **2017**, Depression and other common mental disorders: global health Estimates, 1–24.

Wu, X, He, W, Zhang, H, Li, Y, Liu, Z & He, Z **2014**, Acteoside: A lipase inhibitor from the

Chinese tea Ligustrumpurpurascens kudingcha. *Food Chemistry*, 142, 306–310.

Wu, H, Niu, H, Shao, A, Wu, C, Dixon, B, Zhang, J & Wang, Y **2015**, Astaxanthin as a Potential Neuroprotective Agent for Neurological Diseases, *Marine Drugs, 13(9), 5750– 5766.*

Wu, Q, J & Tymianski, M **2018**, Targeting NMDA receptors in stroke: new hope in Neuroprotection, *Molecular brain*, *11*(1), 15.

Wu, Q, Song, J, Meng, D & Chang, Q **2019**, TPPU, an sEH Inhibitor, Attenuates Corticosterone-Induced PC12 Cell Injury by Modulation of the BDNF-TrkB Pathway, *Journal of molecular neuroscience: MN*, *67*(3), 364–372.

Yang, H, Lou, C, Sun, L, Li, J, Cai, Y, Wang, Z, Li, W, Liu, G & Tang, Y **2019**, AdmetSAR 2.0: web-service for prediction and optimization of chemical ADMET properties, *Bioinformatics,* 35:1067–1069.

Yang, X, Guo, A, L & Pang, Y, P **2019**, "Astaxanthin attenuates environmental tobacco smoke-induced cognitive deficits: a critical role of p38 MAPK," *Marine Drugs*, vol. 17, no. 1, p. 24.

Yamagishi, R & Aihara, M **2014**, Neuroprotective effect of astaxanthin against rat retinal ganglion cell death under various stresses that induce apoptosis and necrosis, *Molecular Vision,* 20, 1796–1805.

Ying, C, J, Zhang, F, Zhou, X, Y, Hu, X, T, Chen, J, Wen, X, R, Sun, Y, Zheng, K, Y, Tang, R, X & Song, Y, J **2015**, Anti-inflammatory Effect of Astaxanthin on the Sickness Behavior Induced by Diabetes Mellitus, *Cellular and Molecular Neurobiology*, doi:10.1007/s10571015-0197-3.

Yokoyama, A, Izumida, H & Miki, W **2014**, "Production of astaxanthin and 4-keto zeaxanthin by the marine bacterium, agrobacterium aurantiacum," *Bioscience, Biotechnology, and Biochemistry*, vol. 58, pp. 1842–1844.

Yu, D, Hennebelle, M, Sahlas, D, J, Ramirez, J, Gao, F, Masellis, M, Cogo-Moreira, H, Swartz,

R, H, Herrmann, N, Chan, P, C, Pettersen, J, A, Stuss, D, T, Black, S, E, Taha, A, Y & Swardfager, W **2019**, Soluble Epoxide Hydrolase-Derived Linoleic Acid Oxylipins in Serum Are Associated with Periventricular White Matter Hyperintensities and Vascular Cognitive Impairment, *Translational stroke research*, *10*(5), 522–533.

Zeynab, K, Tahereh, F, Micheal, A, Ali, M, P-S & Saeed, S **2022**, Anti-inflammatory action of astaxanthin and its use in the treatment of various diseases, *Biomedicine & Pharmacotherapy*, Volume 145, 112179, 0753-3322.

Zhang, D, H & Lee, Y, K **1997**, "Enhanced accumulation of secondary carotenoids in a mutant of the green alga, Chlorococcum sp.," *Journal of Applied Phycology*, vol. 9, no. 5, pp. 459–463.

Zhang, J, C, Yao, W & Hashimoto, K **2016**, Brain-derived neurotrophic factor (BDNF)-TrkB signalling in inflammation-related depression and potential therapeutic targets, *Current Neuropharmacology*, 14(7), 721–731.

Zhang, X, S, Zhang, X, Wu, Q, Li, W, Wang, C, X, Xie, G, B, Zhou, X, M, Shi, J, X & Zhou, M, L **2014a**, Astaxanthin offers neuroprotection and reduces neuroinflammation in experimental subarachnoid hemorrhage, *Journal of Surgical Research*, 192, 206–213.

Zhang, X, S, Zhang, X, Wu, Q, Li, W, Zhang, Q, R, Wang, C, X, Zhou, X, M, Li, H, Shi, J, X & Zhou, M, L **2014b**, Astaxanthin alleviates early brain injury following subarachnoid hemorrhage in rats: Possible involvement of Akt/bad signalling, *Marine Drugs*, *12*, 4291–4310.

Zhang, Z, Robinson, L, Jia, T, Quinlan, E, B, Tay, N, Chu, C, Barker, E, D, Banaschewski, T, Barker, G, J, Bokde, A, Flor, H, Grigis, A, Garavan, H, Gowland, P, Heinz, A, Ittermann, B, Martinot, J, L, Stringaris, A, Penttilä, J, van Noort, B & Desrivières, S **2020**, Development of Disordered Eating Behaviors and Comorbid Depressive Symptoms in Adolescence: Neural and Psychopathological Predictors, *Biological Psychiatry*,

S00063223(20)31672-3.
Zheng, Y, Yang, W, Sun, W, Chen, S, Liu, D, Kong, X, Tian, J & Ye, X **2020**, Inhibition of Porcine Pancreatic α-Amylase Activity by Chlorogenic Acid, *Journal of Functional Foods*, 64, 103587.