AN INVESTIGATION INTO THE ANTIDEPRESSANT ACTIVITY OF *Hypericum perforatum*

A Thesis
Submitted in fulfillment of the
requirements for the Degree of

DOCTOR OF PHILOSOPHY
PHARMACY

of

RHODES UNIVERSITY

by

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November 2004

ABSTRACT

Hypericum perforatum is a herbal medicine that has been used for centuries for the treatment of depression. Many studies have been conducted in the Northern hemisphere on the efficacy of the HP extracts produced there. These studies include clinical trials and pharmacological investigations using a standardised HP extract or a fraction of the HP extract containing certain compounds, such as hypericin, pseudohypericin, hyperforin and several of the flavonoids thought to be responsible for the antidepressant activity. The mechanism of action of HP and its constituents is still not completely clear and it is speculated that the antidepressant activity is the result of several of the compounds acting synergistically.

HP is indigenous to and also cultivated in the Western Cape of South Africa. Extracts from these plants are sold in the local health shops and there are no previous studies evaluating the efficacy of these products. The aim of this thesis is to investigate the antidepressant activity of one of these products and two of its constituents, quercetin and caffeic acid, to gain further insight into their mode of antidepressant action and to compare these results with similar studies which used a standardised extract produced in the northern hemisphere.

The first study investigated the effect of HP, quercetin and caffeic acid on pineal metabolism. Changes in the synthesis of melatonin produced by the pineal gland have been implicated in depression. The results showed an increase in the level of melatonin produced in the animals treated with quercetin, which suggests that this compound may mediate antidepressant activity through such a mechanism. There are no previous reports on the *in vivo* effects of HP or any of its constituents on pineal metabolism.

The second study investigated the effect of HP, quercetin and caffeic acid on the activity of the liver enzyme, tryptophan-2,3-dioxygenase (TDO). Inhibition of this enzyme has been shown to increase plasma levels of tryptophan, a precursor of serotonin and thereby result in increased serotonin levels in the brain. Low levels of serotonin in the brain have been implicated in depression. This study revealed significant inhibition of TDO by caffeic acid and this suggests that this constituent of HP could be contributing to its antidepressant activity through such a mechanism. There are no previous reports investigating the *in vivo* effect of HP or any of its constituents on TDO activity.

Modulation of the levels of indoleamines, serotonin (5-HT) and dopamine (DA) as well as the metabolites, 3,4 dihydroxyphenyl acetic acid (DOPAC), 5-hydroxyindole acetic acid (5-HIAA) and homovallinic acid (HVA) in the brain have been implicated in the neuropharmacology of depression. Different studies using enzyme-linked immunosorbant assay (ELISA), high performance liquid chromatography with electrochemical detection (HPLC-ECD) and liquid chromatography-mass spectrometry (LC-MS) were used to determine changes in the levels of these indoleamines brought about after treatment with HP caffeic acid and quercetin. The results of the ELISA study showed significant increases in 5-HT levels in the brains of the animals treated with caffeic acid and quercetin. The results of the HPLC-ECD studies also revealed significant increases in 5-HT levels and a decrease in the turnover of 5-HT in the animals treated with quercetin. A significant increase in DA levels in the animals treated with quercetin was shown in both the HPLC-ECD and LC-MS studies. There was also an increase in DA turnover in the animals treated with HP shown in the HPLC-ECD and LC-MS studies. These results suggest that HP and its constituents, quercetin and caffeic acid mediate their antidepressant effects through serotonergic and dopaminergic neurotransmission.

Adaptive changes in the density of β-adrenergic (β-AR), 5-HT_2 and *N*-methyl-D-aspartate (NMDA) receptors have been implicated in depression. Several studies, investigating the effect of treatment with HP and quercetin on these different receptor densities, were undertaken using radioactive binding assays. Treatment with HP resulted in significant down regulation of β-AR and NMDA receptor densities and up-regulation of $5HT_2$ receptors. The effects on the β-AR and 5-HT_2 receptors are similar to the results reported using HP in the Northern hemisphere, but the effect on the NMDA receptors is novel providing insight into the mode of action of HP.

Apoptosis of neuronal cells has been implicated in neuro-degenerative and depressive disorders. Detection of apoptosis, using fluorescent microscopy observed through the labelling of DNA strand breaks, showed a decrease in the amount of apoptosis in the animals treated with HP and quercetin. This adds further support for the use of HP as an antidepressant and these results are similar to results reported from the Northern hemisphere. The results of all these studies suggest that the quality of the locally produced tincture is similar in efficacy to that of the standardised product of the Northern hemisphere.

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ABBREVIATIONS

Ag/AgCl	Silver/Silver chloride
aMT	Melatonin
ANOVA	Analysis of variance
APES	Aminopropyltriethoxysilane
Bmax	Maximum number of binding sites
BSA	Bovine Serum Albumin
Ca^{2+}	Calcium ion
CNS	Central Nervous System
CPM	Counts per minute
CSF	Cerebrospinal fluid
DA	Dopamine
DNA	Deoxyribonucleic acid
DPM	Disintegrations per minute
EDTA	Ethylenediaminetetraacetic acid
Em	Emission
Ex	Excitation
GABA	γ-aminobutyric acid
GCE	Glassy carbon electrode
GSH	Reduced glutathione
HAMD	Hamilton rating scale for Depression
5-HIAA	5-Hydroxyindoleacetic acid
5-HT	5-hydroxytryptamine (serotonin)
5-HTOH	5-Hydroxytryptophol
HCl	Hydrochloric acid
H_2O	Water
H_2O_2	Hydrogen peroxide
HP	*Hypericum perforatum*
HPLC	High performance liquid chromatography
Kd	Dissociation constant
LC-MS	Liquid chromatography-Mass spectrometry
5-MIAA	5-Methoxyindoleacetic acid

5-MTOH	5-Methoxytryptophol
mg/kg	Milligrams per kilogram
min	Minute
MK-801	Dibenzocycloheptenеimine
ml	Millilitre
mM	Millimolar
MAO	monoamine oxidase
NA	noradrenaline
NMDA	*N*-methyl-D-aspartate
PBS	Phosphate buffered saline
rpm	revolutions per minute
s	seconds
SD	Standard deviation
SEM	Standard Error of the Mean
SSRI	Selective Serotonin Reuptake Inhibitor
TCA	Trichloroacetic acid
TdT	Terminal deoxynucleotidyl transferase
TESPA	3-aminopropyl triethoxysilane
Tris	Tris(hydroxymethyl)-aminomethane
TUNEL	TdT-mediated dUTP nick end labelling

ACKNOWLEDGEMENTS

I would like to thank the following people for their contributions to my thesis:

My supervisor, Professor Santy Daya, for his invaluable insight, support and guidance throughout my research.

Dr Edith Antunes, for her assistance and contribution to the HPLC and LC-MS work.

Professor Ted Botha, for his time and expertise in assisting me with the use of the fluorescence microscope.

The staff and fellow postgraduates in the Faculty of Pharmacy, especially Dave and Sally Morley, who have been ready to assist me when needed.

My family: my mother, Pam Stephens who never failed to believe in me, though she never really understood "why" I chose to follow an academic career later in life. My late father, Bill Stephens, who sadly never lived to see the fulfillment of my dream, but always believed that I would achieve it. My two children, Paul and Vicky, who have had to survive a mother who changed careers in midlife and "dared" go back into academic research. My brother, Brent, who provided me with a laptop computer which has been 'invaluable' to attaining this goal.

My best friends, Ruth Jackson and Janet Brown, who have assisted me with editing this thesis and who have really 'been there' for me during the rough times and without whom, I truly believe this thesis would not really have been possible!

Lastly, to those people 'out there' who dare 'to dream the impossible dream' – all things are truly possible for those who dare to go there!

CHAPTER 1

LITERATURE REVIEW

INTRODUCTION

Depression is primarily characterised as an illness with emotional symptoms such as anxiety and vegetative symptoms such as sleep disturbances (Stahl S.M. 2000). It can be a persistent, recurring illness that can cause enormous personal suffering for individuals and their families. At present, disability caused by depression is estimated to be the fourth most important cause of worldwide loss of life years and it is possible that it will increase to the second most important within 20 years (Mulrow C.D. *et al.* 1998). This has led to a continued search for effective treatments, including antidepressant drugs, herbal remedies, psychotherapy and electro-convulsive shock therapy.

1.1 THE NEUROBIOLOGY AND PHARMACOLOGY OF DEPRESSION

1.1.1 Neurotransmitter Systems

The catecholamines, adrenaline, noradrenaline and dopamine form the adrenergic systems within the central nervous system (CNS). Some of these adrenergic neurons radiate from the ancient limbic system (emotional centres) and discharge catecholamines into the frontal cortex. The catecholaminergic pathways are thus responsible for alertness, mood and stress (fight or flight) responses. Serotonin is the primary neurotransmitter modulating the excitatory catecholamine systems of the CNS. Serotonin neurons are responsible for the control of memory, mood, sex drive and appetite (Stahl S.M. 2000).

The serotonin and noradrenaline systems have their most important cell bodies in small areas of the brain stem that serve as headquarters for sending axonal projections throughout the brain in specific pathways that mediate specific functions (See Figure 1.1 for an illustration of the serotonin projections and Figure 1.2 for an illustration of the noradrenergic projections).

Multiple serotonergic and noradrenergic pathways may be dysfunctional in depression, generating many different symptoms (Stahl S.M. 2000).

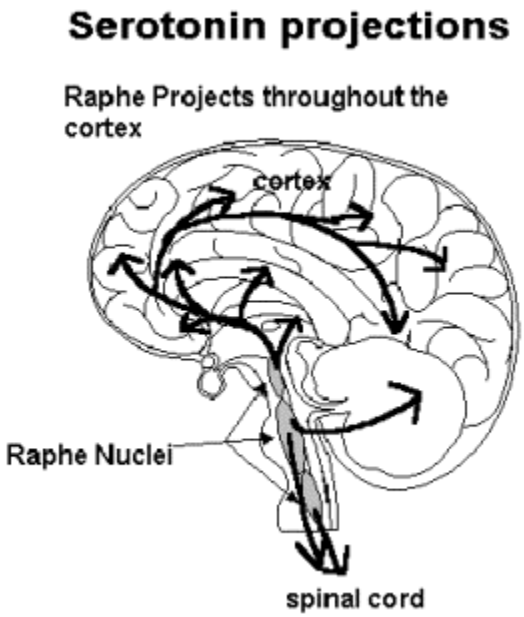

Figure 1.1 The projections of the serotonin system (www.cellscience.com/CCA.htm)

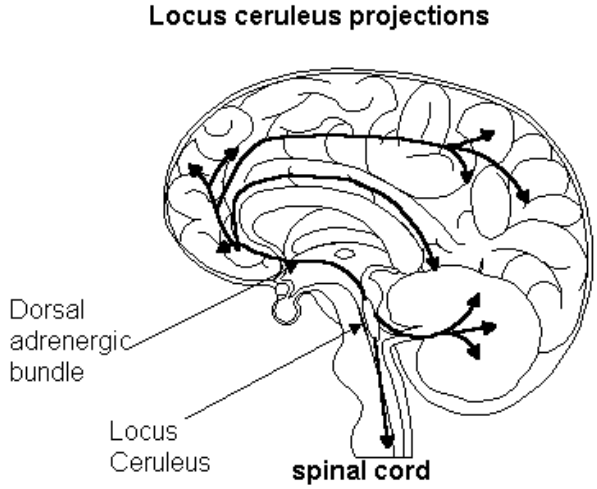

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Figure 1.2 The projection of the noradrenaline system (www.cellscience.com/CCA.htm)

The projections of the serotonin system arise from the nuclei of the dorsal raphe and the raphe magnus. The serotonin receptors (5-HT) have been identified into various sub-types with the 5-HT_1 and 5-HT_2 sub-types being of greater interest in psychiatry. The most important of the

5-HT_1 subclass is 5- HT_{1A} which is concentrated in the raphe and hippocampus. This receptor is implicated as an autoreceptor that modulates 5-HT release from the presynaptic neurons. The 5-HT_2 rceptors occur in high concentrations in the frontal cortex and nucleus accumbens (Van Oekelen D. *et al.* 2003).

1.1.2 Hypotheses of Depression

Several hypotheses of the biological determinants of depression have emerged over the past century. The most important of these and the implications thereof are reviewed below. Today it is generally accepted that depression is not necessarily due to a shortage of one vital brain neurotransmitter, but rather to a disruption in the equilibrium between different regulatory systems.

1.1.2.1 The monoaminergic hypothesis of depression

The monoaminergic hypothesis of depression assumes that the main symptoms of depression are the result of insufficient concentration of noradrenaline (NA) and serotonin (5-HT) in the synaptic clefts of the neurons in the brain (Cordi A.A *et al.* 2001). This hypothesis has evolved to consider the possibility that depression may be the result of a deficiency in signal transduction from the monoamine neurotransmitter to its postsynaptic neuron, even with normal levels of neurotransmitter and receptor being present (Stahl S.M. 2000). Emerging theories that link genetic and environmental risk factors for depression suggest that stress can cause depression by down-regulating certain genes, resulting in less key gene products, such as the brain-derived neurotrophic factor (BDNF), being produced. BDNF sustains the viability of neurons, so if the encoding gene is repressed the result may be atrophy or even apoptosis of neurons (Stahl S.M. 2000).

1.1.2.2 The dopamine hypothesis of depression

The original hypothesis was formulated in the late nineteen seventies by Solomon Snyder and linked schizophrenia with dopamine (DA) activity. Later, this hypothesis was extended to include

depression following the observation that many antidepressants influence the metabolism of dopamine. Following chronic antidepressant treatment, the presynaptic DA receptors become subsensitised and this results in an increase in DA release. A decrease in homovallinic acid (HVA), the main metabolite of dopamine, in the cerebral spinal fluid (CSF) of depressed patients who demonstrate marked motor retardation has also been reported (Van Praag H.M. 1982). Therefore, a decrease in the ratio of HVA to DA is indicative of decreased turnover of DA. This hypothesis is also supported by reports of significantly reduced dopamine turnover in depressed suicide victims (Bowden C. *et al.* 1997).

1.1.2.3 The permissive hypothesis of depression

This hypothesis emphasizes 5-HT as a neuro-modulator and its importance as a target for antidepressant action. According to this theory, a lowered concentration in the central nervous system (CNS) of 5-HT results in an affective state regulated by NA. Decreased 5-HT and NA levels will give rise to depression. This means that 5-HT may act as a 'permissive' modulator of neurotransmitter function through connections between serotonergic pathways and make connections with noradrenergic and dopaminergic pathways via the associated receptors (Harvey B.H. 1997).

1.1.2.4 The glutamatergic N-methyl-D-aspartate hypothesis

Recent findings indicate that the dysfunction of CNS glutamatergic pathways may play a role as one of the mechanisms involved in depression. Several studies have independently confirmed that compounds which reduce activity at the NMDA receptors produce similar effects to clinically active antidepressants. It is therefore hypothesized that adaptive changes in the NMDA receptor complex could be a common pathway affected by all antidepressants (Heresco-Levy and Javitt, 1998).

1.1.2.5 The kynurenine hypothesis

This hypothesis emerges from the premise that depression arises from altered levels of serotonin (5-HT) in the brain. Serotonin is a metabolite of the essential amino acid tryptophan (TRP) and

all 5-HT required by the neurons in the brain is synthesized in the brain because 5-HT is unable to cross the blood brain barrier (BBB). Therefore, the synthesis of 5-HT is heavily dependent upon the availability of TRP to the CNS. The production and subsequent transport of TRP from the bloodstream into the CNS can be compromised by several factors:

1. Stress, elevated cortisol levels, vitamin B6 deficiency and even high doses of TRP (2000mg of TRP), which all stimulate the conversion of TRP to kynurenine, lowering TRP level (Green A.R. *et al.* 1980).
2. Elevated serum levels of kynurenine inhibit the transport of TRP into the CNS and this results in reduced 5-HT levels (Gal E.M. *et al.* 1980)
3. Transport of TRP across BBB requires binding to a transport molecule which TRP shares with five other amino acids (tyrosine, phenylalanine, valine, leucine and isoleucine).
4. TRP is used by the body for other metabolic purposes in addition to 5-HT production, including synthesis of proteins and niacin.

Therefore, inhibition of the liver enzyme tryptophan 2,3-dioxygenase (also known as tryptophan pyrrolase) during the first and rate-limiting step of the kynurenine pathway would enhance circulating levels of TRP and thereby lead to increased neural production of 5-HT (Badawy A.A.B. *et al.* 1981).

1.1.3 Treatments for Depression

Tricyclic antidepressants (TCAs) and monoamine oxidase inhibitors (MAOIs) were introduced more than 40 years ago. These were found to have many side effects and to be highly toxic in the treatment of depression. This led to the development of the selective serotonin reuptake inhibitors (SSRIs) and the selective noradrenaline reuptake inhibitors (SNRIs) which are better tolerated and safer. However, these have not been shown to be conclusively superior to the TCAs and MAOIs (Muller & Kasper 1997).

The chemical structures of antidepressant drugs vary significantly and therefore cannot be considered to be the most important factor in the search for new drugs with antidepressant

activity. However, the mechanism of action of these drugs has provided insights into the pathology of depression. The basic chemistry and possible mechanism of action of major classes of antidepressant drugs are discussed below.

1.1.3.1 Tricyclic Antidepressants (TCA)

These drugs all have a characteristic three ring structure (See Figure 1.3) and are chemically similar to the phenothiazines. The discovery of their antidepressant action was fortuitous when imipramine, originally considered as a neuroleptic, was found to have antidepressant activity. Thereafter, first generation antidepressants emerged which display activity as mixed noradrenaline and serotonin reuptake inhibitors (Hollister L.E. & Potter W.Z. 1998). Most TCAs inhibit the reuptake of monoamine neurotransmitters into the presynaptic neuron by competitive inhibition of the ATPase in the membrane pump. Some TCAs are more selective than others but this has not been shown to influence the efficacy of the drug (Waller D.G. *et al.* 2001). The different monoamine reuptake properties can also include an increase in dopaminergic activity via a presynaptic mechanism for amitriptyline and a post synaptic mechanism for desipramine and imipramine (Besson A. *et al.* 1999).

The major drawback of the TCA drugs is the side effects which result from their antimuscarinic, antihistaminic and alpha adrenoceptor-blocking activity.

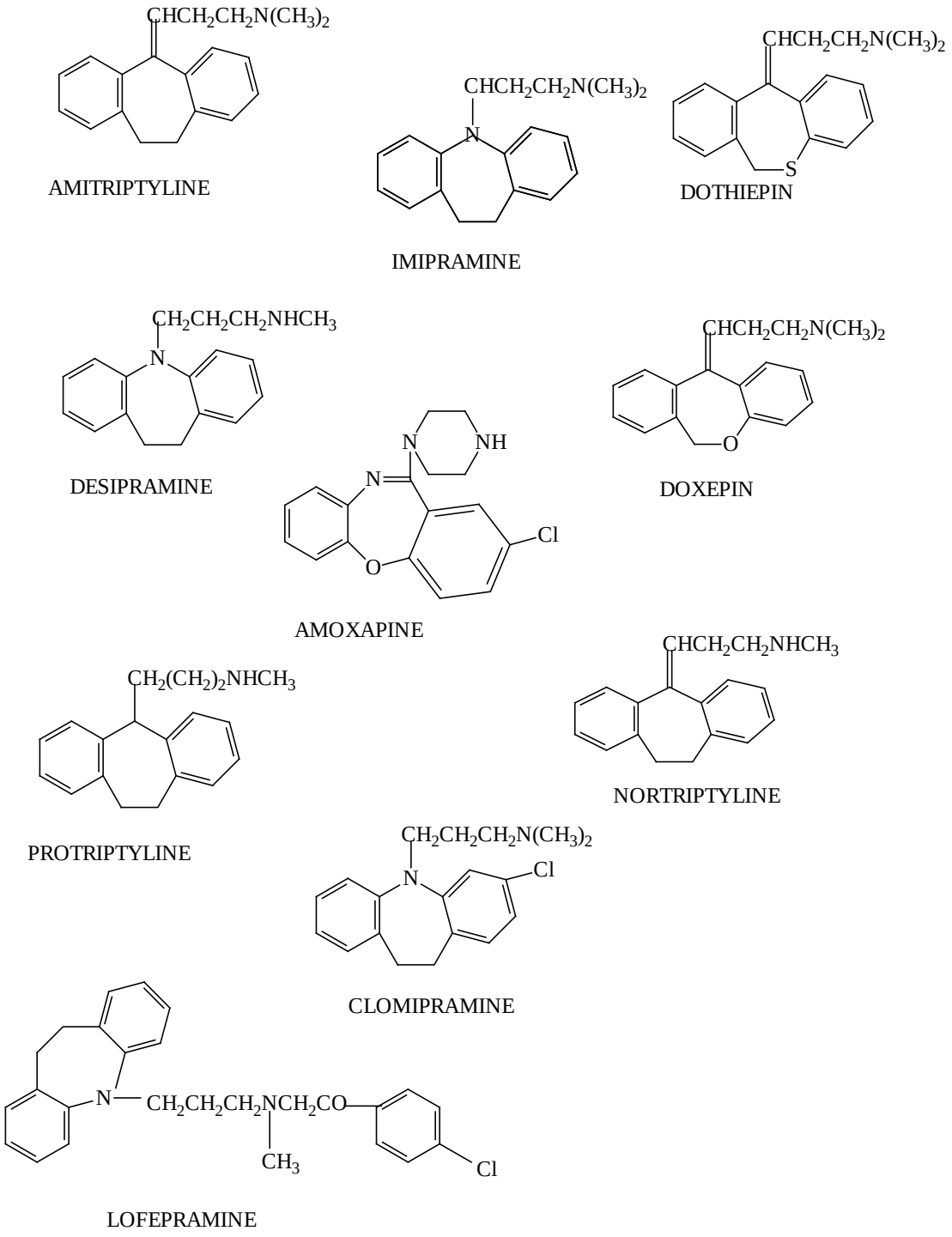


Figure 1.3 Chemical structures of some tricyclic antidepressants

1.1.3.2 Heterocyclics

Between 1980 and 1996 “heterocyclic” antidepressants were discovered. Examples of these can be seen in Figure 1.4 shown below. Amoxapine and maprotiline resemble the structure of the TCAs while trazadone is distinctly different. Maprotiline is similar to the TCA, desipramine in being a potent noradrenaline reuptake inhibitor and it has less sedative and antimuscarinic side effects. Amoxapine is a metabolite of the antipsychotic drug loxapine and displays some dopamine receptor antagonism. Trazadone has shown unpredictable efficacy in the clinical setting (Potter W.Z. & Hollister L.E., 1998).

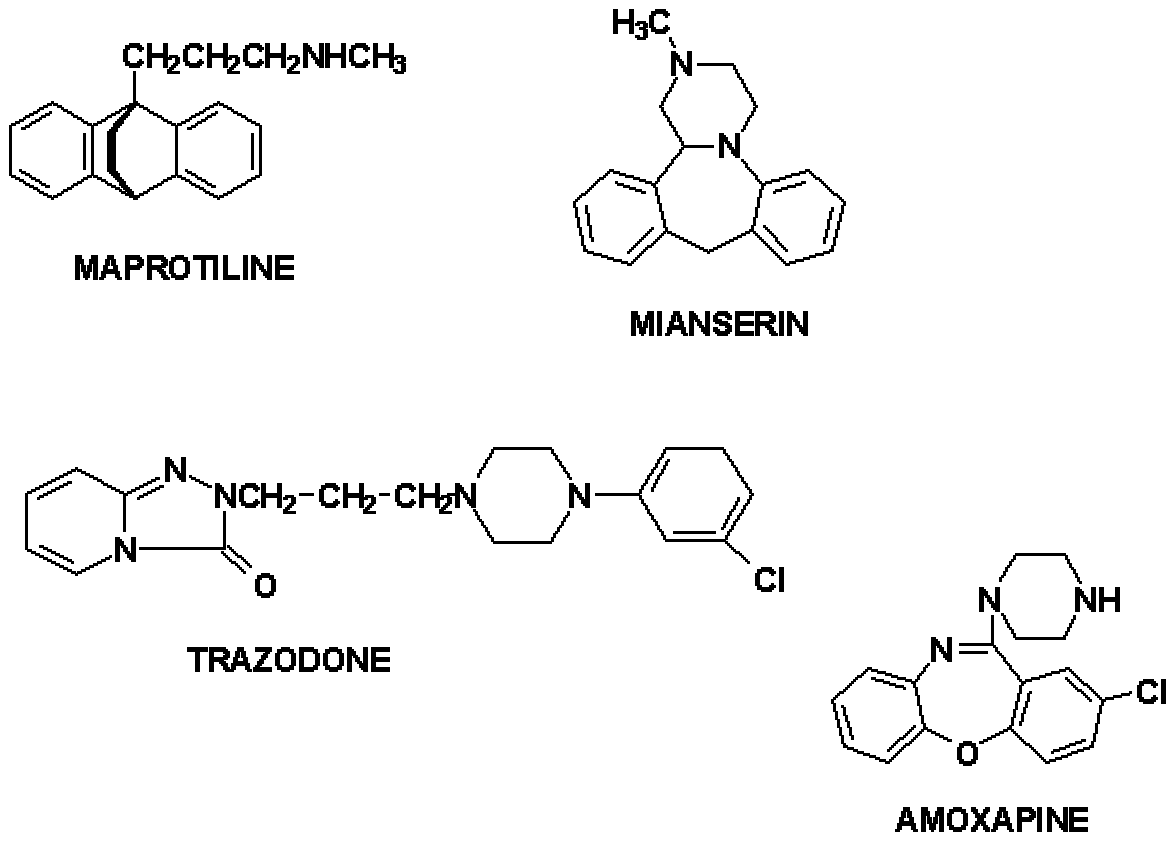


Figure 1.4 Chemical Structures of Some Heterocyclic Antidepressants

1.1.3.3 Selective Serotonin Reuptake Inhibitors (SSRI).

Unlike the tricyclic antidepressants, the SSRIs reduce the neuronal uptake of serotonin but have no effect on noradrenaline. Therefore the SSRIs have a better side effect profile in comparison with TCAs because these drugs have a low affinity for muscarinic, histaminergic and adrenergic receptors (Waller D.G. *et al.* 2001).

Fluoxetine was the first SSRI to be used clinically followed by paroxetine and sertraline. The latter two have shorter half lives and different potencies as inhibitors of specific P450 isoenzymes (Potter W.Z. and Hollister L.E., 1998). The chemical structures of these SSRIs are shown in Figure 1.5 below.

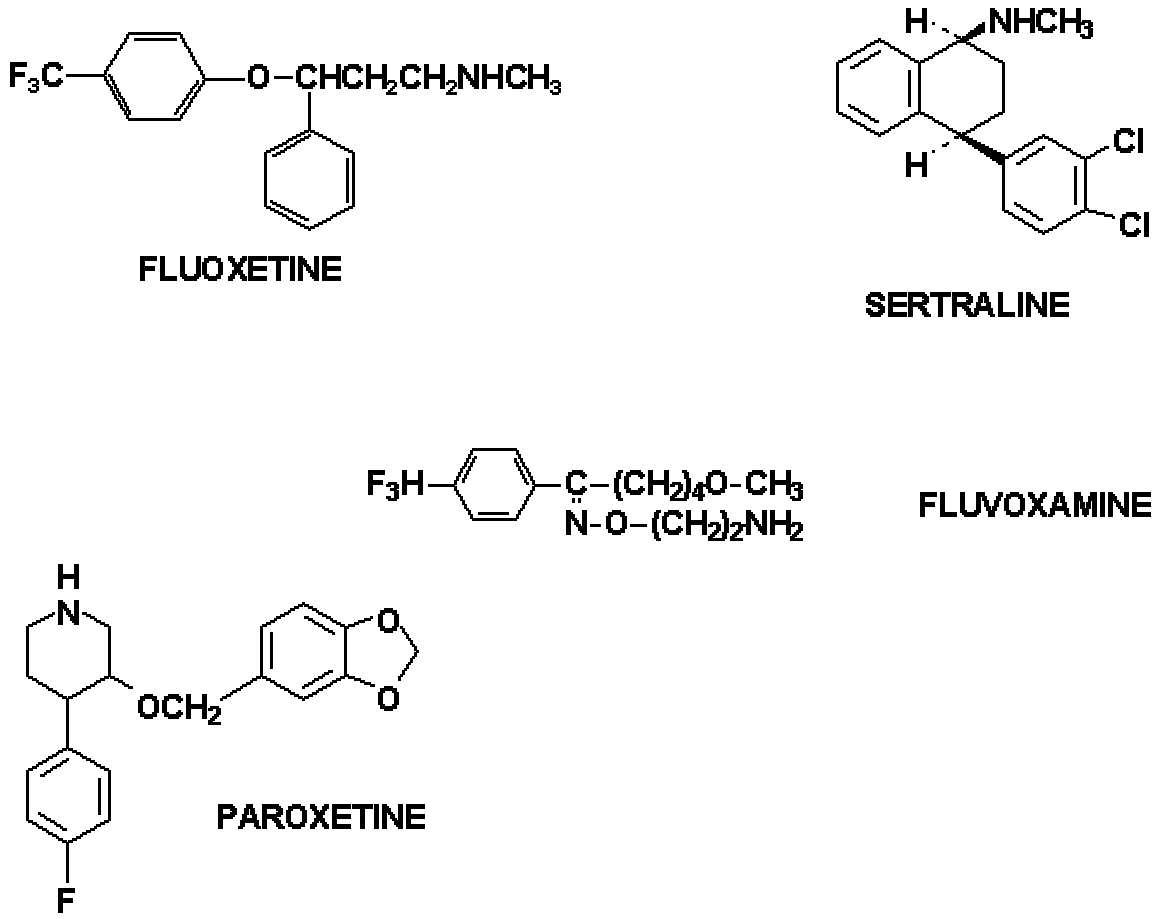


Figure 1.5 Chemical Structures of Some SSRI Antidepressants

1.1.3.4 Monoamine Oxidase Inhibitors (MAOI)

The mechanism of action of MAOIs is complex, but their primary action is to inhibit the enzyme, monoamine oxidase (MAO), which is responsible for degrading free monoamines. There are two isoforms of MAO, designated MAO-A and MAO-B, with MAO-B being the predominant in many parts of the brain. MAO inhibitors have many side effects, but recently the reversible MAO-A inhibitor, moclobemide, was introduced which has fewer side effects (Waller D.G. *et al.* 2001).

Today, many people are searching for natural remedies to overcome depression. These have fewer side effects and are easily obtainable.

1.2 Hypericum perforatum

Figure 1.6 *Hypericum perforatum* flowers.

1.2.1 Historical background

Hypericum perforatum (HP), more commonly known as St. John's wort, is a plant which has been used for centuries as a medicinal herb. HP has five-petalled yellow flowers (see Figure 1.5) and is a common weed in Europe, Asia and the USA which usually flowers around 24 June (St John's day). The word *Hypericum* comes from the Greek word *hyper* (above) and *eikon* (icon or image) because ancient Greeks and Romans placed branches of *Hypericum* above images or statues in their homes, believing that this plant had mystical powers providing protection from evil spirits (Miller A.L. 1998).

The term *perforatum* refers to the tiny translucent excretory glands seen on the underside of the leaves which look like perforations (Di Carlo *et al.* 2001). Wheatley (1997) refers to the oil from the perforations in the leaves being known to promote the healing of burns and that the tea made from the dried herb has a "mood-brightening" effect. According to Miller (1998), HP has traditionally been used for wound healing, as an anti-inflammatory as well as for analgesic activity. The crude drug is usually extracted in approximately 70% ethanol solution, from the dried herbs' aerial parts, which are harvested shortly before or during the flowering period.

In Germany, as an alternative to TCAs, high dosage HP extracts are prescribed and at present these are more prescribed than SSRIs and make up 25% of all prescriptions of antidepressants (Müller W.E. & Kasper S. 1997a). This increased usage of HP has led to an upsurge in the research being done into the efficacy of HP as an antidepressant. Initially, most research reported came from clinical trials and in the last few years more results from biochemical research are being reported.

1.2.2 Clinical Studies

Clinical studies with different extracts of HP have focused mainly on the effects in patients with depression. There are also studies investigating the use of HP in other conditions, such as seasonal affective disorder, chronic fatigue and premenstrual syndrome.

1.2.2.1 Depression

Linde K. *et al.* (1996) published a review and meta-analysis of randomised controlled trials using HP extracts, involving 23 studies and 1757 patients with depression. This has been updated to include new studies and published as a Cochrane review of 27 randomised controlled trials of HP extract in patients with "neurotic depression" and mild to moderately severe depressive disorders (Linde K. and Mulrow C.D. 2001).

Several trials have been conducted in an effort to compare the efficacy of HP to placebo and other antidepressant medications. Some of these clinical studies have shown that HP has superior efficacy when compared to placebo and that it has comparable efficacy to standard antidepressants in the treatment of mild to moderate depression. (Volz 1997; Wheatley D. 1997.) One four-week double-blind study involving 105 mildly depressed patients compared the antidepressant effects of *hypericum* (Jarsin®300) with placebo. Over half the patients on the *hypericum* therapy responded positively (according to the Hamilton Depression Scale criteria), whereas only about one-fourth of the patients responded to placebo. Only two patients experienced side effects while on the *hypericum* preparation, complaining of skin reddening, itching, and tiredness (Sommer H. and Harrer G. 1994). One of the advantages of HP over other antidepressants, is that it has very few side effects and these are generally mild. The majority of

these side effects involve fatigue, gastrointestinal symptoms, dizziness and confusion. Photosensitivity appears to be an extremely rare event with the recommended doses (Ernst E. *et al.* 1998).

Most of the clinical trials with HP have been done over short periods of time of 2 - 6 weeks duration in patients with mild to moderate depression, but there are a few studies that compare the efficacy of HP with TCAs in patients with severe depression. One such study is by Vorbach E.U. *et al.* (1997), where high dosage HP was found to be as effective as a standard TCA, but this trial was also only done over 6 weeks, so there is still a need for an evaluation of HP in long-term use and efficacy.

A double-blind, placebo controlled, cross-over study in 12 healthy male volunteers investigated the effects of a single dose of HP extract (LI 160) 2700mg (9 x 300mg tablets standardised to hypericin 0.3%) on plasma concentrations of growth hormone, prolactin and cortisol. A significant increase in plasma growth hormone concentration and a significant decrease in plasma prolactin concentration were observed following HP administration. Plasma cortisol levels were unchanged. These findings suggest that this dose of HP extract may increase aspects of brain dopamine function in humans, although further studies are required to confirm this to assess the dose-reponse relationships and to determine whether there is evidence for effects on dopaminergic systems in patients with depression treated with HP (Franklin M. 1999).

Another study, which utilised a randomised, 3-way, cross-over design, investigated the effects of a single dose of HP extract (LI 160S) 600mg, 300mg or placebo, on hormone concentrations in 12 healthy volunteers. HP extract (600mg) increased cortisol secretion between 30 and 90 minutes after dosing, indicating an influence of HP on certain CNS neurotransmitters. There was no difference between the 3 groups with regard to adrenocorticotrophic hormone, growth hormone and prolactin secretion (Laakman G. *et al.* 2000).

In several recent reviews on the efficacy of HP, there is still no consensus on the mechanism of action of HP extracts nor the validity of the clinical trials conducted thus far. Vitiello (1999), in his review for example, questions the validity of the clinical trials that have not included placebo control groups because placebo response rate has been shown to be high among depressed

patients. A large study has been launched in the United States of America in which the efficacy of HP extracts is being compared with the SSRI, sertraline and placebo. The author suggests that while HP extracts have been reported to be safe, without serious side effects, adverse reactions cannot be excluded when HP is used in combination with other drugs.

1.2.2.2 Seasonal Affective Disorder (SAD)

There are two studies that investigated the efficacy of HP in the treatment of SAD. Kasper S. (1997) compared the efficacy of 300mg HP (LI 160 extract) three times a day with or without bright light therapy. After four weeks treatment there were no significant differences between the groups and both groups had significant reductions in the Hamilton Rating Scale for Depression (HAMD) scores. Similar results were obtained from a study by Wheatley D. (2001) done over a period of eight weeks treatment.

1.2.3 Animal Studies

Animal studies suggest that HP is effective in three major biochemical systems relevant for antidepressant activity. These are the inhibition of the synaptic re-uptake system for serotonin (5-HT), noradrenaline (NA) and dopamine (DA). It is suggested that HP is the only antidepressant capable of doing this with similar potencies (Muller W. E. *et al.* 1997). This is not surprising because the crude extract is made up of several compounds and it is possible that the clinical efficacy could be attributed to the combined contribution of several mechanisms, each one too weak by itself to be responsible for the overall effect (Bennett D.A. *et al.* 1998).

1.2.3.1 Effects on Serotonin Receptors

Teufel-Mayer R. and Gleitz J. (1997) investigated the long term effects of HP extracts on the affinity and density of 5-HT_{1A} and 5-HT_{2A} receptors. This was because it had been noted by these authors that HP extracts, similar to TCAs, have only been shown to improve depression after a lag phase of 10-14 days. This led them to believe that adaptations of the 5-HT receptors must occur, in other words, this delay may indicate that treatment with HP causes long-term changes in the central nervous system. After treating rats with HP extract for 27 weeks, using receptor

binding assays it was found that the $5HT_{1A}$ and 5-HT_{2A} receptors increased by more than 50% in the rats treated with HP as compared with the control rats. However, the affinity for the receptors remained unchanged, suggesting that there was an increase in the regulation of these serotonin receptors as a result of dosing with HP over a long period of time. The increase in the concentration of 5-HT_{1A} receptors is consistent with the effect of TCAs, indicating a change in neurotransmitter metabolism. However the increase in 5-HT_{2A} receptor density is opposite to the effect of TCAs, which these authors suggest may indicate a different mode of action for HP. In addition, these authors report that an increased density of 5-HT_{2A} receptors has been correlated with depression in the past and also has been seen to occur after electroconvulsive shock treatment (ECT). The latter treatment for depression is also known to have a 10-14 day delay which may support the theory that an adaptation of the density of neurotransmitter receptors occurs. These authors suggest that the mode of action of HP in the treatment of depression is similar to that of ECT.

1.2.3.2 Effects on analgesic activity

Ozturk (1997) investigated the antidepressant effects of various *Hypericum* species, including *perforatum*, on animal models. The author's findings confirm the antidepressant activity of HP and that it also has analgesic activity. The results of this study also suggest that the antidepressant effects of HP are not related to endogenous opioid mechanisms, but that the analgesic activity does appear to involve δ-opioid mechanisms.

1.2.3.3 Effects on Forced Swimming Test (FST)

Butterweck V. *et al.* (1997), in a study using rats, made use of the forced swimming test (FST) and the tail suspension test to evaluate antidepressant activity. In order to evaluate effects on the central nervous system, body temperature and ketamine-induced sleeping time was used. Using HPLC, the crude HP was separated into 14 fractions and each of these were also tested on the animals. From the results of the tests, a decrease in ketamine-induced sleeping time and increased body temperature after oral dosing with the extracts was found. This led these authors to suggest that HP does not exert any adrenergic activity, but there is involvement of the dopaminergic system. Two fractions of the HP extract, one containing mostly flavonoids and the other

naphthodianthrones, showed significant results in the FST by reducing immobility time. From this study, these authors suggest that several of the compounds found in HP are involved in the pharmacological effects.

In other studies utilising the rat forced swimming test, an experimental model of depression, HP extracts induced a significant decrease in immobility (De Vry J. *et al.* (1999); Panocka I. *et al.* 2000). The latter study, which involved a dry extract containing 0.3% hypericin and 3.8% hyperforin, suggested that the antidepressant activity may be mediated by interaction with sigma receptors and increased serotonergic transmission (Panocka I. *et al.* 2000).

1.2.3.4 Anxiolytic Effects

In other experimental models of depression, including acute and chronic forms of escape deficit induced by stressors, HP extract was shown to protect rats from the consequences of unavoidable stress (Gambarana C. *et al.* 1999). HP extract was also found to increase locomotor activity in an open-field test, and to have an anxiolytic–like effect in a light-dark test, whereas individual constituents did not show any such effects. The anxiolytic activity was blocked by pre-treatment with the benzodiazepine receptor antagonist flumazenil (Vandenbogaerde A. *et al.* 2000).

Oral treatment of a standardised 50% ethanolic extract of Indian HP 100 and 200 mg/kg once daily for three days in rat models of learning and memory, resulted in significant attenuation of scopolamine and sodium-nitrate-induced impaired retention of active avoidance. This was comparable to that seen with intraperitoneal peracetam (a nootropic agent) (Kumar V. *et al.* 2000).

1.2.3.5 Effects on Alcohol Intake

Several other pharmacological actions have been documented for *hypericum*, some of which may be related to its antidepressant effect. Depression and alcoholism are thought to have some neurochemical similarities, such as low brain serotonin concentrations (Ballenger J.C. *et al.* 1979). Oral treatment of a single dose of HP (100, 200, 400, 600 or 800 mg/kg) to two strains of alcohol preferring rats significantly reduced alcohol intake in both strains (Rezvani A.H. *et al.*

1999). In another study, acute intraperitoneal treatment of HP 10 to 40 mg/kg, fluoxetine 1-10 mg/kg and imipramine 3-30mg/kg reduced alcohol intake in a dose dependent manner in a 12 hour limited access two bottle choice (ethanol/water) procedure (De Vry J. *et al.* 1999).

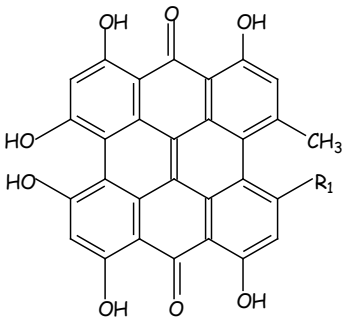
1.2.4 Biologically active compounds

1.2.4.1 Introduction

Nahrstedt A. and Butterweck V. (1997) reviewed the possible biologically active compounds and other chemical constituents of HP. According to these authors, seven groups of bioactive structures have been identified. The structures of some of these are shown in Figure 1.7.

The main constituents of the crude extract are phenylpropanes, flavonol glycosides, biflavones and oligomeric proanthocyanidins which are biogenetically related. Xanthones and naphthodianthrones usually occur in smaller quantities (less than 1%) while phloroglucinols can be more than 5%. The leaves and petals also contain essential oils. Recently a fraction containing free amino acids was investigated, and one of these, γ-aminobutyric acid (GABA), which has a sedative effect, was isolated. However, it was found in such small quantities that it is unlikely to make more than a small contribution to HPs effectiveness.

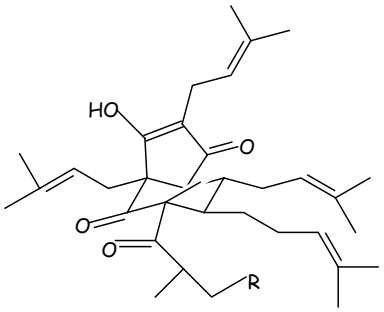
Naphthodianthones



Hypericin $R_1 = CH_3$

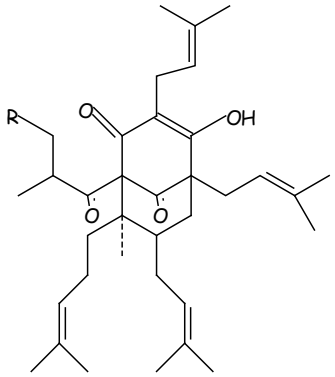
Pseudohypericin $R_1 = CH_2OH$

Phloroglycinols

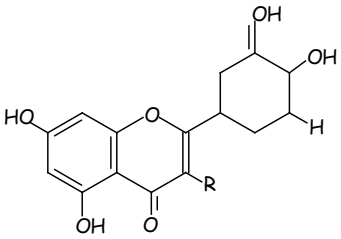


Hyperforin R = H

Adhyperforin $R = CH_3$

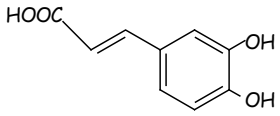


Flavonol Glycosides

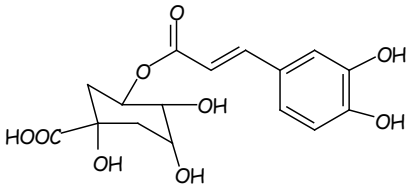


Quercetin	R = OH
Quercitrin	R = a-L-rhamanosyl
Isoquercitrin	R = β-D-glycosyl
Luteolin	R = H
Rutin	R = β-rutinosyl

Phenylpropanes

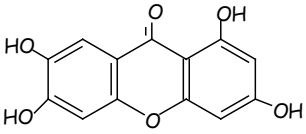


Chlorogenic acid



Caffeic acid

Xanthones



1,3,6,7-Tetrahydroxyxanthone

Figure 1.7 Structures of some of the compounds found in *Hypericum perforatum* (Nahrstedt and Butterweck 1997).

The main components of raw HP have been shown to vary in concentration from product to product. Therefore, different extracts might induce different behavioural effects, depending on whether or not natural active constituents of the plants are present (Guilhermano L.G. *et al.* 2004). Hypericum extracts and some of its constituents from plants in the northern hemisphere have been shown to decrease immobility in the forced swimming test (FST) (Butterweck V. *et al.* 1997, 1998, 2000). The majority of commercially available HP extracts are standardised to contain 0.3% hypericin. Analysis of commercial products such as the one shown in Table 1.1 below, reveal the variability of this constituent and especially for the hyperforin content. The antidepressant activity of HP extracts has been suggested to correlate closely with their hyperforin contents (Chatterjee S.S. *et al.* 1998a,b; Bhattacharya S.K. *et al.* 1998).

Table 1.1 Results of the analysis of some commercially available *Hypericum perforatum* extracts (San Rafael Chemical Services, (www.vitacost.com/.../reviews/stjohns/labtest.html)

Sample Identification		Contents	Hypericin		Hyperforin	
Lab ID	Client ID	wt g	mg/g	mg/cap	mg/g	mg/cap
0276-1	Nature's Answer SJW	liquid	0.079	0.0732	-	-
0276-2	Nature's Herbs SJW	0.401	1.66	0.667	0.804	0.322
0276-3	Nature's Way SJW	0.469	1.77	0.829	8.89	4.17
0276-4	Nature's Way (Standardised)	0.468	1.41	0.658	22.1	10.3
0276-5	Thompson SJW	0.437	1.5	0.655	1.6	0.7
0276-6	Twin Lab TruHerbs SJW	0.659	0.828	0.545	2.98	1.96
0276-7	Nature's Way Mood Aid	0.532	1.11	0.59	12.4	6.6
0276-8	Natrol Mood Support	0.591	1.28	0.755	15	8.89

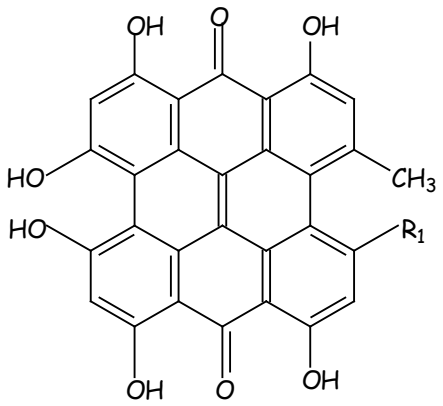
HP is indigenous in some countries in the southern hemisphere, such as South Africa. It has recently been cultivated in Brazil where its medicinal use as an antidepressant is based on studies performed in the Northern hemisphere. Even though the plants are genetically the same, the concentrations of the active constituents could be influenced by climate, geological environment and agricultural techniques (Büter B. *et al.* 1998). Hypericin and hyperforin are unstable under light and high temperatures and so storage of HP can also influence the concentration and activity of the active constituents (Bilia A.R. *et al.* 2001). There are few scientific studies evaluating the

effectiveness of HP produced in countries outside Europe. Very recently, the antidepressant effects in the FST of the hydroalcoholic extracts of HP cultivated or imported and commercially available in Brazil were reported. Neither the locally cultivated nor the imported extracts of HP tested showed efficacy in the FST, even though these extracts were standardised to contain 0.3% hypericin as required by national legislation. These authors suggest that individual extracts ought to be evaluated by quantification of constituents or by experimental screening tests in order that the expected effects will be seen in the clinical setting for all locally produced and imported hypericum extracts (Guilhermano L.G. *et al.* 2004).

HP is indigenous to the Western Cape of South Africa. At present there is no legislation governing the quality of locally produced HP products which are available to the public. There is only one report of a study comparing some of these products using qualitative analysis. The study found high inter-product variability with respect to flavonoid, napthrodianthrones and hyperforin content in ethanolic extracts as well as tablets. Most of the products had much higher than expected quercetin content which the author suggests is the result of degradation of the other glycoside flavonoids. There was a five-fold difference between the highest and lowest concentrations of pseudohypericin and a nine-fold difference for hypericin in one of the cases. Only one of the products analysed was found to have significant hyperforin content (Wild T.J. 2003).

There are no studies investigating the biological activities of the HP extracts produced and available to consumers in South Africa. Almost all the studies evaluating the different possible constituents with antidepressant activity in HP have been conducted in the northern hemisphere, particularly in Europe. The results of some of these studies conducted on different pharmacologically active constituents are reviewed in the following sections.

1.2.4.2 Hypericin and Pseudohypericin



Hypericin $R_1 = CH_3$

Pseudohypericin $R_1 = CH_2OH$

Figure 1.8 Structures of Hypericin and pseudohypericin

1.2.4.2.1 Phototoxicity

Initially, most interest was shown in the compound, hypericin, which has an intense red colour and phototoxic effects. These have been particularly seen to be manifested in cattle which have eaten 3-7g of hypericin while grazing and this has also caused some to die. However, human dosage is usually 30-50 times lower than the phototoxic dose (Nahrstedt & Butterweck, 1997). This was substantiated in a recent study by Schempp C.M. *et al.* (1999a), where the amount of hypericin present in the serum and skin blister fluid of humans after a high single dose (1800mg) or a lower dose (300mg) taken 3 x daily for 7 days, was measured. The levels of hypericin in the skin in both cases, was found to be far below those which are estimated to be phototoxic.

The photoactivating effect of hypericin has been investigated *in vitro* in several cancer cell lines, and *in vivo* in animal models of cancer. Incubation of prostatic adrenocarcinoma cells and a metastatic cell line of human prostate cancer with hypericin 0.001 to 0.3 μg/ml followed by laser irradiation resulted in phototoxic effects in both cell lines, whereas no effect was seen in the absence of irradiation (Colasanti A. *et al.* 2000). Similar studies *in vitro* have demonstrated

photocytotoxicity in human urinary bladder carcinoma cells (Kamuhabwa A.R. *et al.* 2000b) and in pancreatic cancer cells that had been implanted in the pancreas, treated intratumourally with hypericin followed by laser phototherapy, a decrease in cancer cell growth was observed when compared with untreated controls (Liu C. D. *et al.* 2000). Also, in another study, laser intra-tumoral photodynamic therapy with hypericin to tumours grown in mice, following transplantation of human squamous carcinoma cells resulted in a significant induction of tumour necrosis when compared with laser treatment alone (Chung P.S. *et al.* 2000).

1.2.4.2.2 Monoamine oxidase activity

Suzuki O. *et al* (1984) showed that hypericin had weak MAO-A and B activity in rat brain mitochondria *in vitro*. Studies with the whole HP extract, have reported weak or no MAO inhibition as well (Cott J.M. 1997 and Müller W.E. 1997).

1.2.4.2.3 Effect on different receptors

In a study by Raffa (1998), different standard receptor and uptake assays were used to determine the possible pharmacological profile of hypericin. These results show that hypericin had a modest affinity for the muscarinic cholinergic receptors as well as for sigma (σ) receptors. The latter affinity is suggested by this author to be a significant finding because it could provide insight into a possible mode of action of HP. This is because σ receptors have been implicated in other studies which involved the AD action of SSRIs and MAOIs and also the modulation of NMDA-glutamate receptors which it is suggested, forms part of a common pathway for antidepressant action (Debonnel G.& de Montigny C. 1996; Leonard B.E. 1997).

Cott J.M. (1997) tested the crude extract of HP as well as pure hypericin in a battery of 39 receptor assays. Hypericin showed affinity only for the NMDA receptors, while the crude showed significant affinity for adenosine, $GABA_A$, $GABA_B$, $5\text{-}HT_1$, benzodiazepine, inositol triphosphate and MAO-A and B receptors. This author reports that the significance of GABA binding is not known, but that other researchers have found that plasma levels of GABA are low in some depressed patients and that benzodiazepines, which increase GABA activity, may act as antidepressants as well as anxiolytics. Cott has also shown, in previous studies, that GABA

neural systems also have an influence on dopamine and dopamine-induced behaviours (Cott J.M. *et al.* 1976; Cott J.M. and Engel J. 1977).

1.2.4.2.4 Antiviral Activity

Hypericin and pseudohypericin (see Figure 1.7), have also been shown by Kraus G.A. *et al.* (1990), to have anti-retroviral activity against equine infectious anaemia virus (EIAV). Simple quinones which resembled different parts of the hypericin ring system were also tested, but none of these showed the same antiviral activity of hypericin. These authors suggest that hypericin's antiviral effect may be the result of the hydrophobic nature of its ring system and that the quinone is affected by the reduction potential of the hydroxyl groups.

In other similar studies, hypericin and pseudohypericin have been reported to inhibit several encapsulated viruses *in vitro*, including herpes simplex virus types 1 and 2 (Wood S. *et al.* 1990; Weber N.D. *et al.* 1994), and human immunodeficiency virus (HIV)-1 (Meruelo D. *et al.* 1988; Lavie G. *et al.* 1989; Hudson J.B. *et al.* 1991; Lopez-Bazzocchi I. *et al.* 1991). Hypericin has also been reported to inactivate murine cytomegalovirus (MCMV) and sindbis virus. It is suggested that the antiviral activity involves a photoactivation process (Hudson J.B. *et al.* 1991; *American Herbal Pharmacopeia* 1997).

1.2.4.2.5 Effect on Immune and Inflammatory response

Bork *et al.* (1999) investigated hypericin as a possible inhibitor of NF-κB which is an important mediator of the immune and inflammatory response. This is because it has been reported that hypericin delays cell growth and has been shown to inhibit protein kinase C (PKC) (Agostinis *et al.* 1995). The results show that hypericin inhibits NF-κB even at low micromolar concentrations and that hypericin does not have anti-oxidant activity. These authors suggest that this indicates that it inhibits a step in the activation cascade, possibly PKC, upstream from the merging point of the various signaling pathways. However, it is not yet known whether hypericin's inhibitory effects on NF-κB bears any relation to the antidepressant activity of hypericins, but these authors speculate that it is possible that hypericin may prevent the expression of neuronal NF-κB target genes involved in neurotransmission.

In other studies involving immune and inflammatory responses, hypericin has been shown to inhibit specific growth-factor-regulated protein kinases (Takahashi I *et al.* 1989; De Witte P. *et al.* 1993; Agostinis P. *et al.* 1995) and to inhibit the release of arachidonic acid and leukotriene B_4 (Panossian A.G. *et al.* 1996). In a rabbit model of proliferative vitreoretinopathy (PVR), an intravitreal injection of hypericin 0.1ml (10 or 100µM) inhibited the progression of PVR when compared with severity in control eyes, five days after treatment with hypericin. These authors suggested that as protein kinase C is important in the cellular reactions occurring in PVR, modulation of protein kinase C by hypericin may be a factor in this system (Tahara Y.R. *et al.* 1999). Hypericin and pseudohypericin have also been reported to inhibit 12-lipoxygenase activity; the products of lipoxygenase-catalysed reactions, such as leukotrienes and may therefore be involved in inflammatory reactions (Bezakova L. *et al.* 1999).

1.2.4.3 Hyperforin and Adhyperforin

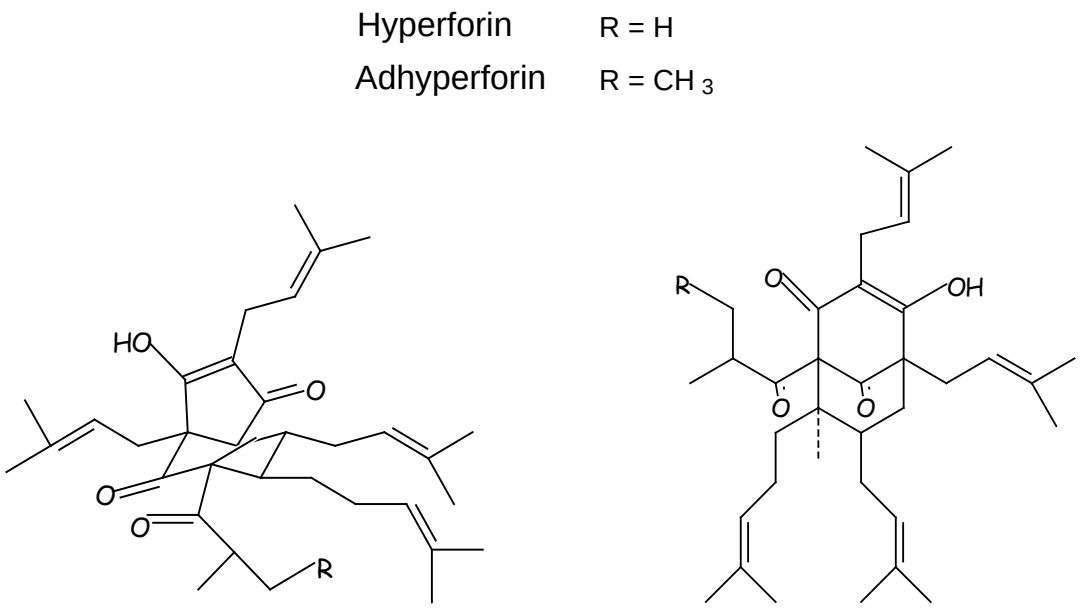


Figure 1.9 Two possible views of the structure of Hyperforin

More recently, several researchers have turned their attention to the phloroglucinol derivative, hyperforin (see Figure 1.9 above) as being the most biologically active component of HP in the treatment of depression. The reason why it was not considered earlier is that it had been shown to

be unstable when exposed to light and air. However, it has subsequently been shown to be very stable in the blood. Erdelmeier C.A.J. (1998), in his study, reviewed the chemistry of hyperforin and its derivatives and the author suggests a method where the crude is extracted with n-heptane, allowing hyperforin to be isolated on a large scale from HP extracts.

1.2.4.3.1 Antibacterial Activity

Hyperforin, a lipophilic compound, is also reported by Russian researchers (Gurevich A.I. *et al.* 1971), to have antibacterial properties. In particular, hyperforin is reported to have antibacterial activity against *Staphylococcus aureus* (Brondz I. *et al.* 1982) as well as multidrug-resistant *S. aureus* and Gram-positive bacteria, including *Streptococcus pyogenes* and *Corynebacterium diphtheriae.* However, these antibacterial effects of hyperforin are only observed at high concentrations and there were no inhibitory effects against Gram-negative bacteria, such as *Enterococcus faecalis, Escherichia coli* and *Pseudomonas aeruginosa,* or against *Candida albicans* (Schempp C. M. *et al.* 1999).

1.2.4.3.2 Monoamine oxidase activity

Both hyperforin and its homologue, adhyperforin, occur exclusively in the reproductive parts of the plant. Several *in vitro* studies suggest that hyperforin and some other, as yet unidentified compound of HP, inhibit synaptosomal biogenic amine uptake and MAO, respectively (Chatterjee *et al.,*1998; Cott, 1997; Wonneman *et al.,* 1997). Muller *et al.* (1998) compared the effects of the methanolic extract with the CO_2 hyperforin (38%) enriched extract in various animal assays. These authors demonstrated that hyperforin-enriched (38%) CO_2 extract had negligible effect in MAO-A and MAO-B assays.

1.2.4.3.2 Effect on different receptors

Chatterjee *et al.* (1998a) found that supercritical extraction of HP by carbon dioxide produced extracts which were high in hyperforin content (38.8%), but did not contain hypericines and other phenolic or flavonic compounds. These authors demonstrated that pure hyperforin could be involved in serotonergic 5-HT_3 and 5-HT_4 receptor mediated processes. From the results of the

behavioural despair test and the elevated maze test, these authors also suggest that hyperforin's immediate effects resemble those of anxiolytics and that the antidepressant effects are only seen after several days, once the acute effects have faded.

The results from the synaptosomal uptake experiments by Chatterjee S.S. *et al.* (1998a), suggest that hyperforin is the major constituent of HP responsible for the inhibition of 5-HT, NA and DA reuptake. These authors showed that subchronic treatment with hyperforin also caused down regulation of the cortical β-receptors to the same degree as the whole HP extract as reported previously by Müller W.E. *et al.* (1997). This is said to be a common property of most, though not all, antidepressant drugs. Interestingly, a high dose hyperforin extract did not elevate 5-HT_2 receptor density as had been found with the whole HP extract by Müller W.E. *et al.* (1997), but the down regulation of these receptors by hyperforin was similar to the results found after subchronic TCA treatment. This led these authors to conclude that other constituents of the crude HP must be responsible for the elevation of the 5-HT_2 receptors which had been previously reported by Muller W.E. *et al* 1997.

1.2.4.3.4 Effects on the digestive system

Chatterjee S.S. *et al.* (1998a) also investigated the effects of hyperforin on the digestive system. Based on reports that HP could be effective in the treatment of bacterial and viral infections as well as dyspepsia, these authors decided to screen their CO_2 extract, which had a high hyperforin content, as well as other HP extracts, which were very low in hyperforin content, on various animal models suitable for detecting effects on digestive organs. The results showed that only the hyperforin CO_2 extract had spasmolytic like activity on guinea pig ileum and that it also inhibits serotonin uptake in rat peritoneal cells. Furthermore, these authors were able to show that extraction under controlled conditions, in the absence of light, air and high temperatures, yielded much more stable extracts with a high hyperforin content which could be stored in dark bottles at room temperature over long periods of time.

1.2.4.3.5 Anti-inflammatory Activity

A study indicating that hyperforin may contribute to the anti-inflammatory properties of HP reported that incubation of human epidermal cells with hyperforin suppressed the proliferation of alloreactive T cells when compared with the control, and also inhibited the proliferation of peripheral blood mononuclear cells in a dose dependent manner (Schempp C.M. *et al.* 2000a).

1.2.4.3.6 Effect on behavioural activity

In another study by Chatterjee S.S. *et al.* (1998b), a comparison was made in standard behavioural tests of the effects of pure hyperforin with the CO_2 extract, which did not contain any hypericin, and an ethanolic extract. The results of the learned helplessness and behavioural despair forced swim tests revealed that the pure hyperforin CO_2 extract and the ethanolic extract possessed imipramine like antidepressant activities. This led them to conclude that it is the hyperforin constituent of the hypericum extract responsible for this activity.

Bhattacharya *et al.* (1998) compared behavioural activity profiles of the whole ethanolic extract with the CO_2 extract of hyperforin in relation to the observed antidepressant activities in both rats and mice. These authors used various behavioural models including reversal of reserpine induced hypothermia, L-DOPA-induced behaviour and apomorphine-induced stereotypy. The results suggest that hyperforin may not be the only active antidepressant component and that other constituents of the extract may affect its efficacy. In particular, these authors demonstrated that hyperforin is not the constituent of HP which produces “dopaminergic activity”, but that it is most likely the constituent responsible for the serotonin reuptake inhibition. It was also noted that the effects of 50, 150 and 300mg/kg/day of the whole extract of HP corresponded with those observed with 5, 15 and 30mg/kg/day, respectively, of the CO_2 extracts, except in the case of reserpine induced hypothermia where 300mg of the whole extract of HP and only 5mg of the CO_2 extract was required to completely reverse the effects.

1.2.4.3.7 Effects on neurotransmitter release

The effects on synaptosomal uptake of neurotransmitters and MAO inhibition by the CO_2 hyperforin enriched extract and the ethanolic extract was also compared by Chatterjee S.S. *et al.* (1998b). The results of the these experiments enabled the authors to rank the order of the potencies on the various neurotransmitters as:

hyperforin : NA > DA > GABA > 5-HT >> Glutamate
the ethanolic and CO_2 extracts : DA > GABA > NA = 5-HT >> Glutamate

From these results, the authors suggest that hyperforin is a potent inhibitor of neurotransmitter uptake, but it is not the only active ingredient and it is also possible that other compounds found in HP modulate the efficacy of hyperforin. Only a high concentration of the ethanolic extract and not the hyperforin extract showed weak MAO-A and B, indicating that, although MAO inhibitors are present in the herb, the potencies are too low to be responsible for inhibition of neurotransmitter uptake in synaptosomes.

It has been reported that the mode of action of hyperforin in 5-HT uptake inhibition can be associated with the elevation of free intracellular sodium ion concentrations (Singer A. *et al.* 1999) and that this may be secondary to activation of the Na^+/H^+ exchange as a result of a decrease in intracellular pH (Singer A. *et al.* 2000). Hyperforin was shown to inhibit 5-HT reuptake in washed platelets, but not in fresh platelet-rich plasma, suggesting that plasma-protein binding could be a limiting factor for 5-HT uptake inhibition *in vivo* (Uebelhack R. and Frank L. 2000).

Kaehler S.T. *et al.* (1999) used the push-pull superfusion technique to investigate whether hyperforin, isolated from HP, influences the release of catecholamines, 5-HT, 5-hydroxyindoleacetic acid (5-HIAA) and amino acids in the rat locus coeruleus, *in vivo.* The rats were injected i.p. with 10mg/kg hyperforin and immediately the concentration of NA increased. After 60 minutes, the concentration of 5-HT increased and after 45 minutes, the extracellular concentration of dopamine increased. 5-HT and DA levels remained high while that of NA decreased after 150 minutes. 5-HIAA concentration was not affected and the only amino acid

which significantly increased in concentration was the excitatory amino acid, glutamate. These authors suggest that the increased concentrations of these compounds, is mainly due to uptake inhibition especially in the light of the finding that 5-HIAA concentration did not increase. Interestingly, GABA extracellular concentrations did not increase, which these authors speculate could either be due to GABA being taken up by non-neural cells or to modulation of GABA turnover by hyperforin.

1.2.4.3.8 Effects on serotonergic, noradrenergic and glutaminergic pathways

Dimpfel W. *et al* .(1998) studied the effects of dosing rats with the whole ethanolic extract compared with dosing with the hyperforin CO_2 extract on intracerebral field potentials in freely moving rats. This was measured by a Tele-Stereo-EEG. Both extracts contained the same amount of hyperforin and both groups were dosed orally. Previous studies as reviewed by Saletu B. (1998) have shown that some TCAs cause theta and delta power frequency increases and others lead to increases in alpha frequencies. In a previous study, Dimpfel W. *et al.* (1989) showed that manipulations in the noradrenergic system resulted in theta power changes and pharmacological manipulations in the serotonergic system, to alpha-1 power changes. In a more recent study, Dimpfel W. (1997) correlated manipulations in the cholinergic system with delta frequency power changes, using tele-stereo EEG. In this study, the authors' results show that initially both extracts reflected patterns of alpha-1 electrical power changes similar to SSRIs. After 2 hours, however, only the whole extract developed increases in delta activity similar to that of NMDA antagonists, which suggests the interaction of another compound with the glutamatergic system.

1.2.4.3.9 Effects on electroencephalography (EEG) readings

The quantitative EEG has been used for more than 20 years for analysing pharmacological actions of drugs on the CNS. Schellenberg R. *et al.* (1998) compared the effects of different concentrations of hyperforin (0.5% and 5%) in HP extracts (900mg/day) on the electroencephalography (EEG) readings of healthy volunteers. The results show that the extract containing 5% hyperforin was clinically superior when compared to 0.5% of hyperforin. The 5% showed a marked tendency to produce higher increases in EEG baseline power performances at the delta, which was most obvious after a single-dose was administered. Theta and alpha-1

frequency values were more pronounced after repetitive dosing than with the 0.5% hyperforin. The authors suggest that there is a "therapeutic window" for drugs acting on catecholaminergic transmitter systems as a result of presynaptic versus postsynaptic interactions of drugs with receptors. The presynaptic action after low drug dosage would be reflected as an increase in alpha-1 on the EEG, while higher doses may act postsynaptically (as seen with SSRIs) and result in alpha-1 decreases.

1.2.4.3.10 Oral bioavailability

Biber A. *et al.* (1998) investigated the oral bioavailability of hyperforin from *Hypericum* extracts in rats and human volunteers. In rats, the plasma levels were determined for 8 hours after oral dosing of 300mg/kg HP (containing 5% hyperforin), and these results show rather slow rates of absorption and elimination with the half-life estimated at 6 hours. The results on human volunteers show that hyperforin is an orally bioavailable constituent of HP and that concentration in the blood reaches steady-state and is easily maintained with 3 times a day dosing. Plasma levels up to 300ng/ml of hyperforin were well tolerated and only after mean plasma levels exceeded 400ng/ml was an adverse side effect of a mild headache reported.

1.2.4.3.11 Clinical study

Laakman G. *et al.* (1998) conducted the only clinical trial reported in the literature to compare HP with 0.5% and 5% hyperforin content respectively, with patients treated with placebo over a 6 week period. The sample size consisted of approximately 46 patients per group and each patient was initially rated for severity of depression according to the Clinical Global Scale (CGS) and on the Hamilton Rating Scale for Depression (HAMD). The patients did self-assessments using the Depression Self-Rating Scale. Eighty percent of the patients were rated as moderately depressed. The results at the end of the trial period show that the HP with 5% hyperforin content was the most effective treatment of depression.

1.2.4.4 Flavonoids

The flavonoids include the flavonols, flavonol glycosides and biflavones. Flavonoids are common compounds found in many plants and plant products. Quercetin is the simplest flavonoid being the aglycone of the flavonols found in HP. The structures of these compounds are illustrated in Figure 1.10 below.

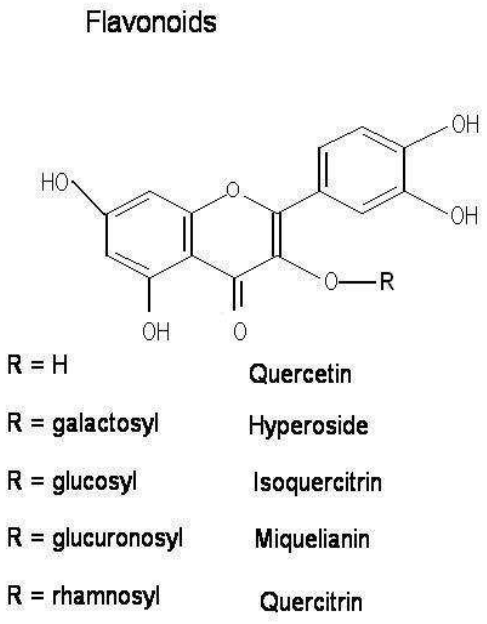


Figure 1.10 Structure of some of the flavonol glycosides present in *Hypericum perforatum* extracts.

There have only been a few studies investigating the possible role of the flavonoids in contributing to the pharmacological activity of HP.

1.2.4.4.1 Neurotransmitter effects

Calapai G. *et al* (1999) evaluated the role of flavonoids in the antidepressant action of HP that contained the same amount of hypericin (0.3%) but differing amounts of flavonoids (6% and 50%). The rats were dosed orally, 24 hours and 1 hour prior to decapitation, with either one of these extracts or the SSRI, fluoxetine (see Figure 1.11 below for structure). The effects on the

5-HT content in different regions of the brain were studied using High Performance Liquid Chromatography (HPLC). Both extracts of the flavonoids (6% and 50%) and the fluoxetine treated rats showed a significant increase in 5-HT content in the cortex, but only the extract with 50% flavonoids showed increased 5-HT in the diencephalon. In the brain stem, the 50% flavonoid extract and fluoxetine increased 5-HT content. The authors suggest that the increased levels of 5-HT in the brain stem could be important because manipulation of the hypothalamic-pituitary-adrenal axis by 5-HT may provide a new approach in the treatment of depression. Both HP extracts produced significant increases in NA and DA in the diencephalon, while only the 50% flavonoid extract produced a significant increase in NA content in the brainstem. From these results the authors concluded that the two extracts of HP act differently and that this difference can be attributed to the flavonoid content.

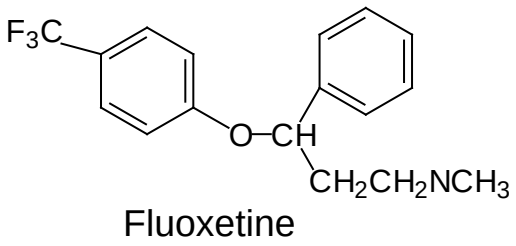


Figure 1.11 Structure of the SSRI Fluoxetine.

1.2.4.4.2 Antiviral Activity

Flavonoid and catechin-containing fractions (flavonoids often associated with condensed tannins) have also exhibited antiviral activity against the influenza virus (Mishenkova E.L. *et al.* 1975).

1.2.4.4.3 Amentoflavone

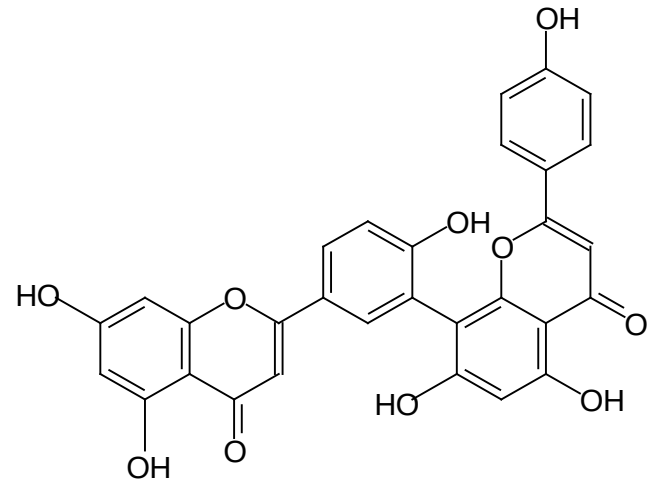


Figure 1.11 Structure of Amentoflavone

Amentoflavone (see Figure 1.11 above), one of the biflavones, was shown by Baureithel K.H. *et al.* (1997) to inhibit the binding of [^{3}H]-flumazenil to the benzodiazepine binding sites of the $GABA_A$ complex of rat brain, *in vitro.* This compound was the only one out of several isolated from HP including hypericin that showed this type of activity. The authors suggest that amentoflavone might be an important compound involved in the antidepressant effects of HP. Anti-inflammatory and anti-ulcerogenic properties have also been documented for amentoflavone (Berghofer R. and Holzl J. 1987).

1.2.4.4.4 Quercetin

Quercetin is one of the flavonoids found in HP in relatively high concentration. Quercetin has been shown to exhibit a variety of biological effects including antioxidant and free radical-scavenging activities such as protection against hydrogen peroxide-induced DNA damage in cells (Aherne S.A. and O'Brien N.M. 1999). Excessive production of nitric oxide (NO) in the brain has been associated with neurotoxicity and with the development of neurodegenerative disease. Quercetin has been shown to inhibit NO production with an IC50 less than 10^{-3}M (Soliman K.F. and Mazzio E.A. 1998). In a more recent study, Luo L. *et al.* 2004 extracted quercetin from air dried stems of HP grown in China and demonstrated this compound's inhibitory effects on NO

synthase in serum and cerebral homogenate. The authors suggest that there must be a relationship between NO synthase inhibition by quercetin and the antidepressant effect of HP.

The actual concentration of quercetin, as is found with all the other constituents of HP, varies from product to product. The amount of flavonoids present in the tincture supplied by Flora Force® which is produced from HP plants grown in the Western Cape, South Africa, has been analysed and the electrophenogram depicting these results can be seen in Figure 1.12 below.

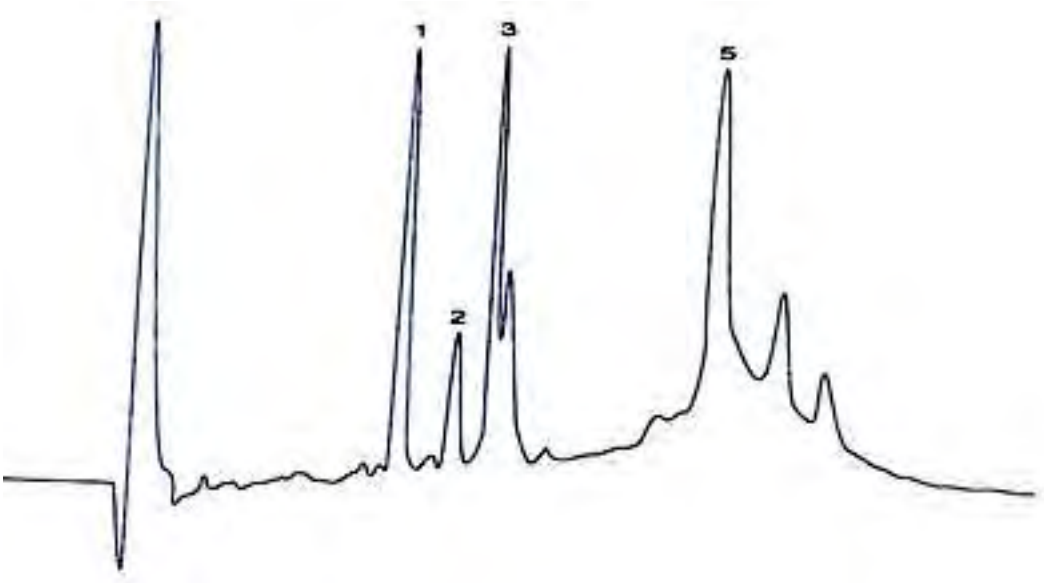


Figure 1.12 Electrophenogram illustrating the separation of flavonoid components of the Flora Force® tincture.

1 – Rutin 2 – Isoquercitrin 3 – Hyperoside 5 – Quercetin

Conditions: 25mM sodium borate with 50mM SDS, pH 8.9. Applied voltage 15 kV, Injection 200mbar for 0.1 minutes. Capillary 50μm ID, total length 82cm, effective length 53cm (Wild T.J. 2003).

1.2.4.5 Caffeic acid

Caffeic acid is one of the phenylpropanes, which was considered to be a main constituent of HP, most probably arising from hydrolysis of caffeic acid esters. There is considerable variability in the amount of this compound detected in HP extracts and more recent investigations have found that it is almost undetectable in HPLC runs of crude drug extracts (Nahrstedt A. & Butterweck V. 1997). The antioxidant effect of caffeic acid has been shown to be comparable to that of the endogenous antioxidant, glutathione (Kerry N. & Rice-Evans C. 1999). Excessive nitric oxide (NO) production in the brain has been correlated with neurotoxicity and the pathogenesis of several neurodegenerative diseases. The suppression of NO production may be beneficial in retarding many of these disorders. Caffeic acid has been shown to have a dose-dependent suppressive effect on NO production (Soliman K.F. and Mazzio E.A. 1998).

1.3 MELATONIN AND DEPRESSION

Melatonin (aMT), an indoleamine produced by the pineal gland from tryptophan, is an indicator of circadian rhythms in mammals. Changes in the circadian rhythms such as diurnal and seasonal variation, have been associated with depression since 1979 (Arendt J. 1979; Wetterberg L. 1979). Exposure to light influences aMT levels (Wetterberg L. 1979). Seasonal variation of depressive symptoms is usually associated with changes in the length of daylight hours. Patients with seasonal affective disorder (SAD) have recurring episodes of depression in winter (Checkley S.A. 1993).

It is also possible that patients with bipolar disorder, characterised by episodes of depression followed by mania, may result from disruptions in the circadian rhythms. Several studies reviewed by Pacchierotti C. *et al.* (2001) have shown that the secretion of melatonin in depressed patients may be significantly altered and the majority of these studies show a decrease in the amount of aMT secreted. In a more recent study by Crasson M. *et al.* (2004), the time of the nocturnal peak for aMT secretion was significantly delayed in patients suffering from major depression compared to healthy controls. Chronic treatmeant with antidepressants that inhibit noradrenaline reuptake have been shown to down regulate β-adrenergic receptors while increasing aMT output (Banoo S. 1991).

In animal studies predictive of antidepressant-like activity of drugs in humans, such as the tail suspension test (TST) and the forced swimming test (FST), aMT has been shown to have antidepressant properties (Mantovani M. *et al.* 2003; Raghavendra V. *et al.* 2000). Recent evidence indicates adaptations in the *N*-methyl-D-aspartate (NMDA) receptor complex following antidepressant treatment (Skolnick P. *et al.* 1996). Mantovani M. *et al.* (2003) reported that aMT exerts an antidepressant-like effect in the TST and that this seems to be mediated through an interaction with NMDA receptors and the L-arginine-nitric oxide pathway.

1.4 APOPTOSIS

Cell death occurs naturally when developing neurons undergo an active process of dying and this is seen as a critical phase in the development of the nervous system. Different types of programmed cell death have been described, with apoptosis and necrosis being the most common. Neuronal apoptosis can also be considered to be pathological and is implicated in diseases such as Alzheimer's, Parkinson's and Huntington's (Martinou I. *et al,* 1997). Necrosis is usually the result of severe insult causing cell damage that is characterised by a breakdown of the membranes and disruption of organelles. Inflammation is usually a result of necrosis and this distinguishes it from apoptosis which is the result of programmed cell death.

Recent findings indicate that dysfunction of the CNS glutamatergic pathways may represent one of the mechanisms involved in the pathophysiology of depression (Heresco-Levy U. and Javitt D.C. 1998). The *N*-methyl-D-aspartate receptor is a highly Ca^{2+}-permeable, ligand-gated ion channel in neurons and a member of the ionotropic glutamate receptor family. Excessive stimulation of the NMDA receptors leads to excessive intracellular Ca^{2+} influx, generation of free radicals such as nitric oxide and reactive oxygen species, collapse of the mitochondrial membrane potential, loss of ATP, and eventually neuronal apoptosis. This process is termed excitotoxicity and is considered to be an integral component in a final common pathway to neuronal injury in neurodegenerative disease (Kaul M. and Lipton S.A. 2000). It has been suggested that recurrent depressive episodes inflict cumulative neuronal injury, particularly in the hippocampus where there is a high concentration of glutamatergic synapses, as a result of the neurotoxic actions of glucocorticoids, glutamate and nitric oxide (Sapolsky R.M. 2000).

Figure 1.13 below illustrates the NMDA receptor signaling pathways that lead to neuronal apoptosis which may contribute to neurodegenerative disease. These pathways can be targeted to prevent neuronal apoptosis by:

1. Antagonizing NMDA receptors
2. Modulating the activation of the p38 mitogen activated kinase (MAPK) – MEF2C (transcription factor) pathway

3. Preventing toxic reactions of free radicals such as nitric oxide (NO) and reactive oxygen species (ROS), and
4. Inhibiting apoptosis-inducing factors including caspases (Kaul M. and Lipton S.A. 2000).

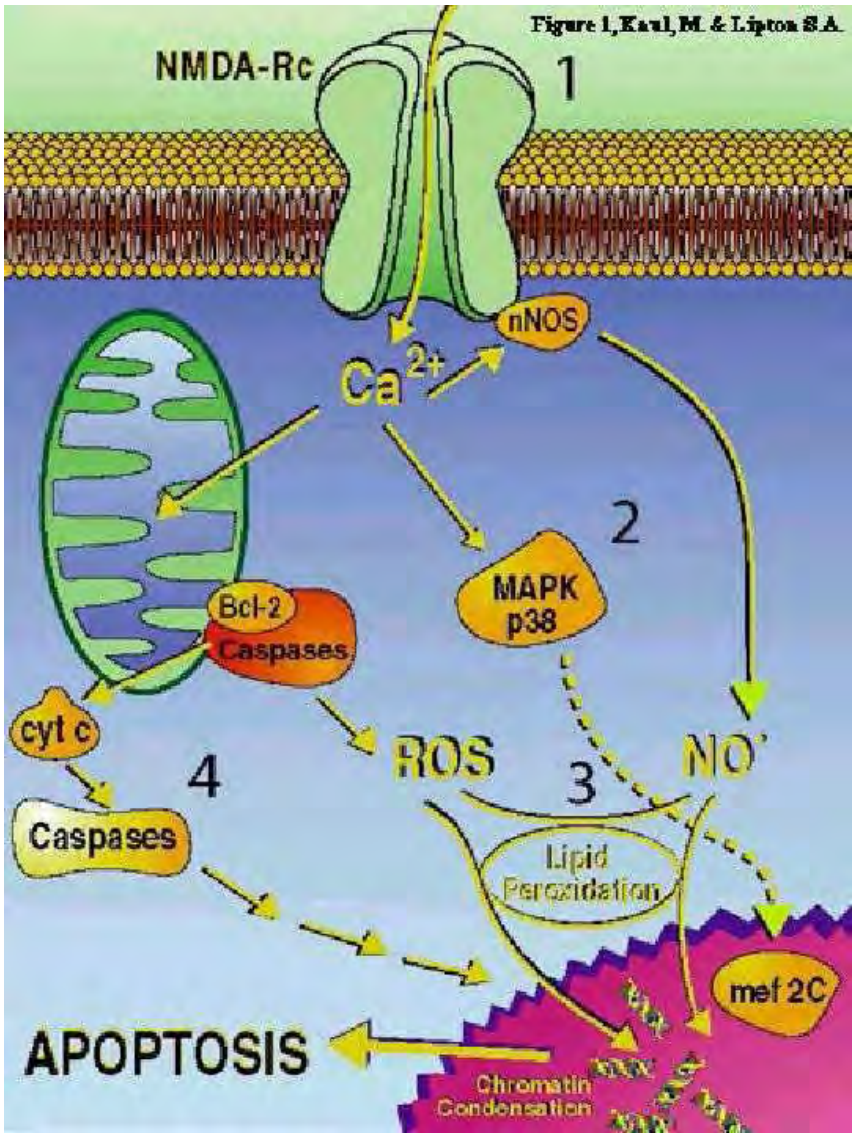


Figure 1.14 Current model of NMDA receptor-associated neuronal injury (Kaul M. and Lipton S.A. 2000).

1.5 OBJECTIVES

The pharmacological properties of *Hypericum perforatum* produced in the Northern hemisphere are well documented and it is clearly one of the leading psychotherapeutic phytomedicines on the market. The crude extract has been shown to affect a wide range of neuronal receptors and while researchers have tried to show that one compound, for example hypericin or hyperforin, is responsible for its antidepressant effects, it is now becoming clear that this is more likely the result of several of the compounds found in HP, acting synergistically.

HP is also indigenous to the Western Cape of South Africa. The medicinal use of HP as an antidepressant in this country is based on the results of studies conducted in the Northern hemisphere, particularly Europe, where standardised locally cultivated products were used. There is only one study that compares some of the products produced in South Africa using quantitative analysis and the results of this study revealed high inter-product variability (Wild T.J. 2003). There are no studies investigating the pharmacological activity of any of the HP extracts produced from the indigenous plants grown in the Western Cape.

The purpose of the present study is to investigate the effect of the crude extract of HP grown in the Western Cape of South Africa, and some of its constituents in several biological assays indicative for antidepressant activity. These assays include the effect on pineal metabolism, tryptophan pyrrolase and receptor binding. Brain serotonin concentrations are measured using enzyme-linked immunosorbent assay (ELISA) and brain concentrations of monoamine neurotransmitters, dopamine, serotonin and their metabolites are determined using high performance liquid chromatography (HPLC). All the assays compare the effects of HP, quercetin and/or caffeic acid with that of the SSRI, fluoxetine (see Figure 1.11, page 31 for structure), a drug known for its antidepressant activity. Finally, the effect of HP and one of its constituents, quercetin, on neuronal apoptosis is investigated.

CHAPTER 2

PINEAL ORGAN CULTURE STUDIES.

INTRODUCTION

The pineal gland, believed by Descartes to be the seat of the soul, is now established as an integral component of the neuroendocrine system. (Reiter R.J. 1989.) The pineal arises from the roof of the third ventricle under the posterior end of the corpus callosum and is connected by a stalk to the posterior commissure. The pineal stroma contains neuralgia and parenchyma cells with secretory functions (Ganong W.F. 1991).

The ability of the pineal gland to convert a nervous input into hormonal output characterises the gland as a neuroendocrine transducer. Pineal innervation comes from the autonomic as well as the parasympathetic nervous system. The rat pineal gland is able to use tryptophan to synthesise a number of indoleamines. The pathway for tryptophan metabolism is shown in Figure 2.1.1 on page 42. Serotonin (5-HT) is an important intermediate that can be metabolised by three different pathways:

(i) oxidative deamination by monoamine oxidase (MAO)
(ii) N-acetylation and
(iii) O-methylation.

5-HT is metabolised by MAO to 5-hydroxyindole acetaldehyde, an intermediate that is either oxidised to 5-hydroxyindole acetic acid (5-HIAA) or reduced to 5-hydroxytryptophol (5-HTOH). These metabolites are substrates for the enzyme hydroxyindole-O-methyltransferase (HIOMT) that metabolises 5-HIAA and 5-HTOH to 5-methoxyindole acetic acid (5-MIAA) or 5-methoxy tryptophol (5-MTOH) (Reiter R. J. 1989).

5-HT conversion to melatonin (aMT) is a major pathway involving two steps. The primary step is the N-acetylation of 5-HT by the enzyme N-acetyl transferase (NAT) to yield N-acetylserotonin (NAS) and the acetyl group is provided by acetyl co-enzyme A hydrolase. In the second step, a methoxy group is transferred from S-adenosylmethionine (SAM) to the 5-hydroxy position of NAS yielding aMT. This conversion is catalysed by HIOMT (Reiter R.J. 1989).

Changes in the synthesis of melatonin have been suggested to be involved in depression. Reduced levels of the neurohormone in major depression have been associated with a deficiency of noradrenaline (Checkley S.A. *et al.*, 1986). aMT is released from the pineal gland with a circadian rhythmicity (Reiter R.J., 1991) and depression is often associated with disturbances in circadian rhythms such as sleep. It has been suggested that the initiation of aMT synthesis depends on the level of activity of the noradrenergic system and that the peak of aMT rhythm could be determined by the availability 5-HT and/or aMT turnover (Skene D.J. *et al.*, 1994).

The following studies were undertaken to determine the effect of fluoxetine, *Hypericum perforatum*, quercetin and caffeic acid (two of the constituents of HP) on the metabolism of 5-HT in the pineal gland of the rat. The rats were dosed orally for a minimum of eighteen days since it is well known that there is a two to three week lag period before the therapeutic effects of antidepressants are manifested (Leonard B.E. 1997).

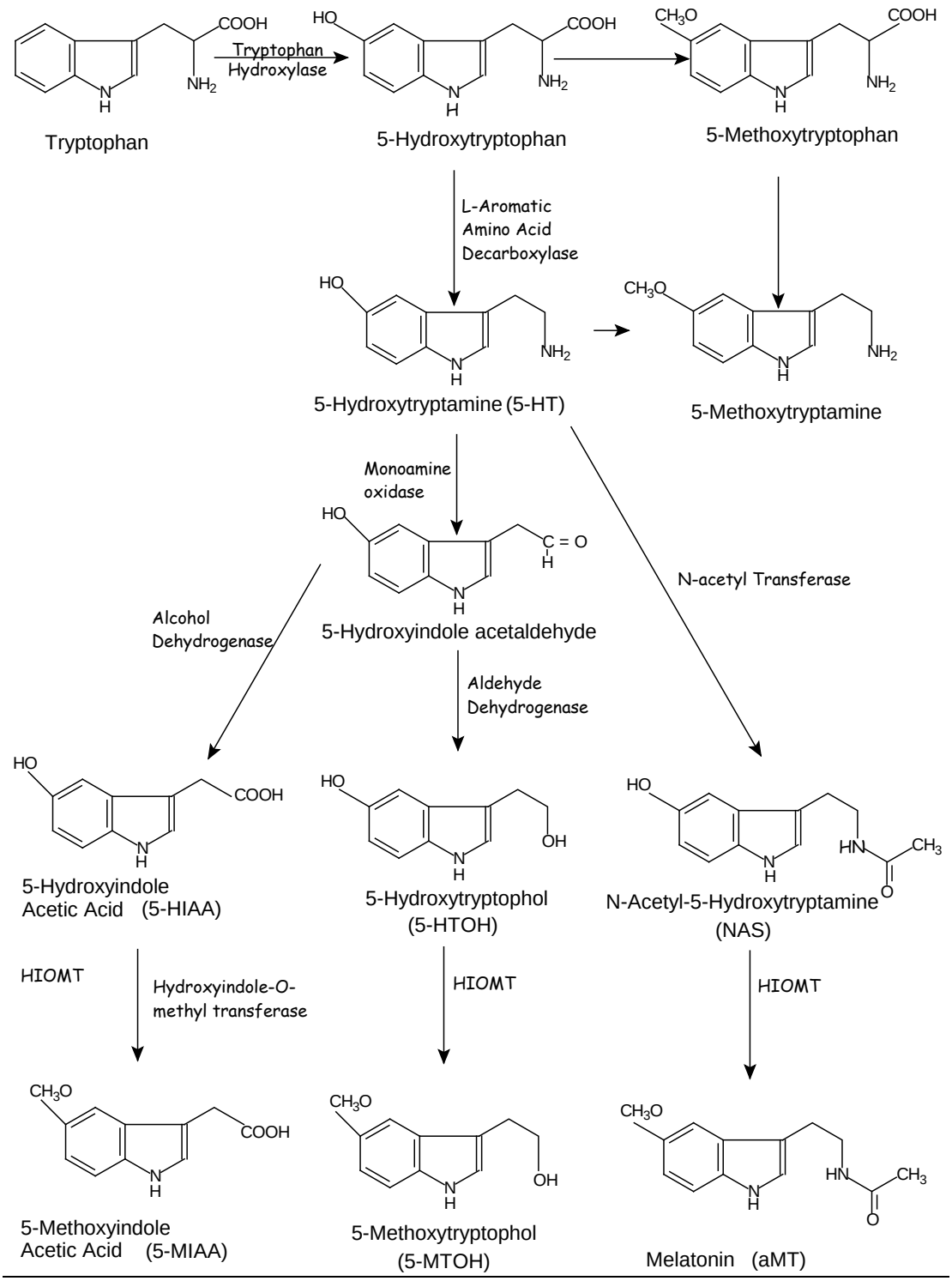


Figure 2.1.1: The Pathway of Indole Metabolism in the Pineal Gland

2.1.1 EFFECT OF *Hypericum perforatum* AND FLUOXETINE ON PINEAL INDOLE METABOLISM.

2.1.2 Introduction

Organ culture techniques are widely used in research because these are convenient and not time consuming. The pineal gland is especially suitable because it is small, easily accessible and able to maintain its metabolic function. It is able to synthesise various indoleamines by utilising a radioactive exogenous precursor such as [^{3}H]-tryptophan or [^{14}C]-serotonin and 95% of the indoles synthesised during the incubation period are secreted into the culture media (Khan R.B., 1989; Klein D.C. and Rowe J. 1970). These radioactive indoles can then be isolated from the media, analysed and quantified.

The isolation of the radioactive metabolites was performed using Thin Layer Chromatography (TLC) and quantification, by using liquid scintillometry. The bi-dimensional TLC system first employed by Klein D.C. *et al.* (1969) was used for the separation of pineal indoles. The pineal indoles were separated by two solvent systems: the first one comprised of chloroform, methanol and glacial acetic acid [93:7:1] and the second used ethyl acetate only. The first solvent system separated aMT and NAS and the 5-hydroxyindoles from the 5-methoxyindoles. The glacial acetic acid effectively separated 5-HIAA from 5-MIAA. The second organic solvent, ethyl acetate, optimised the separation of 5-MIAA and 5-MTOH from aMT and the separation of 5-HTOH and 5-HIAA from NAS. Serotonin was unaffected by the two solvents and thus remained at the point of origin. This assay was effective and allowed trace quantities of indoles to be separated, (1pmole/10µl).

In this study, the effect of *Hypericum perforatum* and fluoxetine on serotonin metabolites secreted by the pineal gland was determined using the organ culture technique and thin layer chromatography.

2.1.2 Materials and Methods:

2.1.2.1 Chemicals and Reagents

Chloroform, methanol and glacial acetic acid were from UNILAB (Saarchem, South Africa). Hydrochloric acid from Merck (South Africa) and radiolabelled tryptophan from Amersham Biosciences (United Kingdom). Fluoxetine was obtained from Eli Lily (Pty) Ltd (South Africa) and *Hypericum perforatum* from Flora Force Health Products (Pty) Ltd (Rondebosch, South Africa).

2.1.2.2 Animals

Male rats of the Wistar strain were used in all experiments and were housed five per cage in a well ventilated room with an automatically regulated lighting cycle of 12 hours light (6am – 6pm) followed by 12 hours darkness. The rats were allowed access to a standard diet and water *ad libitum.*

Fifteen male Wistar rats, weighing between 200 and 250g, were used and divided randomly into three groups consisting of five rats each. These were dosed orally twice a day, morning and evening, for eighteen days in the following manner:

1. 5 rats were dosed with 0.4ml 35% ethanol (control group).
2. 5 rats were dosed with 0.4ml *Hypericum perforatum* extracted in 35% ethanol, the resulting dosage being approximately 300mg/kg/day (Hypericum group).
3. 5 rats were dosed with 0.4ml Fluoxetine also diluted in 35% ethanol, the resulting dosage being approximately 1.25mg/kg/day (Fluoxetine group).

The dosage of 300mg/kg/day of HP was the most commonly used dose found in the literature reviewed. It was decided to halve the dosage of fluoxetine that had been previously used in our laboratory because that dosage (2.5mg/kg/day) had been found to be toxic in our laboratory.

2.1.2.3 Tissue preparation

At the end of the dosing period, the rats were sacrificed by neck fracture and decapitated between 10h00 and 12h00. A sharp scissor blade was inserted into the foramen magnum and the skull was cut laterally on both sides to the area just behind the orbit. This part of the skull was lifted upwards and folded back using a clean pair of forceps. The brain was gently peeled away from the dura and the skull, exposing the pineal gland which was often found adhering to the skull. Occasionally, the pineal was found in the groove between the cerebral hemispheres, anterior and cephalic to the colliculi. The pineals were carefully removed with forceps and placed individually into sterile Kimble glass tubes which each contained 56µl BGJb culture medium and 4µl of radiolabelled [^{3}H]-tryptophan (4µCi) was added. The air in the tube was displaced with carbogen (95% O_2/5% CO_2), the tubes were sealed with parafilm and incubated in the dark at 37^0C for 24 hours. The following day the culture medium was assayed for pineal indoles using TLC.

2.1.2.4 Thin Layer Chromatography (TLC)

The TLC technique used was based on the method developed by Klein and Notides (1969), with a few adaptations and consisted of the following steps:

1. 10 x 10 cm silica gel plates (from Merck, West Germany) were spotted with 10µl of the culture medium and evaporated with a gentle stream of nitrogen.
2. 5µl of "cold" indoleamine standards were spotted on top of the culture medium and also evaporated with nitrogen (N_2).
3. The TLC plates were then developed twice in the same direction in a solvent containing the following solvent system: chloroform : methanol : glacial acetic acid (93:7:1), until the solvent front reached 9 cm up the TLC plate. The plates were then dried with N_2 after each run.
4. The TLC plate was then placed in the second tank, containing the solvent ethyl acetate and run at right angles to the first direction. The solvent was allowed to migrate 6 cm up the plate and then the plate was again dried with N_2.
5. The plate was sprayed with Van Urck's reagent (1g of paradimethylbenzaldehyde dissolved in 50ml of 25% HCl followed by the addition of 50ml of 95% ethanol), and the

plates were placed in an oven at 60^0C for 20 minutes to facilitate the visualisation of the compounds.

6. The radioactivity of each spot was determined by scraping off each spot into a scintillation vial containing 1ml absolute ethanol. 3ml of scintillation cocktail was added to each vial and vortexed for 30 seconds. Thereafter, the vials were placed in a Beckman LS 2800 scintillation counter for quantification.

The preparation of the standards involved dissolving 4μg of each indole in 0.5ml of absolute ethanol containing 1% (w/v) vitamin C, to limit oxidation and kept in the dark at 0^0C.

2.1.2.5 Statistical Analysis

All data was statistically analysed using one way analysis of variance (ANOVA) followed by Newman-Keuls test for multiple column comparison. Results were expressed as means±S.E.M. Significance was set at $P<0.05$.

2.1.3 Results

A typical bi-dimensional thin layer chromatogram of the pineal indole metabolites can be seen in figure 2.1.2 below. The six blue-green spots of the pineal metabolites were clearly separated and could be easily identified.

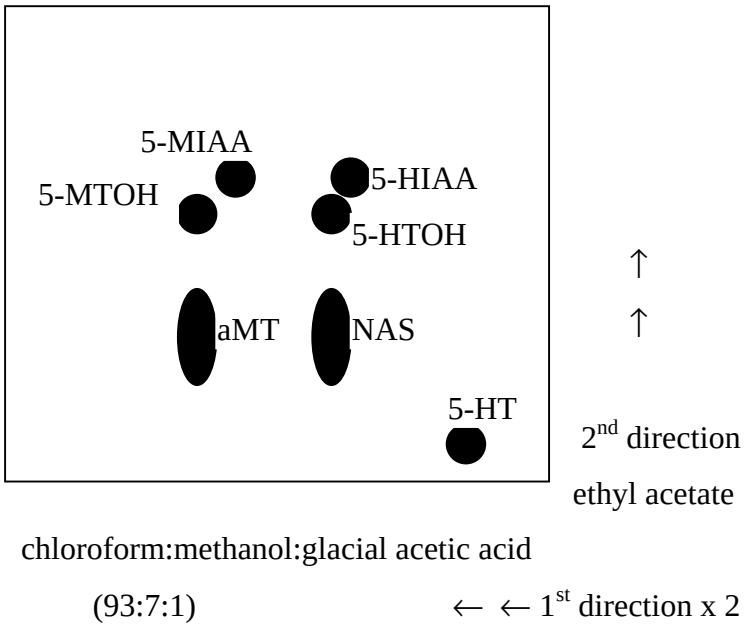


Figure 2.1.2: Bi-dimensional TLC plate of pineal indole metabolism.

Key: 5-HT = serotonin NAS = N-acetylserotonin
aMT = melatonin 5-HTOH = 5-hydroxytryptophol
5-HIAA = 5-hydroxyindoleacetic acid
5-MTOH = 5-methoxytryptophol
5-MIAA = 5-methoxyindoleacetic acid

The results of the liquid scintillometry were expressed as a percentage of total ^{3}H-tryptophan added in dpm's/10µl culture medium/pineal gland/24 hour incubation period (means± S.E.M.) and summarised in Table 2.1 below.

Table 2.1 Effect of treatments with *Hypericum perforatum* and fluoxetine on the percentage of indole levels released in the culture medium after 24hour incubation period (means ±S.E.M). n = 5; *P<0.05;P<0.01; ***P<0.001.**

Indoles	Control	Hypericum	Fluoxetine
aMT	0.18±0.02	0.22±0.02	0.27±0.04*
NAS	2.43±0.12	1.89±0.27	1.82±0.14*
5-HIAA	16.39±0.97	10.52±0.51***	14.68±0.62**
5-HTOH	5.68±0.22	5.95±0.28	5.27±0.24
5-MIAA	0.2±0.02	0.18±0.01	0.14±0.01*
5-MTOH	0.27±0.01	0.28±0.01	0.34±0.02*

The amount of radio-labelled melatonin released into the culture medium after a 24 hour incubation period was significantly increased ($P<0.05$) in the rats treated with fluoxetine when compared with the controls(See Figure 2.1.3 below).

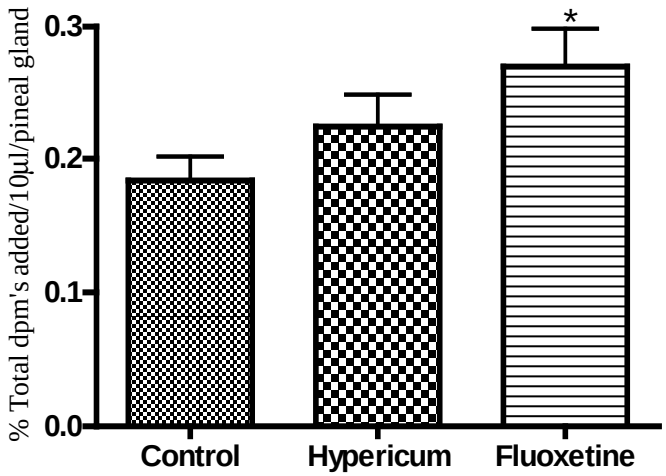


Figure 2.1.3 Effect of *Hypericum perforatum* and fluoxetine on the amount of aMT released into the culture medium by the rat pineal gland after a 24 hour incubation. *P<0.05

The amount of NAS released into the culture medium after a 24 hour incubation period was significantly decreased ($P<0.05$) in the rats treated with fluoxetine when compared with the controls as shown in Figure 2.1.4 below.

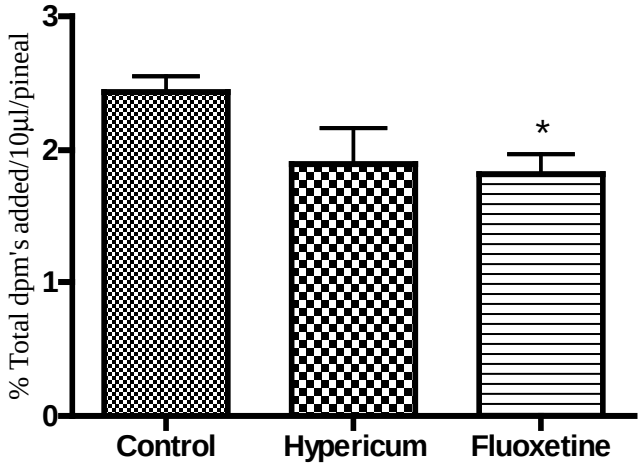


Figure 2.1.4 Effect of *Hypericum perforatum* and fluoxetine on the amount of NAS released into the culture medium by the rat pineal gland after 24 hour incubation. *P<0.05 and n = 5.

The amount of 5-HIAA released into the culture medium after 24 hour incubation period was significantly decreased in the rats treated with HP($P<0.001$) and fluoxetine ($P<0.01$) when compared with the controls, as can be seen in Figure 2.1.5 below.

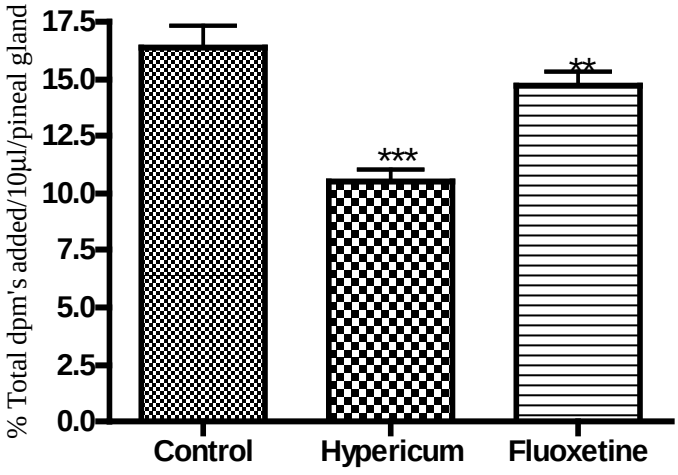


Figure 2.1.5 Effect of *Hypericum perforatum* and fluoxetine on the amount of 5-HIAA released into the culture medium by the rat pineal gland (n = 5) after a 24 hour incubation.**P<0.01, ***P<0.001.

There was no significant difference in the amount of 5-HTOH released into the culture medium by the pineal gland after a 24 hour incubation period (see Figure 2.1.6 below).

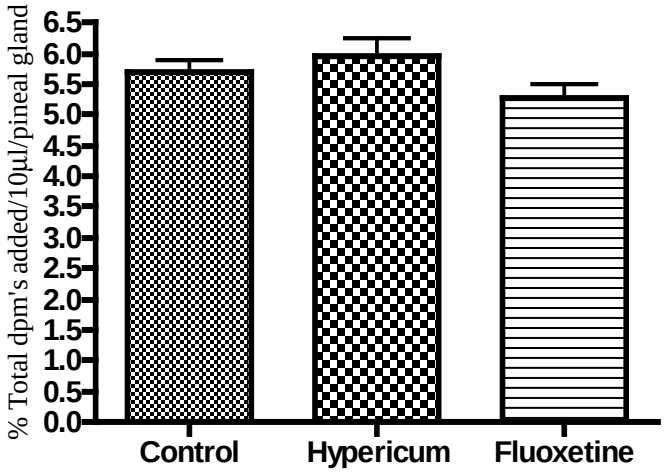


Figure 2.1.6 Effect of *Hypericum perforatum* and fluoxetine on the amount of 5-HTOH released into the culture medium by the rat pineal gland (n = 5) after 24 hours incubation.

There was a significant (P<0.05) decrease in the amount of 5-MIAA released into the culture medium by the pineal glands period from the rats treated with fluoxetine after a 24 hour incubation period, as can be seen in Figure 2.1.7 below.

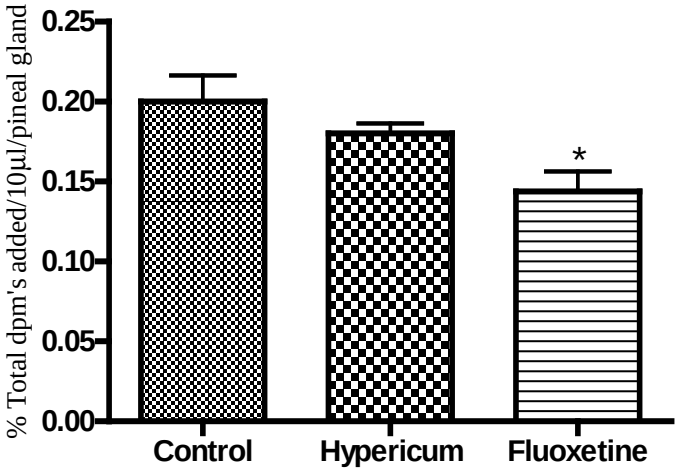


Figure 2.1.7 Effect of *Hypericum perofatum* and fluoxetine on the amount of 5-MIAA released into the culture medium by the rat pineal gland after a 24 hour incubation. *P<0.05

Fluoxetine significantly increased (P<0.05) the amount of 5-MTOH released into the culture medium after 24 hour incubation period as shown in Figure 2.1.8 below.

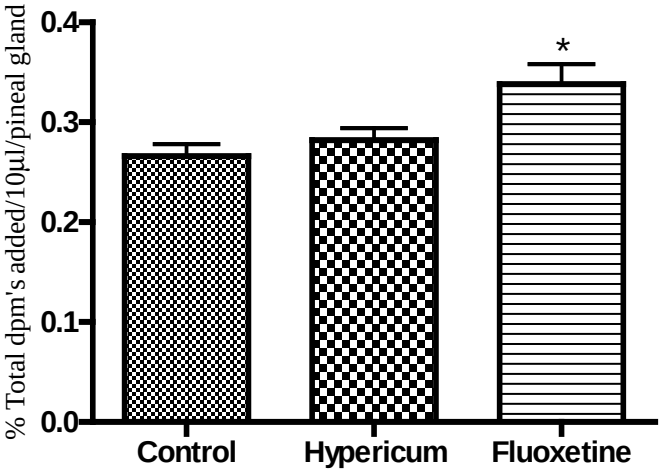


Figure 2.1.8 Effect of *Hypericum perforatum* and fluoxetine on the amount of 5-MTOH released into the culture medium by the rat pineal gland after 24 hour incubation. *P<0.05, n=5.

The sum of the four (5-HIAA, 5-HTOH, 5-MIAA, 5-MTOH) monoamimne oxidase (MAO) metabolites released into the culture medium by the rat pineal gland after 24 hour incubation was not significantly different as shown in Figure 2.1.9 below.

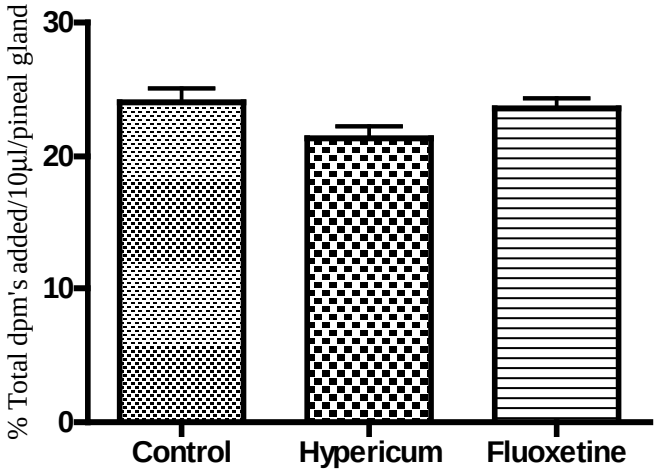


Figure 2.1.9 Effect of *Hypericum perforatum* and fluoxetine on the total amount of MAO metabolites (5-HIAA,5-HTOH, 5-MIAA, 5-MTOH) released into the culture medium by the rat pineal gland after 24 hour incubation. n=5.

2.1.4 Discussion

The results of the pineal organ culture were inconclusive, but the following bears mentioning. The sum of the % of the total dpm's added to the culture medium for the four MAO metabolites (5-HIAA, 5-HTOH, 5-MIAA & 5-MTOH), were not significantly different from that of the control suggesting that neither HP nor fluoxetine antidepressant effects result from the inhibition of the enzyme MAO. This is not surprising for fluoxetine, a selective serotonin reuptake inhibitor, and this finding is consistent with previous studies (Skene D.J. 1985). Prevous studies with whole HP extract have only reported weak or no inhibition of MAO (Cott J.M. 1997; Müller W.E. 1997).

The most significant effects on individual pineal metabolites were caused by fluoxetine. There was a significant decrease in the levels of NAS and a significant increase in melatonin production suggesting increased activity of the enzyme hydroxyindole-O-methyl transferase (HIOMT) on NAS as a substrate. Similarly, increased activity of this enzyme would explain the significant increase in the level of 5-MTOH. The significant decrease in 5-HIAA and 5-MIAA suggests an

increase in the activity of the enzyme alcohol dehydrogenase. The most noteworthy result in the pineals of the rats treated with HP was a very significant decrease in the amount of 5-HIAA released into the culture medium. Again this suggests increased activity of alcohol dehydrogenase.

De Bellis M.D. *et al.* 1993 found significantly decreased 5-HIAA levels in the cerebrospinal fluid of fluoxetine treated patients with major depression. 5-HIAA is the major metabolite of 5-HT (van Praag H. 1982) and the ratio [5-HIAA/5-HT] has been shown to be an indicator of 5-HT turnover. A reduced turnover rate has been reported to occur after treatment with some TCAs and also HP (Butterweck V. *et al.* 2002). The results of this study suggest that some of the antidepressant effects of HP may be brought about by a similar mechanism of action to the SSRI, fluoxetine. It was decided to compare the effect of HP with two of its constituents, quercetin and caffeic acid on pineal indole metabolism in the following study.

2.2 EFFECT OF *Hypericum perforatum*, QUERCETIN AND CAFFEIC ACID ON PINEAL INDOLE METABOLISM

2.2.1 Introduction

The results from the previous study indicate that *Hypericum perforatum* may have similar effects to fluoxetine on the serotonin metabolites produced by the pineal gland. In particular, there were significant decreases in the pineal metabolite 5-HIAA in the rats treated with HP and in the rats treated with fluoxetine. Significant decreases in 5-HIAA levels in cerebrospinal fluid have been reported following fluoxetine treatment in patients with major depression (De Bellis M. D. 1993).

In this study, the effects of *Hypericum perforatum* and two of its constituents, quercetin and caffeic acid, on the amount of serotonin metabolites released by the rat pineal gland, was investigated. The aim of the study was to determine whether quercetin and/or caffeic acid could be the active compound contributing to the effects of HP.

2.2.2 Materials and Methods

2.2.2.1 Chemicals and Reagents

Chloroform, methanol, ethanol, propylene glycol (propane-1,2-diol), glycerol and glacial acetic acid were from UNILAB (Saarchem, South Africa). Hydrochloric acid was from Merck (South Africa) and radiolabelled serotonin from Amersham Biosciences (United Kingdom). *Hypericum perforatum* was from Flora Force (Pty) Ltd (Rondebosch, South Africa), quercetin and caffeic acid were from Sigma Aldrich (Switzerland).

2.2.2.2 Animals

Adult male rats of the Wistar strain, weighing between 200 and 250g were used in this study and divided randomly into four groups consisting of five rats each. It was decided to change the dosing procedure from that outlined in section 2.1.2.2 because the rats had become anxious and

had been aggressive during this dosing procedure. It was decided to reduce the amount of stress that had been induced by the previous form of dosing by using the procedure outlined below.

The rats were placed in individual cages every morning and dosed for twenty one days. Each rat received a quarter digestive biscuit with the drug dissolved on it. The rats remained in their individual cages until the biscuit had been eaten and were then placed in communal cages (five per group).

5 rats in the control group received the vehicle made of ethanol, propylene glycol and glycerol (2:2:1)
5 rats in the hypericum group received 300mg/kg/day HP dissolved in the vehicle.
5 rats in the quercetin group received 5mg/kg/day quercetin dissolved in the vehicle.
5 rats in the caffeic acid group received 5mg/kg/day caffeic acid dissolved in the vehicle.

The same dosage of HP of 300mg/kg/day was used as in the previous study because this was the most common dosage used by other researchers in the literature that was reviewed. There was no indication of dosage required for this study after extensive research of the literature into the acceptable standard dosage of quercetin and caffeic acid. Almost all previous research conducted was *in vitro* and at a molecular level. It was decided to start at a standard acceptable dose of 5mg/kg/day for quercetin and caffeic aid. The next study used lower doses of quercetin (2.5mg vs 1mg/kg/day) and this revealed 5mg/kg/day to be a more effective dose for the biological studies undertaken. Similar subsequent studies with caffeic acid were not undertaken because later HPLC analysis of the HP tincture in our laboratories of the tincture used in these studies showed that this compound was not present at a significant level (results not shown).

2.2.2.3 Tissue preparation

At the end of the dosing period, the rats were sacrificed by neck fracture and decapitated as explained in section 2.1.2.3. Each pineal was placed into a sterile Kimble glass tube that contained 52µl BGJb culture medium and 8µl of radiolabelled [^{14}C]-serotonin (50µCi) was added to each tube. The tubes were sealed with Parafilm after the air had been displaced with carbogen (95%O_2/5%CO_2). The pineals were incubated at 37^{0}C in the dark, overnight. The

following day the pineals were flicked out of the culture medium onto the side of the vial to terminate the synthesis of indoleamines into the culture medium. The vials were frozen until the culture medium could be assayed for pineal indoles using thin layer chromatography.

2.2.2.4 Thin Layer Chromatography (TLC)

The TLC technique and method used were the same as outlined in section 2.1.2.4 for steps (1 – 4).

5. Visualisation of the compounds was carried out under ultra violet light and the spots were circled in pencil.
6. The spots were cut out of the TLC plate and placed individually into scintillation vials containing scintillation cocktail. These were vortexed for 30 seconds and left overnight before being placed in a Beckman LS 2800 scintillation counter for quantification.

2.2.2.5 Statistical Analysis

The statistical analysis was performed as set out in section 2.1.2.5.

2.2.3 Results

A typical TLC plate showing the separation of the pineal indole metabolites is shown in Figure 2.1.2 above. The results of the liquid scintillometry are expressed as the average % of the total dpm's/10µl/pineal gland added for each group ±S.E.M. This is summarised in Table 2.2 below.

Table 2.2 Effect of treatments with *Hypericum perforatum,* caffeic acid and quercetin on the percentage of indole levels released into the culture medium after 24 hour incubation period (means±S.E.M) *P<0.05; **P<0.01; ***P<0.001.

Indoles	Control	Hypericum	Caffeic Acid	Quercetin
aMT	0.46±0.05	0.55±0.09	0.54±0.07	0.77±0.11*
NAS	0.75±0.15	0.55±0.12	0.82±0.1	1.0±0.12
5-HIAA	27.92±1.68	34.93±1.68	32.81±0.94	37.34±1.49**
5-HTOH	7.92±0.17	5.77±18*	3.76±0.72**	5.86±1.25
5-MIAA	0.49±0.09	0.44±0.08	0.49±0.05	0.54±0.07
5-MTOH	0.77±0.11	0.37±0.08	0.63±0.08	0.57±0.11

The comparison of the effects of these compounds on the individual indoleamines is depicted in the following Figures 2.2.1 to 2.2.6.

The amount of radio-labelled melatonin released into the culture medium after a 24 hour incubation period was significantly increased (P<0.05) in the rats treated with quercetin, as shown in Figure 2.2.1 below.

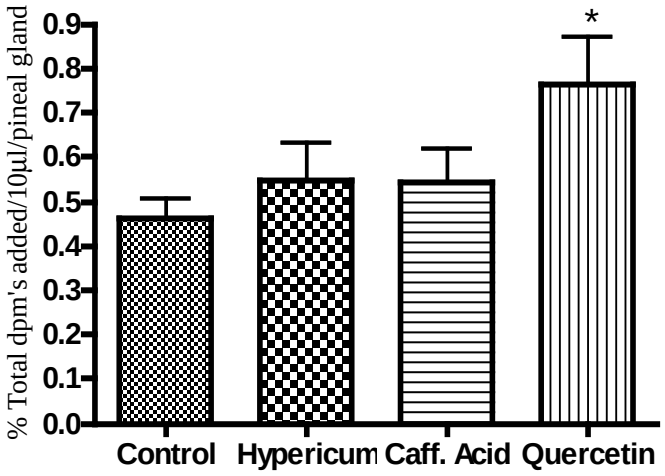


Figure 2.2.1 Effect of *Hypericum perforatum,* caffeic acid and quercetin on the amount of aMT released into the culture medium by the rat pineal gland after 24 hour incubation. *P<0.05

There were no significant differences in the amount of NAS released into the culture medium by the pineal gland after a 24 hour incubation period (see Figure 2.2.2 below).

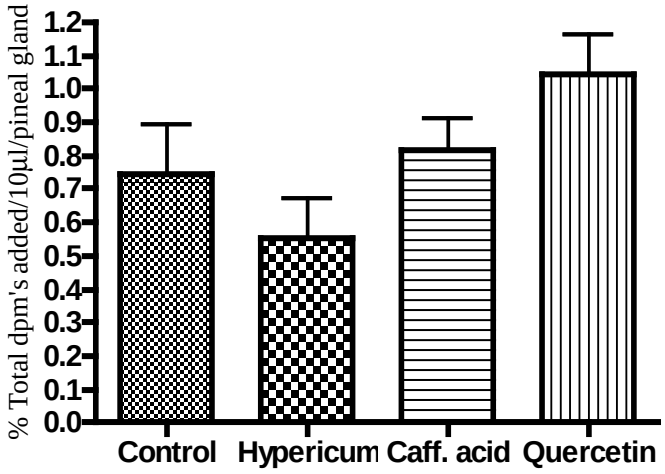


Figure 2.2.2 Effect of *Hypericum perforatum*, caffeic acid and quercetin in amount NAS released into the culture medium by the rat pineal gland after a 24 hour incubation.

There was a significant (P<0.05) increase in the amount of 5-HIAA released into the culture medium by the pineal gland after a 24 hour incubation period (see Figure 2.2.3 below).

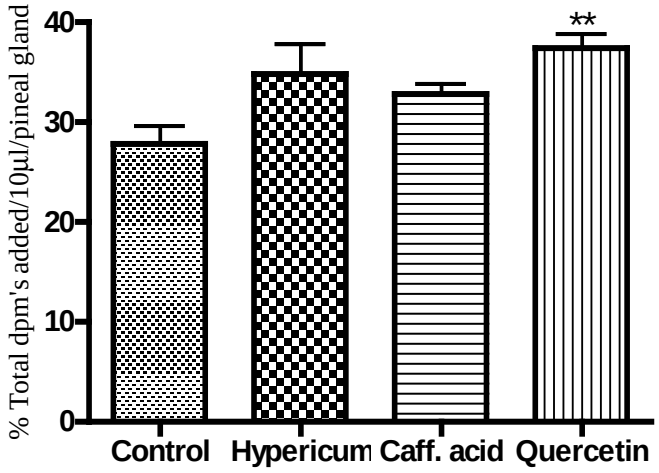


Figure 2.2.3 Effect of *Hypericum perforatum*, caffeic acid and quercetin on the amount of 5-HIAA released into the culture medium by the rat pineal gland after 24 hour incubation.
**P<0.05

There was a significant decrease in the amount of 5-HTOH released into the culture medium by the pineal glands in the rats treated with caffeic acid ($P<0.001$) and HP ($P<0.05$) respectively after a 24 hour incubation period (see Figure 2.2.4 below).

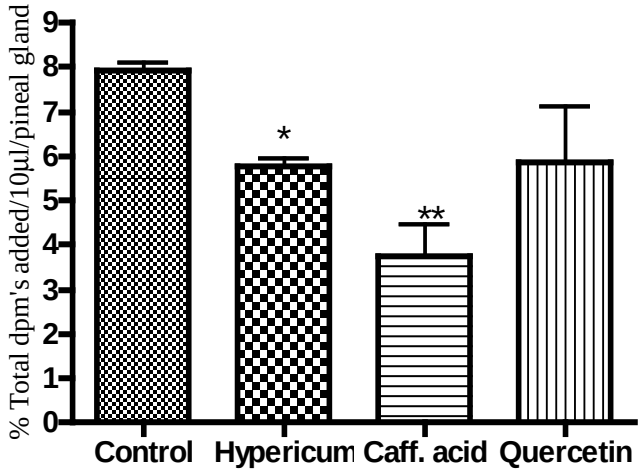


Figure 2.2.4 Effect of *Hypericum perforatum*, caffeic acid and quercetin on the amount of 5-HTOH released into the culture medium by the pineal gland after 24 hour incubation.
*P<0.05; **P<0.01

There were no significant differences in the amount of 5-MIAA and 5-MTOH released into the culture medium by the pineal gland after a 24 hour incubation period (see Figures 2.2.5 and 2.2.6 below).

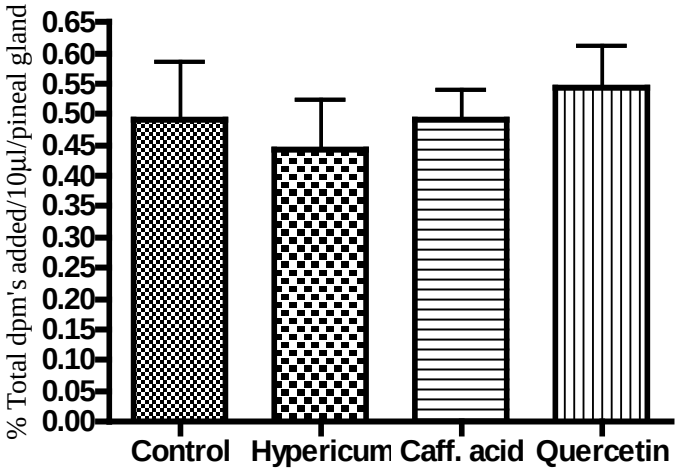


Figure 2.2.5 Effect of *Hypericum perforatum*, quercetin and caffeic acid on the amount of 5-MIAA released into the culture medium by the pineal after 24 hour incubation.

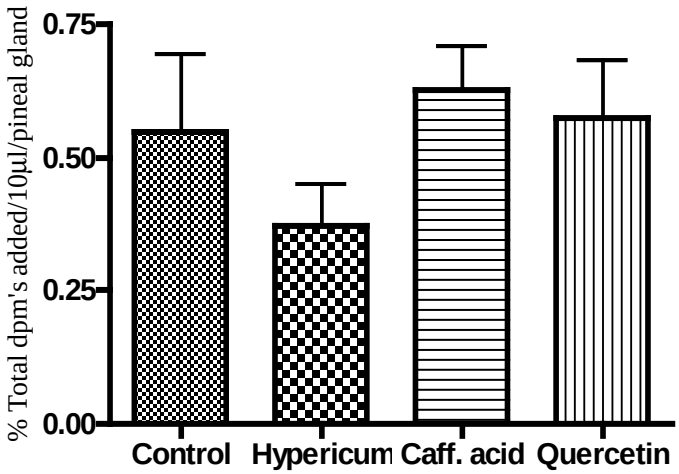


Figure 2.2.6 Effect of *Hypericum perforatum*, caffeic acid and quercetin on amount of 5-MTOH released into the culture medium by the rat pineal gland after 24 hour incubation. n=5

The sum of the four (5-HIAA, 5-HTOH, 5-MIAA, 5-MTOH) monoamine oxidase (MAO) metabolites released into the culture medium by the rat pineal gland after 24 hour incubation were not significantly different as shown in Figure 2.2.7 below.

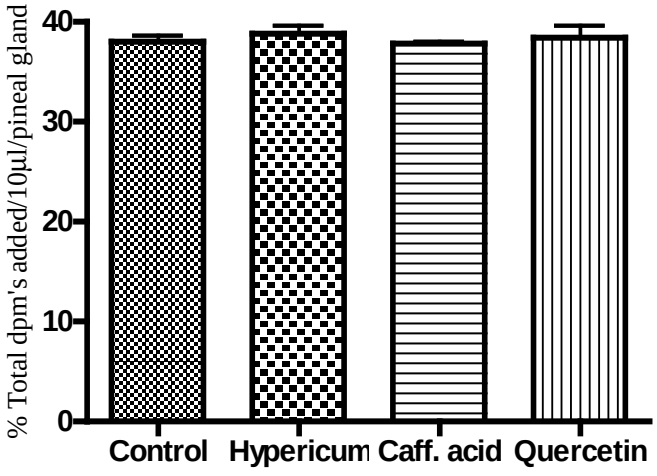


Figure 2.2.7 Effect of *Hypericum perforatum*, caffeic acid and quercetin on the amount of MAO metabolites released into the culture medium by the rat pineal gland after 24 hour incubation.

2.2.4 Discussion

The effects of *Hypericum perforatum*, caffeic acid and quercetin on pineal indole metabolism are dissimilar. Only quercetin produced a significant increase in melatonin levels which suggests that it has a similar effect on melatonin production to that observed with fluoxetine in the previous experiment (see Section 2.1.3). However, the significant increase produced by quercetin in 5-HIAA levels is the converse of that produced by fluoxetine and HP in the previous experiment (see Section 2.1.3). These results also show a significant decrease in 5-HTOH levels by HP and a profound increase by caffeic acid. Banoo S. (1991) reported significant decreases in the level of 5-HTOH produced by the pineal gland after acute treatment with the TCA, desipramine and the heterocyclic, maprotiline. This suggests that HP has a similar effect to these antidepressants on reducing the activity of the enzyme, aldehyde dehydrogenase. There were no significant changes in the percentage total dpm's for the four MAO metabolites suggesting that HP, caffeic acid and quercetin do not inhibit the enzyme MAO.

The most interesting and original finding in this study was the significant increase in melatonin levels in the rats treated with quercetin. This is similar to the effect of fluoxetine on aMT levels reported in the previous study and suggests that quercetin may be one of the active constituents in HP contributing to its antidepressant activity. In the following study, it was decided to investigate whether these effects brought about by quercetin on pineal gland serotonin metabolites including melatonin, are dose dependent.

2.3 EFFECT OF VARYING THE DOSAGE OF QUERCETIN ON PINEAL INDOLE METABOLISM

2.3.1 Introduction

Previous studies have shown that various drugs are capable of affecting pineal indole synthesis and metabolism. The pineal organ culture technique is sensitive enough to detect changes in activity upon drug manipulation (Khan R.B., 1989). The results of the previous study showed that treating rats with 5mg/kg quercetin significantly affects the amount of melatonin and 5-HIAA released into the culture medium after 24 hour incubation. These results were similar to the results observed in the rats treated with fluoxetine in the previous study. The aim of this study is to determine whether treatment with lower dosages of quercetin (1mg/kg and 2.5mg/kg p.o.) would affect the pineal gland indole synthesis and whether these changes are in any way similar to those reported in the previous study.

2.3.2 Materials and Methods

2.3.2.1 Chemicals and Reagents

The chemicals and reagents were the same as set out in Section 2.2.2.1.

2.3.2.2 Animals

Adult male rats of the Wistar strain, weighing between 200 and 250g were used in this study and divided randomly into three groups consisting of five rats each. The animals were housed and cared for as described in Appendix 1. The rats were dosed p.o. over twenty one days according to the procedure explained in Section 2.2.2.2.

5 rats in the control group received the vehicle that was made up of ethanol, propylene glycol and glycerol (2:2:1)

5 rats in the quercetin1 group received 1mg/kg quercetin dissolved in the vehicle.

5 rats in the quercetin 2.5 group received 2.5mg/kg quercetin dissolved in the vehicle.

2.3.2.3 Tissue preparation

At the end of the dosing period, the rats were sacrificed and the pineals removed following the same procedure as described in Section 2.1.2.3.

2.3.2.4 Thin Layer Chromatography (TLC)

The TLC technique and method used were the same as outlined in Section 2.2.2.4.

2.3.2.5 Statistical Analysis

The statistical analysis was performed as set out in Section 2.1.2.5.

2.3.3 Results

The results of the liquid scintillometry were expressed as the percentage of the total dpm's added to each TLC plate. The mean for each indole ± S.E.M. is summarised in Table 2.3 below.

Table 2.3 Effect of treatment with different dosages of quercetin on the percentage of indole levels released into the culture medium after 24 hour incubation. (mean ±S.E.M.). *P<0.05, n = 5.

Indoles	Control	Quercetin(1mg/kg)	Quercetin(2.5mg/kg)
aMT	0.53 ± 0.05	0.52 ± 0.09	0.28 ± 0.1
NAS	0.51 ± 0.04	0.49 ± 0.4	0.46 ± 0.04
5-HIAA	21.78± 1.85	19.33± 0.73	20.75 ± 0.92
5-HTOH	7.29± 0.9	7,86 ± 0.7	9.08 ± 0.4
5-MIAA	0.35 ± 0.02	0.37 ± 0.01	0.43 ± 0.01*
5-MTOH	0.38 ± 0.07	0.36 ± 0.02	0.37 ± 0.05

The effect of different dosages of quercetin (1mg/kg vs 2.5mg/kg) on the amount of indoles released into the culture medium by the rat pineal gland is shown in Figures 2.3.1 to 2.3.7 below. The only significant ($P<0.05$) change that occurred was in the level of 5-MIAA in the rats treated with 2.5mg/kg (see Figure 2.3.5).

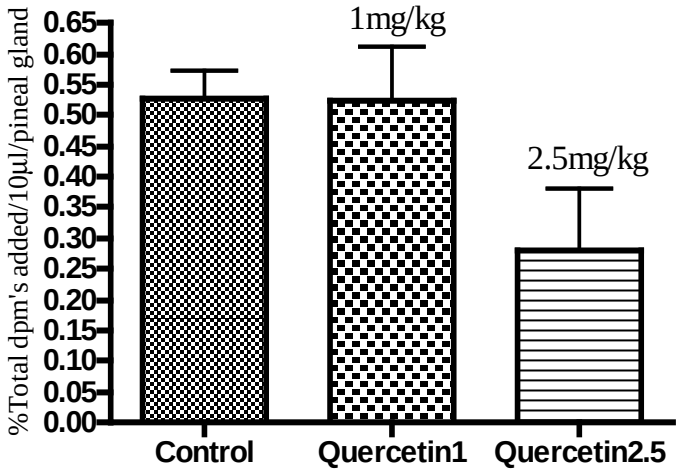


Figure 2.3.1 Effect of different dosages of quercetin (1mg/kg vs 2.5mg/kg) on the amount of aMT released into the culture medium by the rat pineal gland after 24 hour incubation.

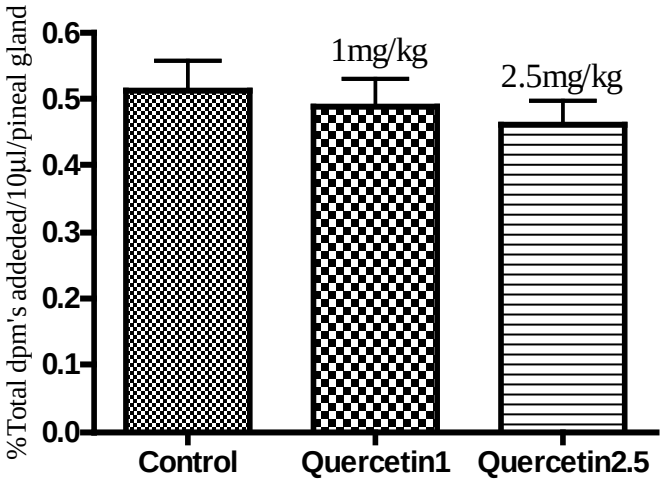


Figure 2.3.2 Effect of different dosages of quercetin (1mg/kg vs 2.5mg/kg) on the amount of NAS released into the culture medium by the rat pineal gland after 24 hour incubation.

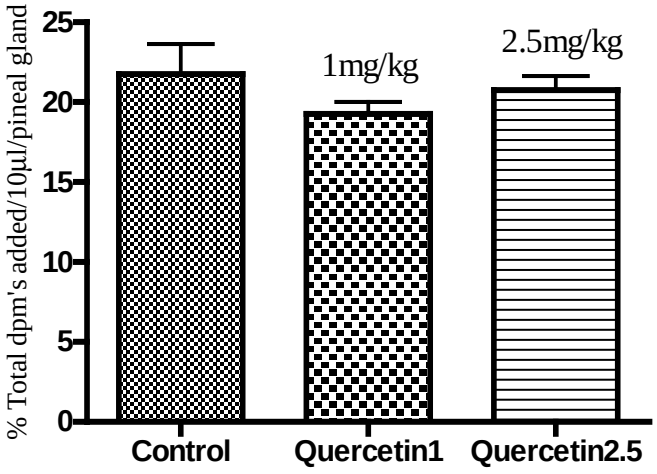


Figure 2.3.3 Effect of different dosages of quercetin (1mg/kg vs 2.5mg/kg) on the amount of 5-HIAA released into the culture medium by the rat pineal gland after 24 hour incubation.

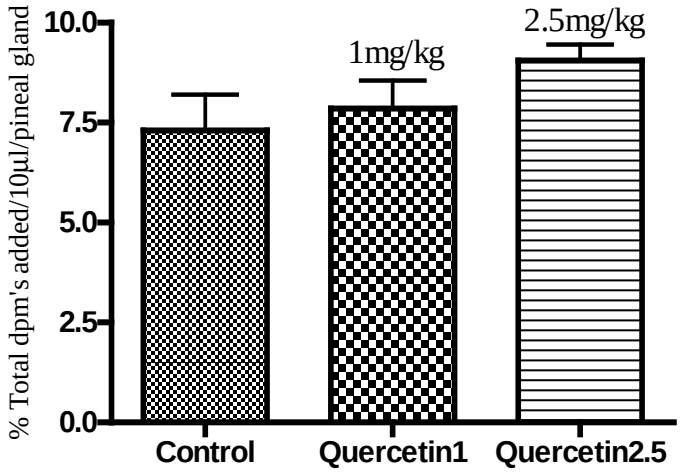


Figure 2.3.4 Effect of different dosages of quercetin (1mg/kg vs 2.5mg/kg) on the amount of 5-HTOH released into the culture medium by the rat pineal gland after 24 hour incubation.

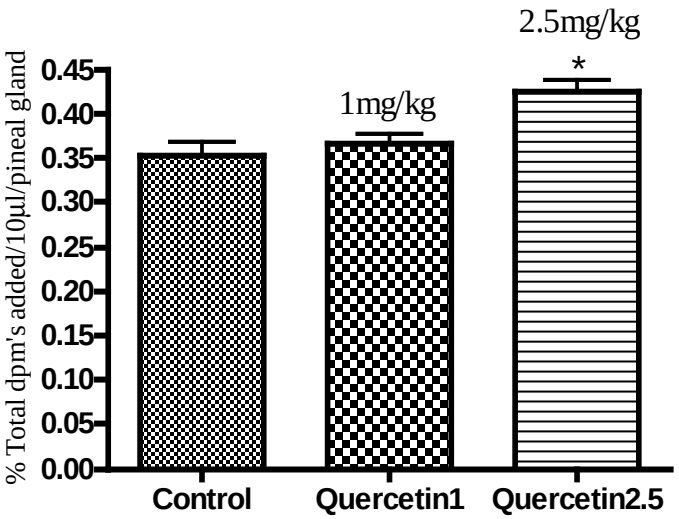


Figure 2.3.5 Effect of different dosages of quercetin (1mg /kg and 2.5mg/kg) on the amount of 5-MIAA released into the culture medium by the rat pineal gland in a 24 hour incubation period.

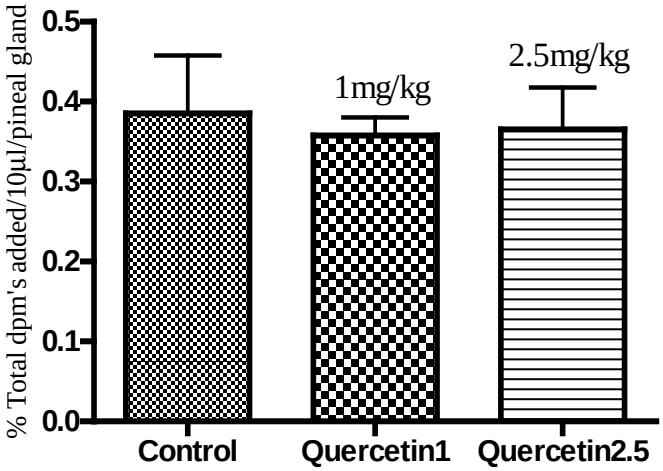


Figure 2.3.6 Effect of different dosages of quercetin (1mg/kg vs 2.5mg/kg) on the amount of 5-MTOH released into the culture medium by the rat pineal gland after 24 hours incubation.

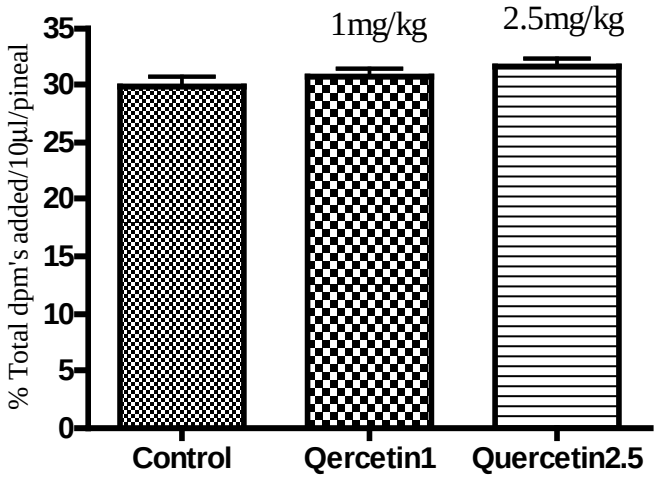


Figure 2.3.7 Effect of different dosages of quercetin (1mg/kg vs 2.5mg/kg) on the amount of MAO metabolites released into the culture medium by the rat pineal gland after 24 hours incubation.

2.3.4 Discussion

The results of this study show that there is no significant difference in the amount of MAO metabolites released by the pineal between dosing the rats at 1mg/kg or 2.5mg/kg when compared with the vehicle treated control. The results are different from those in the previous study where the rats were treated with 5mg/kg quercetin. The previous study showed a significant increase in the amount of melatonin released by the pineal gland, while the results of the present study show no effect in melatonin levels for the rats treated with 1mg/kg or 2.5mg/kg quercetin. This suggests that lower doses of quercetin has no effect on the secretion of melatonin and that a significant increase in aMT levels only occurs at a high dose of 5mg/kg. There were no changes in the amount of 5-HIAA released by the pineal gland in this study which again suggests that significant change only occurs after treatment at 5mg/kg quercetin.

The only significant change that occurred in this study was the increase in the amount of 5-MIAA released into the culture medium by the rat pineal gland (see Figure 2.3.5) in the rats treated with a dose of 2.5mg/kg/day. This is similar to the results of the previous study reported by Banoo S.

(1991) where significant increases in 5-MIAA level were observed in the pineals of rats treated with the TCA, desipramine. This suggests that quercetin may contribute to the antidepressant effect of HP with a similar mode of action as the TCA, desipramine. However, this effect was not observed in the previous study where a dose of 5mg/kg was used and only in the present study at a dose of 2.5mg/kg. This suggests that the effect may be dose sensitive.

CONCLUSIONS

The most significant effects on pineal metabolism in these studies were brought about by fluoxetine and quercetin. A noteworthy result being the increase in the amount of melatonin released, suggesting that both fluoxetine and quercetin may mediate some of their antidepressant activity through this mechanism. This result is consistent with a previous study with fluoxetine (Skene D.J. 1985) and novel for quercetin as there are no previous reports on the effect of quercetin on pineal metabolism in the literature. The results from these studies also reveal that none of the compounds inhibit the enzyme monoamine oxidase, and therefore have a different mode of action from MAO inhibitor antidepressants that bring about their antidepressant effects in this manner. The effect of treatment with quercetin on the release of indoleamines was apparent at a dosage of 5mg/kg/day on aMT and 5-HIAA levels and a lower dose of 2.5mg/kg/day on 5-MIAA level. These effects were similar to the TCA, desipramine and the heterocyclic, maprotiline reported by Banoo S. (1991) and suggests that HP and quercetin may have similar mechanism of action to these antidepressants.

It is possible that the effect of these different compounds on pineal metabolism reported in these studies may be mediated by β-adrenergic receptor activity. The pineal gland has a well- defined noradrenergic system that controls melatonin synthesis and release. The pineal gland has been utilised in research as a model in which to examine the noradrenergic effects of antidepressant treatment and this has been associated with increased melatonin output. It has been shown that there are antidepressant drugs that inhibit the uptake of NA or stimulate β-adrenergic receptors resulting in increased melatonin synthesis (Banoo S., 1991). Therefore, further studies on the effect of HP and quercetin on β-adrenergic receptor densities were undertaken and the results of these studies are reported in Chapter 5.

CHAPTER 3

LIVER TRYPTOPHAN 2,3-DIOXYGENASE STUDIES

INTRODUCTION

Tryptophan is an essential amino acid that serves as the precursor for the biogenic amines serotonin and melatonin (Walsh H. *et al.* 1994). In the liver, the amino acid tryptophan (TRP) is catabolised by the enzyme tryptophan-2,3-dioxygenase (TDO). Serotonin is synthesised from tryptophan in the brain. One way to increase serotonin in the brain would be to increase the amount of tryptophan available for uptake to the brain (Badawy A. A.-B. *et al.* 1980, Badawy A.A.-B. & Evans M. 1981). This could be made possible by inhibiting TDO, also known as Tryptophan pyrrolase, in the Kynurenine pathway (see Figure 3.1 on page 67), in the liver. However, the transportation of TRP to the brain is dependent on a carrier system to cross the blood-brain barrier (BBB). There is also competition between the neutral amino acids for attachment to this carrier molecule, thus affecting the amount of TRP getting into the brain (Wurtman R.J. 1982). It would therefore appear that the two most important factors contributing to increasing TRP availability to the brain for 5-HT synthesis are:

(i) inhibiting TDO activity in the liver and
(ii) decreasing the amount of neutral amino acids at the BBB for uptake to the brain (Badawy A.A.-B. *et al,* 1981).

The following studies focus on the first of these options.

Previous studies have demonstrated the importance of TDO as a regulator of tryptophan catabolism and brain levels of TRP and 5-HT and suggest that antidepressant efficacy might be achieved with inhibitors of TDO (Salter M. *et al.* 1995b). Tricyclic antidepressants (TCAs), have been shown by Badawy A.A.-B. & Evans M. (1981), to inhibit TDO in the liver and, via increased tryptophan concentration in the blood and brain, to increase the synthesis of 5-HT. A

later study by Badawy A.A.-B *et al.* (1991) showed that acute and chronic treatment of rats with the antidepressants lofepramine and its metabolite desmethylimipramine enhances brain 5-HT synthesis secondarily to inhibition of liver TDO by increasing the availability of circulating TRP to the brain. Furthermore, Salter M. *et al.* (1995a), suggest that superior antidepressant efficacy can be achieved with a drug that has a combined TDO inhibitor/5-HT reuptake mechanism of action.

Stone T. W. (2000), in the review of the implications for psychiatry of manipulation of the kynurenine pathway of tryptophan metabolism, suggests that changes in the concentration of tryptophan metabolites probably alter 5-HT metabolism. Conversely, this author suggests that the treatment of depression by selective 5-HT reuptake inhibitors (SSRIs) is likely to change the balance between the amount of tryptophan used for 5-HT synthesis and that available for kynurenine synthesis.

The aim of the following study was to compare the effects of the SSRI, fluoxetine, with *Hypericum perforatum* on tryptophan metabolism in the kynurenine pathway.

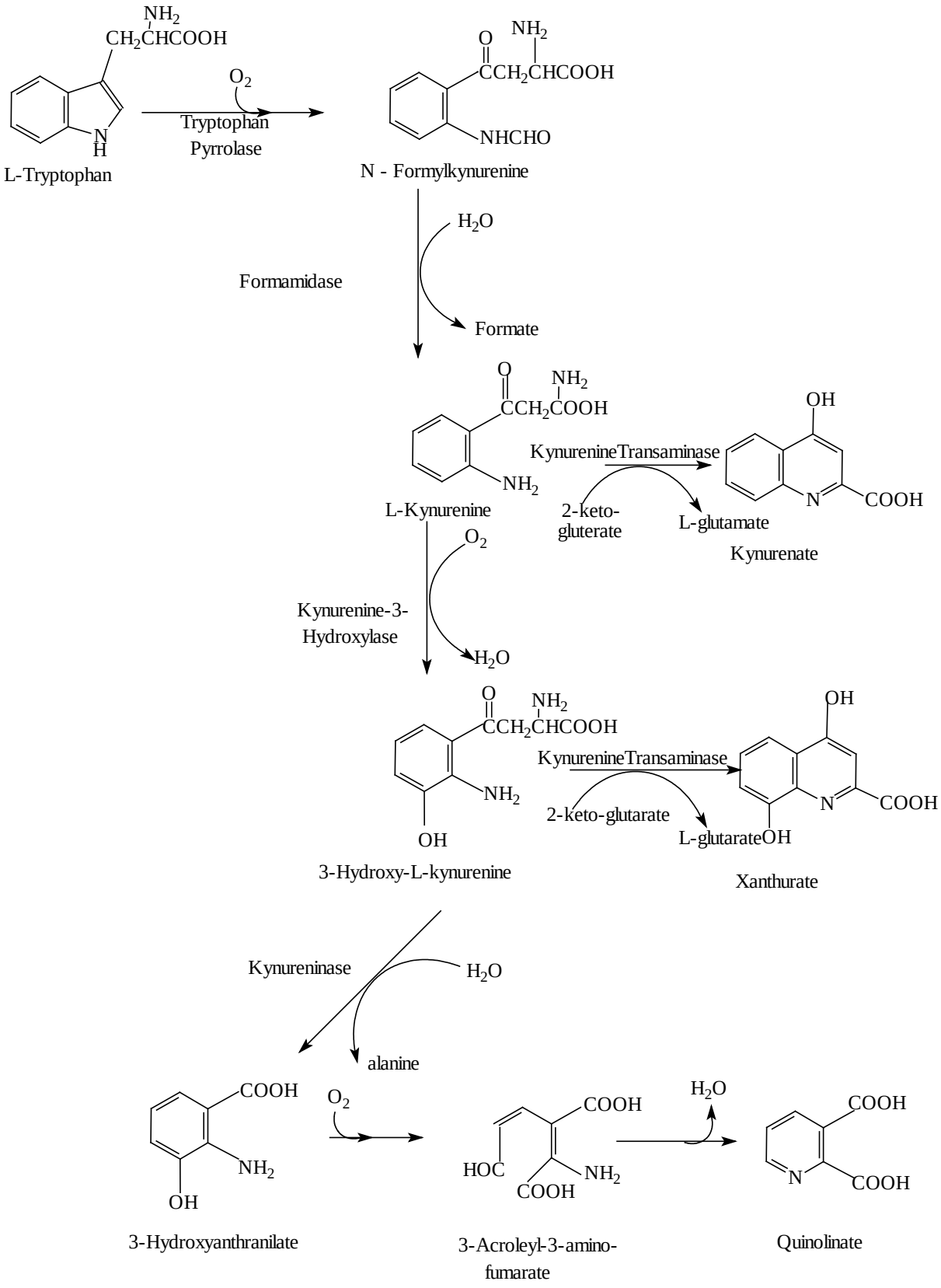


Figure 3.1: The Kynurenine Pathway in the liver (Martin *et al.* 1992).

3.1 A COMPARISON OF THE EFFECTS OF *Hypericum perforatum* WITH FLUOXETINE ON TRYPTOPHAN 2,3-DIOXYGENASE ACTIVITY IN RAT LIVER.

3.1.1 Introduction

TDO is a haem-containing oxygenase that is able to cleave the indole ring of both the L- and D-isomers of TRP. The D-isomer is poorly absorbed and metabolised in both humans and animals. In the Kynurenine pathway, TDO catalyses the conversion of TRP to N-formylkynurenine. In rats and humans, the enzyme consists of two forms:

(i) the holoenzyme which is already active and does not require the presence of exogenous haematin

(ii) the apoenzyme which is inactive and the haem-free form of the enzyme requires the presence of exogenous haematin.

The enzyme can be induced by:

(i) glucocorticoids, which involves the synthesis of new apoenzyme, and its substrate, TRP.

(ii) haem, a cofactor of TDO, which is loosely associated with the apoenzyme (Badawy A.A.-B. *et al.* 1975).

This study investigates whether HP and fluoxetine are able to inhibit TDO activity, *in vivo,* in rat liver. The TDO enzyme assay previously described by Badawy A.A.-B. & Evans M. (1981) was used. The enzyme activity was determined by measuring the formation of kynurenine from TRP, either in the presence (total activity) or the absence (holoenzyme) of added haematin. The difference between the absorbance readings recorded at 365nm of the total activity and the holoenzyme, represents the amount of kynurenine present.

3.1.2 Materials and Methods

3.1.2.1 Chemicals and reagents

L-Tryptophan was purchased from Merck (South Africa) and haematin was purchased from Sigma Chemical Company (St Louis, MO, USA). Trichloroacetic acid and sodium hydroxide were obtained from UNILAB (Saarchem Pty (Ltd), South Africa). *Hypericum perforatum* was purchased from Flora Force Health Products, Rondebosch, South Africa and fluoxetine from Eli Lily (Pty) Ltd (South Africa). All other chemicals and reagents were obtained locally and were of the highest purity available.

3.1.2.2 Animals

Adult male Wistar rats used in this study were the same animals as in section 2.1. The animals were maintained and dosed as described in section 2.1.2.2.

The livers from the rats described above were immediately removed after decapitation. Each liver was perfused with 0.9% NaCl to remove excess blood, immediately frozen in liquid N_2 and stored at -70^0C until required for the assay.

3.1.2.3 Preparation of the liver homogenate

Prior to the commencement of the experiment, the following chemical solutions were prepared in deionised water:

1. L-tryptophan (0.3M) was prepared in 4mM NaOH
2. Trichloroacetic acid (0.9M) in deionised water
3. NaOH (0.6M) in deionised water
4. Sodium phosphate buffer (pH 7.0)
5. 0.14M KCl - 2.5mM NaOH
6. Haematin chloride (2μM) dissolved in 0.1M NaOH.

Fresh solutions of haematin chloride and tryptophan (TRP) were made up every time just prior to the experiment being run. All the livers were treated in exactly the same manner.

On the day of the experiment, the rat liver was slowly thawed on ice and then homogenised in a final 10% w/v of half 140nM KCl - 2.5mM NaOH and half 0.2M sodium phosphate buffer pH 7.0. The method showing how the assay was run is outlined in Table 3.1 below.

Table 3.1: Method for the assay for Tryptophan 2,3-Dioxygenase activity.

Holoenzyme	**Reagents**	**Total Activity**
13.5ml	Water	13.5ml
15.0ml	Homogenate (10%w/v)	15.0ml
0.0ml	Haematin (2µM)	100µl
2.5ml	Tryptophan (0.3M)	2.5ml
3.0ml	Samples transferred to series of test tubes	3.0ml
The air in each test tube was displaced by carbogen (5%CO_2:95% O_2) & sealed		
Incubation for a period of 1 hour with shaking in a water bath at 37^0C		
2ml	Trichloroacetic Acid	2ml
Shake for a period of 4 minutes	Filter through Whatman no.1 filter paper	Transfer 2.5ml of filtrate to another test tube
1.5ml	NaOH (0.6M)	1.5ml
Read absorbance at 365nm using a blank of 2ml TCA and 1.5ml NaOH		

3.1.2.4 Protein assay

The estimation of protein content was carried out using the method described by Lowry O.H. *et al.* (1951) as described in Appendix 2.

3.1.2.5 Statistical analysis

The results were analysed using one way analysis of variance (ANOVA) and comparison of the mean±SEM was performed using the Newman-Keuls multiple comparison test. Statistical significance between the groups was set at $P<0.05$.

3.1.3 Results

The concentration of kynurenine was calculated using Beer-Lambert Law with the molar extinction coefficient, $\varepsilon = 4540 l.mol^{-1}.cm^{-1}$ and the results expressed as nmoles kynurenine formed/minute/mg protein. The holoenzyme activity increased, though not significantly, and the total enzyme activity remained unaltered. The average for the apoenzyme activity showed a small but insignificant decrease for both HP and fluoxetine treated rats as shown in Figure 3.1.2 below.

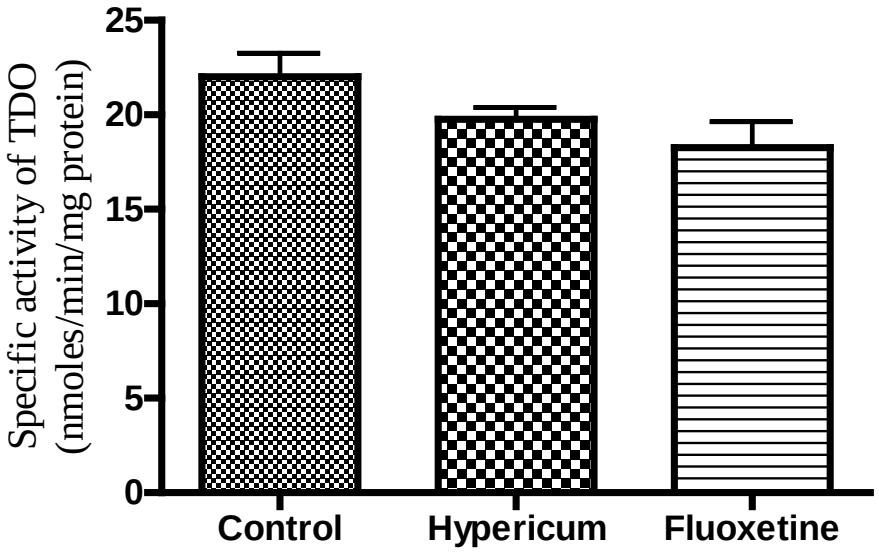


Figure 3.1.2: The effect of *Hypericum perforatum* and fluoxetine on TDO activity in rat liver. n = 5; mean ± SEM

3.1.4 Discussion

The results show that neither HP nor fluoxetine have a significant effect on TDO activity in the rat liver. A possible explanation by Badawy A.A.-B. (1980) is that the ability of compounds to inhibit TDO may depend on the extent of haem saturation of the apoenzyme and so depend on the amount of haem present *in vivo.* This suggests that the amount of haem present in determining the total enzyme activity may have been high enough to decrease the amount of inhibition by HP and fluoxetine.

The fluoxetine results from this study are similar to those reported by Walsh H.A. and Daya S. (1998) where it was shown that fluoxetine had no observable effect on basal hepatic TDO activity in the liver of the rat. In the following study, the effect of two of the constituents of HP, caffeic acid and quercetin, was compared with that of HP on TDO activity in the liver of the rat.

3.2 THE EFFECT OF *Hypericum perforatum*, QUERCETIN AND CAFFEIC ACID ON TRYPTOPHAN 2,3-DIOXYGENASE ACTIVITY IN RAT LIVER

3.2.1 Introduction

The results from the previous study show that *Hypericum perforatum* has no significant inhibitory effect on the activity of the liver enzyme, tryptophan 2,3-dioxygenase. The implication of the inhibition of TDO enzyme is that this could lead to an increase in plasma levels of tryptophan and subsequently induce an increase in brain tryptophan and serotonin levels (Daya S. *et al.* 1989). This has been shown to occur after treatment with several antidepressants and therefore could be seen as contributing to their mechanism of action.

The aim of this study is to investigate the effects of two of the constituents of HP, caffeic acid and quercetin, as compared with that of HP on the activity of TDO in the liver of the rat. It was decided to use a different, less stressful form of dosing as described in section 2.2.2.2 and that more care would be taken in perfusing the livers to remove excess blood that may have interfered with the results of the assay in the previous experiment.

3.2.2 Materials and Methods

3.2.2.1 Chemicals and reagents

The chemicals and reagents used in this study are the same as those described in Section 3.1.2.1 for the TDO assay. *Hypericum perforatum* was obtained from Flora Force (Pty) Ltd (Rondebosch, South Africa), quercetin and caffeic acid were from Sigma Aldrich (Switzerland).

3.2.2.2 Animals

Adult male rats of the Wistar strain used in this study are the same animals as in section 2.2. The animals were cared for as described in Appendix 1 and dosed according to the procedure as described in Section 2.2.2.2.

3.2.2.3 Tissue preparation

The tissue preparation was the same as described in Section 3.1.2.3 and more care was taken with the removal of the excess blood.

3.2.2.4 Statistical analysis

The statistical analysis was performed as described in Section 3.1.2.4.

3.2.3 Results

The results of this study show a significant inhibition of TDO activity by both HP ($P<0.01$) and caffeic acid. ($P<0.05$) (See Figure 3.2.1 below).

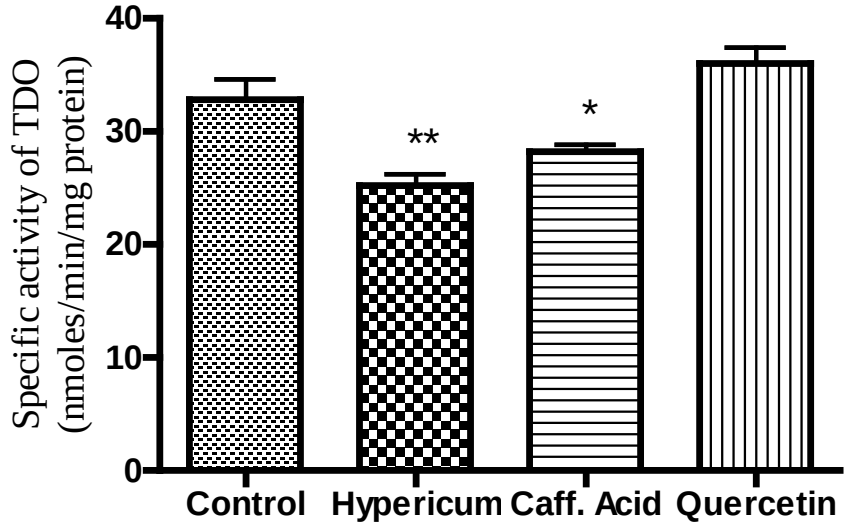


Figure 3.2.1: Effect of *Hypericum perforatum*, caffeic acid and quercetin on TDO activity in rat liver.
n = 5; mean ± SEM *P<0.05; **P<0.01

3.2.4 Discussion

The results of this study show that treatment with both HP (300mg/kg) and its constituent caffeic acid (5mg/kg), have a significant inhibitory effect on TDO activity. The inhibition appears at the level of the apoenzyme, which implies that HP and caffeic acid exert their inhibitory action through partial inhibition of the binding of this enzyme component to its cofactor haem, as this conjugation is necessary for the activation of the apoenzyme. This suggests that treatment with HP and caffeic acid would increase the level of plasma tryptophan, which would result in increased tryptophan being available for uptake into the brain. This, in turn, would affect the levels of 5-HT and aMT in the brain. The results of the effects on 5-HT levels are discussed in Chapter Four and Six, while the affects on aMT levels are discussed in Chapter Two and Six.

Another possible mechanism of action of the inhibition by drugs of TDO is that suggested by Walsh H. A. *et al.* (1994) The authors' argue that the binding of tryptophan to TDO induces an increase in the haem binding affinity of the enzyme which has both a regulatory and a catalytic site. Binding at both sites occurs at high concentrations of tryptophan while at low concentrations this only occurs at the catalytic site. The implication is that HP and caffeic acid could also

possibly prevent the binding of tryptophan to TDO, thus reducing its ability to bind haem, preventing the synthesis of the apoenzyme, and so reducing the activity of TDO.

CONCLUSIONS

The results of the first study demonstrate that HP and fluoxetine treatment had no significant inhibitory effect on TDO in the liver of the rat. The results of the second study showed significant inhibition of TDO by both HP and caffeic acid. A possible explanation for the discrepancy between the two studies in terms of the amount of inhibition shown for the HP treated animals not being significant in the first study, is that the dosing procedure in the second study was more effective.

The significant inhibition of TDO by HP and caffeic acid shown in the second study was a novel finding, and suggests that caffeic acid may be the active constituent of HP contributing to the inhibition of TDO and its antidepressant mechanism of action. The inhibition of TDO also implies that there would be an increase in plasma tryptophan and therefore this could result in increased 5-HT levels in the brain. The concentration of 5-HT in the forebrain of these animals was determined in the following chapter using serotonin-ELISA.

CHAPTER 4

SEROTONIN-ELISA STUDIES

INTRODUCTION

Serotonin (5-HT) was discovered by physiologists a century ago, as a vasoconstrictor compound in serum after blood clotting and was called vasotonin. Later, in the 1940's, this was isolated as a crystalline complex, and renamed serotonin. Subsequent research revealed several important functions for this bioamine including the role it plays in the central nervous system (CNS). In the brain, serotonin pathways originate from neurons in the raphe or midline regions of the pons and upper brainstem. Inhibition and excitation can occur in the same neuron and are mediated by different classes of 5-HT receptors (Nicoll R.A. 1998).

The 'biogenic amine' hypothesis suggests that the role serotonin, noradrenaline and dopamine play in depression can be found in disturbances in the levels of these bioamines. Further evidence of the role of serotonin in depression was shown by the antihypertensive drug reserpine, which induced depression by depleting CNS stores of 5-HT, noradrenaline (NA) and dopamine (DA). This suggests that depression results from decreased 5-HT levels and a lowering of amine-dependent nerve transmission (Potter W.Z. & Hollister L.E., 1998).

Serotonin has also been suggested as being an important neuro-modulator in the 'permissive' hypothesis. This theory postulates serotonin as the major target of antidepressant therapy and that decreased 5-HT and NA are the hallmarks of depression (Harvey B.H. 1997).

Extracts from the herb *Hypericum perforatum* (HP) have gained popularity as 'nature's Prozac'. Increased 5-HT levels in the cortex of rat brain have been shown to occur after acute treatments with HP and fluoxetine (Calapai G. *et al.* 1999). The aim of the following studies is to compare the effects of HP, fluoxetine, caffeic acid and quercetin on 5-HT levels in the forebrain of the rat.

4.1 THE EFFECT OF *Hypericum perforatum* AND FLUOXETINE ON FOREBRAIN SEROTONIN LEVELS USING ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA).

4.1.1 Introduction

Serotonin (5-HT) is an intermediate product of tryptophan metabolism and one of the primary locations for 5-HT metabolism is in the serotonergic neurons in the brain. A substantial body of evidence supports a more than thirty year old hypothesis that modifications in serotonin neural function is a core feature of depression. It has been shown that a deficiency of 5-HT in the brain can be linked with depression and that increasing 5-HT availability alleviates depression. Both the tricyclic antidepressants (TCAs) and the selective serotonin reuptake inhibitors (SSRI) antidepressants have been shown to increase the levels of 5-HT and NA in the CNS (van Praag H.M. 1982 & Daya S. 1994).

This study was undertaken to determine whether HP (300mg/kg), when administered to rats, would alter the serotonin concentration in the forebrain. This could then be compared with fluoxetine (1.25mg/kg), a SSRI, which is known to increase 5-HT in the brain. The 5-HT levels in the forebrain were determined using a Serotonin-ELISA kit manufactured by Immuno Biological Laboratories (Hamburg, Germany) and distributed by Research Diagnostics INC (Flanders, NJ, USA).

The assay procedure follows the basic principle of competitive ELISA where there is competition between a biotinylated and a non-biotinylated antigen for a fixed number of antibody binding sites. The amount of biotinylated antigen bound to the antibody is inversely proportional to the analylate concentration of the sample. Quantification of the unknowns is achieved by comparing the enzyme activity of the unknowns with a response curve prepared by using known standards.

4.1.2 Materials and Method:

4.1.2.1 Chemicals and Reagents

All chemicals and reagents used in the ELISA assay were provided with the diagnostic kit from Research Diagnostics INC. (Flanders, NJ, USA). Fluoxetine was obtained from Eli Lily (Pty) Ltd (South Africa) and *Hypericum perforatum* from Flora Force Health Products (Pty) Ltd (Rondebosch, South Africa). All other chemicals and reagents were obtained locally and were of the highest purity available.

4.1.2.2 Animals

Male Wistar rats weighing between 200 and 250g were used in this study. The animals were the same as described in section 2.1, were housed as described in Appendix 1 and dosed as described in Section 2.1.2.2.

4.1.2.3 Tissue preparation

The forebrains from the rats described above were excised immediately after decapitation, frozen in liquid N_2 and stored at -70^0C until needed. On the day of the assay, these brains were thawed on ice, individually weighed and homogenised in 0.9% NaCl (10% w/v). The test tubes containing the homogenate were then centrifuged at 1500xg for 10 minutes.

4.1.2.4 The ELISA assay

The reagents required for the assay were supplied in the serotonin-ELISA kit and these were all made up to the correct concentrations according to the instructions given. The test procedure as outlined in the kit is summarised as follows:

1. 50µl of each standard and homogenate was pipetted, in duplicate, into the designated wells of the ELISA plate.

2. 50µl of serotonin-biotin was pipetted into each well.
3. 50µl of antiserum added to each well.
4. The plate was carefully shaken before incubation overnight (16 - 20hrs) at 2-8^{0}C.
5. Each well was washed three times with Wash Buffer.
6. 150µl of Anti Biotin AP was added to each well.
7. The ELISA plate was incubated for 120 minutes at room temperature on an orbital shaker (200rpm).
8. Each well was washed three times with Wash Buffer.
9. 200µl of PNPP substrate solution was added to each well.
10. The ELISA plate was sealed and incubated at room temperature on an orbital shaker (200rpm).
11. 50µl of PNPP Stop Solution was pipetted into each well.
12. After gently shaking the plate to mix the contents, the optical density of each well was read at 405nm, using a Power Wave Micro-Plate Reader (BIO-TEK INC. USA).

4.1.2.5 Statistical analysis

All data was statistically analysed using one way analysis of variance (ANOVA) followed by the Newman-Keuls test for multiple column comparison. Results were expressed as means ± S.E.M. Significance was set at $P<0.05$.

4.1.3 Results

The standards used in this assay were used to generate a standard curve (see Figure 4.1.1 below). From this, the serotonin concentration in the forebrain of each rat was determined as nanograms of serotonin per mg of tissue, after correcting for dilution and acylation of the samples. These results are summarised in Table 4.1 and the graph of this data is shown in Figure 4.1.2. The level of serotonin for the rats treated with HP did not increase significantly while there was a very significant increase in serotonin levels for the rats treated with fluoxetine, according to the Newman-Keuls test for multiple column comparison.

Table 4.1: Effect of treatments with *Hypericum perforatum* and fluoxetine on rat forebrain serotonin levels. Mean ± SEM; n = 5. ***P<0.001

Control	Hypericum	Fluoxetine
0.22 ± 0.032	0.27 ± 0.024	0.59 ± 0.029***

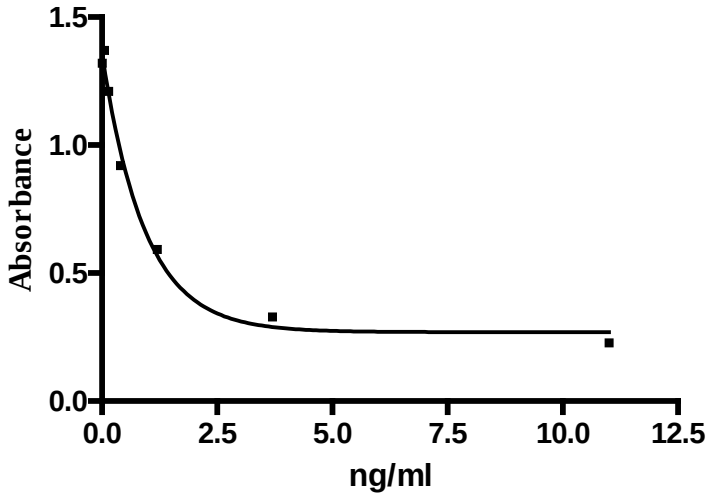


Figure 4.1.1: Serotonin Standard Curve as determined by the Power Wave Microplate Reader from the standards provided in the Serotonin ELISA kit. r^2= 0.9954

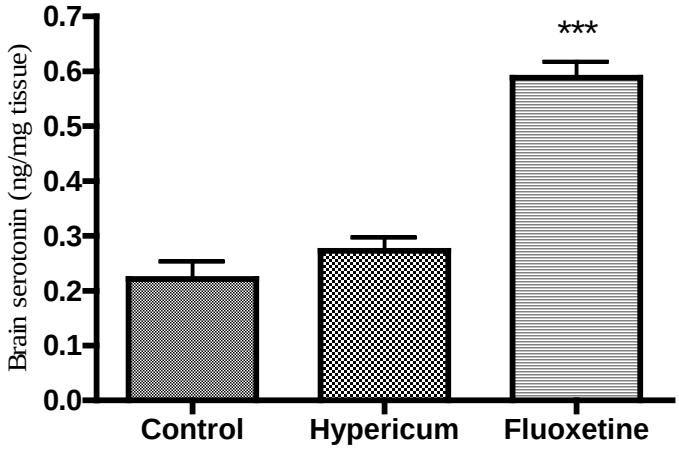


Figure 4.1.2: Effect of *Hypericum perforatum* and fluoxetine on rat forebrain serotonin levels. Mean ± SEM; n = 5. ***P<0.001

4.1.4 Discussion

The level of serotonin determined in the forebrain of the rats treated with HP did not show a significant increase when compared with the control rats. There was a significant increase in the level of serotonin in the rats treated with fluoxetine (1.25mg/kg). There are previous studies such as the one done by Calapai G. *et al.* 1999, which report that HP and fluoxetine increase the serotonin levels in different areas of the brain of the rat. It was therefore unexpected that a significant increase in serotonin levels was not observed in the rats treated with HP in this study. The reason for this was not clear but it was decided to repeat this study using another less stressful form of dosing.

The results of other research reported in the literature attributes SSRI activity to the presence of the compound hyperforin in *Hypericum perforatum* extracts (Chatterjee S.S. *et al.* 1998b, Kaehler S.T. *et al.* 1999). A possible reason why the serotonin levels in the rats treated with HP in this study did not increase similarly to the rats treated with fluoxetine is that the HP extract had very low hyperforin content. A sample of the HP extract was subjected to High Performance Liquid Chromatography (HPLC) and the results obtained suggest that the HP extract used to treat the rats in this study had very low hyperforin content. However, the HPLC analysis did show that the extract had a high concentration of quercetin (Wild T., 2002). The results of the study undertaken by Calapai G. *et al.* (1999) also showed that it was the HP extract with 50% flavonoid content that increased 5-HT levels in the cortex of rat brain.

It was decided to investigate the effect of two other constituents of *Hypericum perforatum,* caffeic acid and quercetin, on the serotonin levels in the forebrain of the rat in the following study.

4.2 THE EFFECT OF *Hypericum perforatum*, CAFFEIC ACID AND QUERCETIN ON FOREBRAIN SEROTONIN LEVELS USING ELISA.

4.2.1 Introduction

The main function of serotonin as a neurotransmitter has been shown to be associated with mood disorders such as depression since certain antidepressants enhance the availability of 5-HT at central receptors (Harvey B. H. 1997). The results of the previous study showed that treatment with HP did not have a significant effect on 5-HT levels. The reason for this unexpected result was not clear, but possibly could be attributed to low concentrations of the constituents in HP that have SSRI activity (Chatterjee S.S. *et al.* 1998b, Kaehler S.T. *et al.* 1999). The following study evaluates the effect of two of these constituents, caffeic acid and quercetin, to determine whether either of them affect 5-HT levels in the forebrain of the rat and therefore may have SSRI activity.

4.2.2 Materials and Methods

4.2.2.1 Chemicals and Reagents

All chemicals and reagents used in the ELISA assay were provided with the diagnostic kit from Research Diagnostics INC. (Flanders, NJ, USA). *Hypericum perforatum* was from Flora Force (Pty) Ltd (Rondebosch, South Africa), quercetin and caffeic acid were from Sigma Aldrich (Switzerland). All other chemicals and reagents were obtained locally and were of the highest purity available.

4.2.2.2 Animals

Adult male Wistar rats were used in this study. The animals were the same as described in section 2.2, were housed as described Appendix 1 and dosed as described in Section 2.2.2.2.

4.2.2.3 Tissue preparation

The tissue preparation was carried out in the same manner as described in Section 4.1.2.3.

4.2.2.4 The ELISA assay

The test procedure for the ELISA assay was carried out according to the instructions in the kit and in the same manner as described in Section 4.1.2.4.

4.2.2.5 Statistical analysis

The statistical analysis was performed as set out in Section 4.1.2.5.

4.2.3 Results

The results of the concentrations of the samples were obtained from the standard curve generated by the ELISA plate reader according to the standards supplied in the serotonin-ELISA assay (see Figure 4.2.1 below). The final values were expressed as ng/mg brain tissue after being corrected for dilution and acylation (see Table 4.2 below) The graph of these results is shown in Figure 4.2.2.

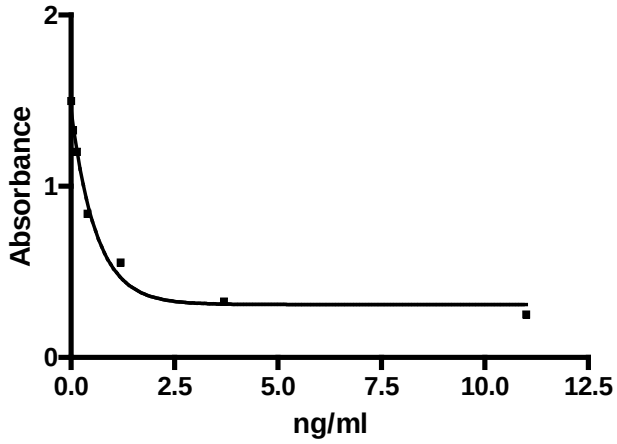


Figure 4.2.1 Serotonin Standard Curve as determined by the Power Wave Microplate Reader from the standards provided in the serotonin-ELISA kit. $R^2 = 0.9982$

Table 4.2 Effect of *Hypericum perforatum,* caffeic acid and quercetin on rat forebrain serotonin levels (ng/mg tissue). Mean ± SEM; n = 5. ***P<0.001

Control	Hypericum	Caffeic Acid	Quercetin
0.11 ± 0.007	0.18 ± 0.01	0.32 ± 0.038***	0.30 ± 0.015***

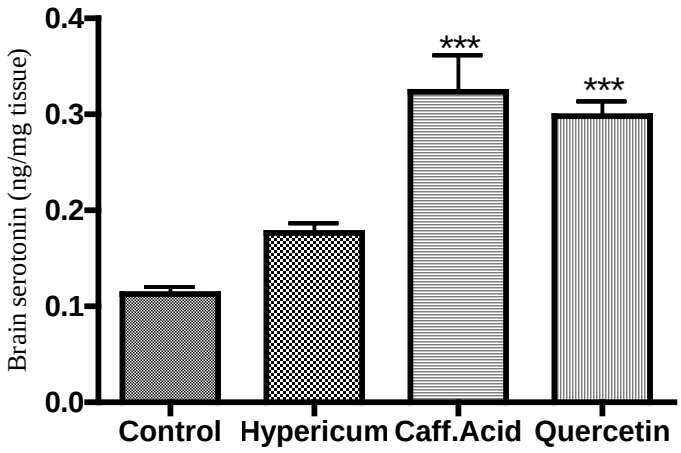


Figure 4.2.2 Effect of treatments with *Hypericum perforatum,* caffeic acid and quercetin on rat forebrain serotonin levels. Mean±SEM; n = 5. ***P<0.001

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4.2.4 Discussion

The results of this study demonstrate that treatment with two of the constituents of *Hypericum perforatum,* caffeic acid (5mg/kg) and quercetin (5mg/kg), causes a significant increase (P<0.001) in forebrain serotonin content. There was no significant increase shown for the rats treated with HP. This suggests that the content of these two compounds in the HP extract is much lower than that used for quercetin and caffeic acid in this study. The possible implication of this novel finding, is that a HP extract with enhanced levels of caffeic acid and quercetin could produce increased SSRI efficacy.

CONCLUSIONS

The rats treated with *Hypericum perforatum* extract in both studies show similar responses in that neither result in a significant increase in serotonin levels in the brain of the rat. The rats treated with fluoxetine, quercetin and caffeic acid do show significant increases in 5-HT levels when compared with the control rats. The increased 5-HT levels in the rats treated with fluoxetine is consistent with previous reports in the literature. However, the results of previous research on the effect of HP on 5-HT levels in rat brain have shown significant increased levels in various areas of the brain (Calapai G. 1999). The insignificant increases in the rats treated with HP in the two studies reported here are therefore surprising. The discrepancy in the values obtained for the controls in the two studies, the one obtained in the first study was lower than the one obtained in the second study, suggests that a third experiment may indicate which is correct. This was later done using high performance liquid chromatography which is a more cost effective method. The results from these experiments (chapter 6) gave control values (0.144±0.002 ng/mg brain tissue) between those obtained here and showed a significant increase in 5-HT levels in the rats treated with HP and quercetin.

The increase in the serotonin levels in the brain could be the result of direct interaction of some of the constituents of HP on 5-HT receptors causing a change in receptor density. The fact that none of the results obtained from HP are statistically significant could be attributed to the concentration of compounds, caffeic acid and quercetin, being too low in the extract at the dosage of HP (300mg/kg) administered in these studies. The significant effects of these compounds was only observed at doses of 5mg/kg. The HPLC results from the analysis of the HP used in these studies, and the results from the second study suggest that caffeic acid and quercetin are the possible constituents of the HP contributing to its overall antidepressant activity. This could be the result of changes that have occurred in 5-HT receptor densities. It was decided to investigate the effect of HP, quercetin and caffeic acid on different receptor densities.

CHAPTER 5

RECEPTOR BINDING STUDIES

INTRODUCTION

The powerful tools of molecular biology have proved useful in the drug discovery process and the discovery of receptor subtypes. The receptor binding assay has greatly facilitated this process. The role of receptors in drug action was proposed over a century ago. This action occurs through different transductional elements such as G proteins, enzymes and ion channels. The activation of these systems can alter cell function as well as affect gene transcription and translation and the post-transcriptional modification of gene products (Williams M. 1991).

The receptor binding assay has advantages over other methods in determining the effect of a drug on the regulation of a particular receptor. The use of a radioligand in the assay provides:

1. Direct quantitative information about the affinity (K_D) of a receptor.
2. The ability to measure the number/density of receptors present (Bmax).
3. Stability for the receptor in storage over several months.
4. The ability to switch from one receptor assay to another since the tissue contains more than one type of receptor (Titeler M. 1981).

The aim of the following studies is to compare the effects of the whole extract of *Hypericum perforatum* and quercetin with fluoxetine, a selective serotonin reuptake inhibitor, on the β-adrenergic, serotonin 2 and the *N*-Methyl-D-Aspartate receptors in rat brain, using radioligand binding assays.

5.1 β-ADRENERGIC RECEPTOR BINDING

5.1.1 EFFECT OF *Hypericum perforatum*, QUERCETIN AND FLUOXETINE ON THE DENSITY OF β-ADRENERGIC RECEPTORS.

5.1.1.1 Introduction

Hypericum perforatum (HP) is used for the treatment of mild to moderate forms of depression. The pharmacological actions of HP have recently been reviewed (Butterweck V. *et al.* 2003) and reports about the possible antidepressant mechanisms of the action of HP and its constituents have been published (Butterweck V. *et al.* 1997, 1998, 2000, 2002; Chatterjee S. *et al.* 1996, 1998; Cott J.M. 1997; Franklin M. *et al.* 1999; Muller W.E. *et al.* 1998). Antidepressant activity has been reported for several compounds found in HP such as the phloroglucinol, hyperforin (Chatterjee S. *et al*, 1998, 1999; Muller W.E. *et al.*, 1998, 2001; Singer A. *et al.* 1999; Wonnemann M. *et al.* 2000, 2001), the naphthodianthrones, hypericin and pseudohypericin (Butterweck V. *et al.* 1998, 2001a,b) and several flavonoids (Butterweck V. *et al.* 2000, Calapai G. *et al.* 1999). However, the mechanism of action of these different compounds is still debated. It appears that these compounds interact with a variety of receptors, ion channels and transporters (Butterweck V. *et al.* 2002). This suggests that the antidepressant activity of HP is most likely the result of several compounds acting synergistically.

Down-regulation of central β-adrenergic receptors has widely been considered a common biochemical marker of antidepressant efficacy. These changes are only observed after two to three weeks of treatment. This corresponds with the clinical findings of the effects of antidepressant drugs (Simbrey K. *et al.* 2004). The following studies investigate the effect of HP, quercetin and fluoxetine on the β-adrenergic receptor density in the cortex of the rat after three weeks treatment.

5.1.1.2 Materials and Methods

5.1.1.2.1 Chemicals and Reagents

Tritiated dihydroalprenolol (DHA) (specific activity 40Ci/mmol) was purchased from Amersham (England). *Dl*-propranolol HCl was from Sigma Chemical Co (USA). *Hypericum perforatum* was from Flora Force (Pty) Ltd (Rondebosch, South Africa) and quercetin was from Sigma Aldrich (Switzerland). All other chemicals were obtained locally and were of the highest quality available.

5.1.1.2.2 Animals

Adult male Wistar rats weighing 200 – 300g were used in this study and housed under the conditions described in Appendix 1. The animals were dosed according to the procedure described in Section 2.2.2.2.

5 rats in the control group received the vehicle composed of ethanol, propylene glycol and glycerol (2:2:1)
5 rats in the hypericum group received 300mg/kg/day HP dissolved in the vehicle.
5 rats in the quercetin group received 5mg/kg/day quercetin dissolved in the vehicle.
5 rats in the fluoxetine group received 1.25mg/kg/day fluoxetine dissolved in the vehicle.

5.1.1.2.3 Tissue preparation

The animals were killed and decapitated between 10h00 and 12h00. The last drug administration was on the previous day. The brain was removed as described in Section 2.1.2.3. The brain, excluding the cerebellum, was homogenized (10% w/v) in 50mM Tris-acetate buffer (pH 7.2). The homogenate was centrifuged at 30 000 rpms for 20 minutes after which the supernatant was discarded. The pellet was re-suspended in the same buffer and centrifuged twice under the same conditions. The final pellet was re-suspended in 0.32M sucrose solution and stored at -70^0C until required.

5.1.1.2.4 Receptor binding assay

On the day of the assay, the homogenate was thawed, re-suspended in the Tris-acetate buffer (pH 7.2) and subjected to a further two steps of centrifugation and washing. The protein concentration was determined according to Lowry O.H. *et al.* (1951) (standard curve shown in Appendix 2) and the final protein concentration used in the assay was approximately 1mg/ml of buffer.

Radioligand receptor binding assays were used to determine the affinity (K_D) and density (Bmax) of the β-adrenergic receptors. As radioligand [^{3}H]-dihydroalprenalol (DHA), in a concentration range of 0.1 to 20nM, was used. Three hundred µl of the homogenate was incubated with six different concentrations of the radioligand for 60 minutes at 25^0C. Non-specific binding was determined by adding 10µM propranolol as the unlabelled ligand to half the tubes. Propranolol is assumed to selectively displace [^{3}H]-DHA from specific β-adrenoceptor sites. A blank, in which tissue was omitted, was also incubated in each series of assays. After incubation the reaction was terminated by adding 4ml of ice cold 50mM Tris-acetate buffer (pH 7.2) and rapidly filtered through Whatman GF/C filters rinsed with ice cold buffer. The bound and free ligand were separated by washing twice with the ice cold buffer.

The filters were then placed in plastic scintillation vials containing 5ml Ready-Solv HP scintillation cocktail (Beckman, USA), vortexed and left to stand overnight. The radioactivity in each sample was quantified the following day using liquid scintillation spectroscopy in a Beckman LS 2500 scintillation counter with a counting efficiency of 61.64%. The counting efficiency was determined by the external channel ratio method of quench correction using computer-assisted analysis. A correction factor was built into the quench correction programme to allow for tritium decay thus enabling the labelled compound to be accurately quantitated. Specific binding of [^{3}H]-DHA was calculated as total bound minus non-specific binding. The blank values were subtracted from the assay values before calculating the results. A schematic representation of the assay procedure is presented in Table 5.1 below.

Table 5.1 Method for the [^{3}H]-DHA receptor binding assay.

Reagents	Total Binding	Non-specific Binding
Homogenate	300µl	300µl
[^{3}H]-DHA (0.1-20nM)	30µl	30µl
dl-Propranolol (10µM)	---	10µl
Buffer	45µl	35µl
Incubate at 25^{0}C for 1 hour		
Buffer	4ml	4ml
Filter rapidly under pressure. Wash filters with 2 x 4ml ice cold buffer.		
Scintillation Fluid	5ml	5ml
Vortex and leave overnight. Count radioactivity.		

5.1.1.2.5 Data analysis and statistics

Radioligand binding data was analysed by the non-linear least square method using GRAPHPAD Prism 4 (GraphPad Software Inc., San Diego, California, USA). Data analysis was performed by analysis of variance (ANOVA) with the Newman-Keuls post-hoc test for multiple comparisons. Data are expressed as means ± SEM of 5 individual animals. Statistical significance was set at $P<0.05$.

5.1.1.3 Results

The results of three weeks treatment with HP, fluoxetine and quercetin, show that the affinity of the receptors as indicated by the K_D values, was not significantly altered (See Figure 5.1.1). However, there was a decrease in the number of binding sites (Bmax) for the rats treated with HP and quercetin (see Figure 5.1.2).

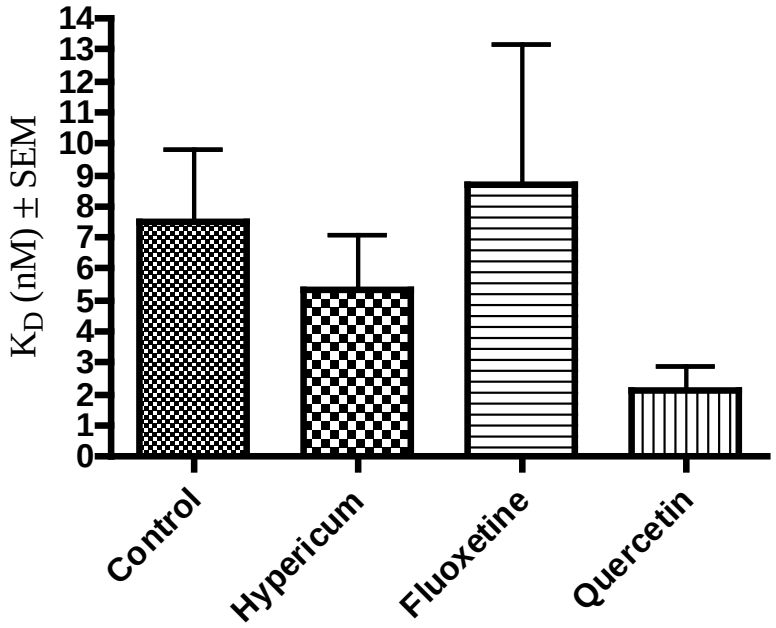


Figure 5.1.1 Effect of *Hypericum perforatum,* fluoxetine and quercetin on the affinity (Kd) of β-adrenergic receptors. Means ± SEM, n = 5.

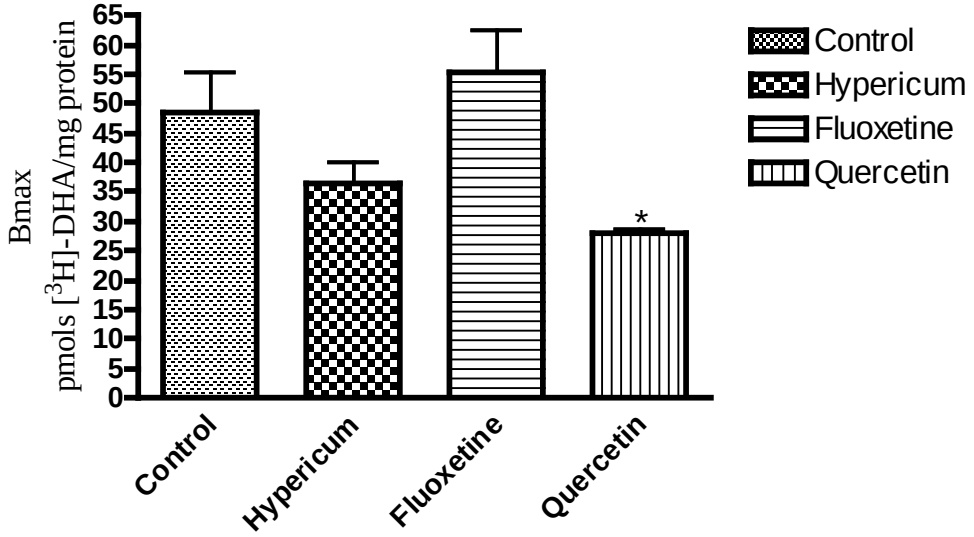


Figure 5.1.2 Effect of treatment with *Hypericum perforatum,* fluoxetine and quercetin on the β-adrenergic receptor density. *P<0.05. means ± SEM, n = 5.

5.1.1.4 Discussion

Clinical studies provide evidence that antidepressant effects become evident after 2–3 weeks treatment. In the present study the effects of *Hypericum perforatum* (HP), quercetin and the selective serotonin reuptake inhibitor fluoxetine on β-adrenergic binding after three weeks treatment were analysed. There were no significant changes in the affinity of the β-adrenergic receptors.

The results of this study show no significant decrease in the receptor density for the rats treated with HP. This finding is surprising because reports from previous studies in the literature show that three weeks treatment with HP (300mg/kg) extract leads to down-regulation of the cortical β-adrenergic receptors (Muller W.E. *et al.* 1997,1998; Simbrey K. *et al.* 2004).

The major novel finding from this study is that quercetin (5mg/kg) significantly decreases the number of *β*-adrenergic receptors in the rat brain. This suggests that quercetin could be the constituent of HP contributing to the antidepressant activity by causing down regulation of the β-adrenergic receptors. The concentration of quercetin in the HP extract used in the study was unknown but had been shown to be relatively high when compared with the other flavonoids and constituents in the extract (Wild T.J. 2003). It was decided to investigate the effect of different doses of quercetin on these receptors in the following study.

5.1.2 EFFECT OF DIFFERENT CONCENTRATIONS OF QUERCETIN ON THE DENSITY OF β-ADRENERGIC RECEPTORS.

5.1.2.1 Introduction

The previous study has shown that three weeks treatment with *Hypericum perforatum* (HP) does not result in a significant decrease in β-adrenergic receptor density. It was shown, however that the effect of treatment with HP's constituent, quercetin (5mg/kg), resulted in a significant decrease in the density of β-adrenergic receptors (β-AR). Down-regulation of β-AR is considered a common biochemical marker of antidepressant efficacy. Previous studies with HP have reported significant decreases in β-adrenergic receptor density (Muller W.E. *et al.* 1998, Simbrey K. *et al.* 2004). However, the constituent of HP responsible for these effects is still a matter of debate. The results of previous research into different constituents of HP have shown that the phloroglucinol derivative hyperforin-TMB and the flavonol-glycoside hyperoside does not affect the density of β-ARs (Simbrey K. *et al.* 2004). However, an earlier study, by Muller W.E. *et al.* (1998), showed that down regulation of these receptors occurrs after administration of a lipophilic HP extract with 38.8% hyperforin content. The results of their experiment suggest that hyperforin may be one, but not the only active compound in HP, since it was administered in a HP extract, in terms of affecting β-adrenergic receptors.

Quercetin is one of the flavonoids found in HP at relatively high concentration. The results of the findings in the previous study show a significant effect on the β-AR's by this compound. In the present study, it was decided to investigate the effect of different dosages of quercetin on the density of these receptors.

5.1.2.2 Materials and Methods

5.1.2.2.1 Chemicals and Reagents

The chemicals and reagents were the same as set out in Section 5.1.1.2.1.

5.1.2.2.2 Animals

Adult male rats of the Wistar strain were the same as described in section 2.3. The animals were housed and cared for as described in Appendix 1.The animals were dosed p.o. over twenty one days according to the procedure explained in Section 2.2.2.2 and the dosages for the different groups were the same as described in Section 2.3.2.2.

5.1.2.2.3 Tissue preparation

The tissue preparation was the same as described in Section 5.1.1.2.3.

5.1.2.2.4 Receptor binding assay

The receptor binding assay was the same as described in Section 5.1.1.2.4.

5.1.2.2.5 Data analysis and statistics

The data analysis and statistics were the same as described in Section 5.1.1.2.5.

5.1.2.3 Results

Figures 5.1.3 and 5.1.4 show the effects of treatment with different dosages of quercetin (1mg/kg vs 2.5 mg/kg) on β-adrenergic receptor status in rat brain after three weeks treatment.

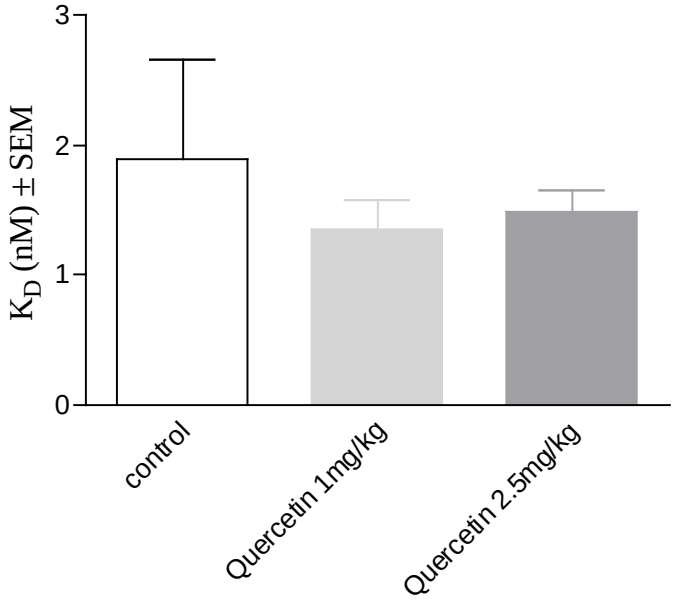


Figure 5.1.3 Effect of different dosages of quercetin (1mg/kg vs 2.5 mg/kg) on the affinity (K_D) of β-adrenergic receptors.
Mean ± SEM, n = 5.

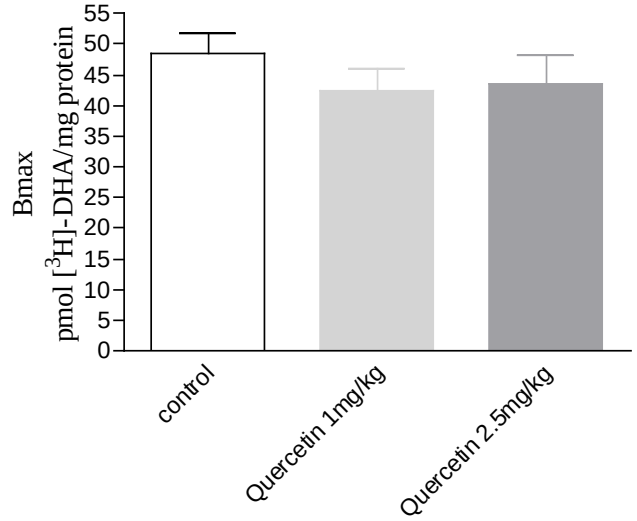


Figure 5.1.4 Effect of different dosages of quercetin (1mg/kg vs 2.5mg/kg) on the density (Bmax) of β-adrenergic receptors in rat brain.
Means ± SEM, n = 5.

5.1.2.4 Discussion

In the present study, the effects of different dosages of quercetin (1mg/kg and 2.5mg/kg) on β-adrenergic binding after three weeks treatment were analysed. The results of this study show that quercetin does not cause significant down-regulation of cortical β-adrenergic receptors (β-ARs) at dosages of 1mg/kg and 2.5mg/kg, because the decrease in the number of receptors (Bmax) shown in this study, were too small to be significant (See Figure 5.1.4). It was also shown that there was no change in receptor affinity (K_D) (See Figure 5.1.3). The significant decrease in β-AR density that was shown to occur in the previous study at a dose of 5mg/kg, suggests that the lower dosages of 1mg/kg and 2.5mg/kg are not sufficiently high enough to produce this effect. It would appear that a much higher dose of quercetin (5mg/kg) is required to affect β- adrenergic status.

The results of these experiments suggest that quercetin at sufficiently high enough concentration in HP extract may contribute to the down-regulation of β-AR receptors and this finding supports previous research reported in the literature (Müller W.E. *et al.* 1998, Simbrey K. *et al.* 2004).

5.2 SEROTONIN 2 RECEPTOR BINDING

5.2.1 EFFECT OF *Hypericum perforatum*, QUERCETIN AND FLUOXETINE ON THE DENSITY OF SEROTONIN 2 RECEPTORS.

5.2.1.1 Introduction

Serotonergic neurons and the neurohormones released by these neurons have been shown in numerous studies to play a role in depression. Several serotonin (5-HT) receptor subtypes have been identified and the 5-HT_2 subclass consists of the 5-HT_{2A}, 5-HT_{2B} and 5-HT_{2C} receptors. These three 5-HT_2 receptor subtypes are positively coupled through the G_q-protein to phospholipase C and phospholipase A_2 , and their activation leads to increased accumulation of inositol phosphates and intracellular Ca^{2+} and stimulation of these receptors leads to excitation (Van Oekenlen D. *et al.* 2003). 5-HT_{1c} and 5-HT_2 receptors have also been found to be closely related because both activate the phosphoinositide second messenger system and the antagonist, ketanserine, binds to both these receptors (Van de Kar L.D. 1991). Post mortem and brain imaging studies on depressed patients have shown a significant decrease in $5HT_2$ binding in the frontal, temporal, parietal and occipital cortical regions of the brain (Yatham L.N. *et al.* 2000). 5-HT_{2A} receptors occur in high concentration in the frontal cortex and the nucleus accumbens, 5-HT_{2C} receptors occur with moderate density throughout the forebrain and hind brain and 5-HT_{2B} receptors are scarce in the brain (Van Oekelen D. *et al.* 2003).

Previous studies report that subchronic treatment of rats, with a methanolic extract of *Hypericum perforatum*, leads to a significant increase in the density of cortical 5-HT_2 receptors (Muller W.E. *et al.* 1997; 1998). This finding was also supported by Teufel-Mayer R. and Gleitz J. (1997), although these authors used a much higher dose of the methanolic HP extract and a much longer treatment period. Interestingly, Muller W.E. *et al.* (1998) found that the CO_2 extract, which contained a much higher concentration of hyperforin, decreased 5-HT_2 receptor density. This suggests that hyperforin is unlikely to be the constituent in HP responsible for the increase in 5-HT_2 receptor density.

The following study investigated the effect of three weeks of treatments with HP, fluoxetine and quercetin on the 5-HT_2 receptors in the cortex of rat brain.

5.2.1.2 Materials and Methods

5.2.1.2.1 Chemicals and Reagents

Tritiated ketanserine (^{3}H-K) (specific activity 63.3Ci/mmol) was purchased from NEN™ Life Science Products, Inc. (Boston, MA, USA). Quercetin and cyproheptadine were purchased from Sigma Aldrich (Switzerland). All other chemicals were obtained locally and were of the highest quality available.

5.2.1.2.2 Animals

Adult male Wistar rats, weighing between 200 and 250g, were housed and cared for as described in Appendix 1. The animals were were the same as those described in section 2.2, dosed in the same way as described in Section 2.2.2.2 and the dosages given are the same as described in Section 5.1.1.2.2.

5.2.1.2.3 Tissue preparation

The tissue preparation was the same as described in Section 5.1.1.2.3.

5.2.1.2.4 Receptor binding assay

The method used for the receptor-binding assay was the same as that described in Section 5.1.1.2.4, except that the radioactive ligand and cold ligand used in this study were [^{3}H]-ketanserine and cyproheptadine, respectively. The method used is outlined in Table 5.2 below.

Table 5.2 Method for the [^{3}H]-ketanserine (K) receptor binding assay.

Reagents	Total Binding	Non-specific Binding
Homogenate	300µl	300µl
[^{3}H]-K (0.25-25nM)	25µl	25µl
cyproheptadine (10µM)	---	50µl
Buffer	70µl	20µl
Incubate at 25^{0}C for 1 hour		
Buffer	4ml	4ml
Filter rapidly under pressure. Wash filters with 2 x 4ml ice cold buffer.		
Scintillation Fluid	5ml	5ml
Vortex and leave overnight. Count radioactivity.		

5.2.1.2.5 Data analysis and statistics

The data analysis and statistics are the same as described in Section 5.1.1.2.5.

5.2.1.3 Results

Figures 5.2.1 and 5.2.2 show the effects of treatment with *Hypericum perforatum*, fluoxetine and quercetin on [^{3}H]-ketanserine binding to serotonin 2 receptors (5-HT$_2$). HP as well as fluoxetine induced a significant decrease in the affinity (K_D) of the 5-HT$_2$ receptor (See Figure 5.2.1). HP, fluoxetine and quercetin all induced significant increases in the density (Bmax) of the 5-HT$_2$ receptors (See Figure 5.2.2).

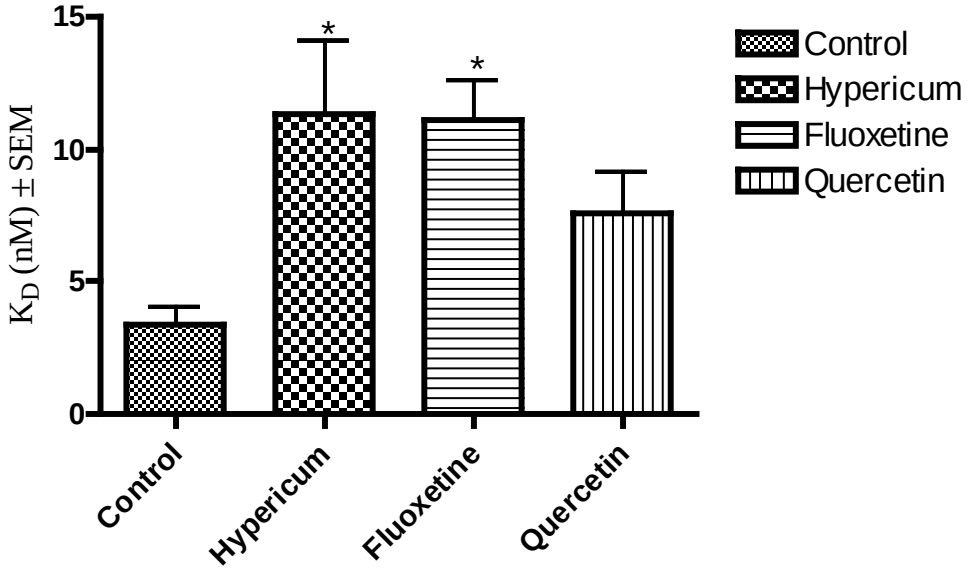


Figure 5.2.1 Effect of *Hypericum perforatum*, fluoxetine and quercetin on the affinity of 5-HT_2 receptors.
*P<0.05, means ± SEM, n = 5.

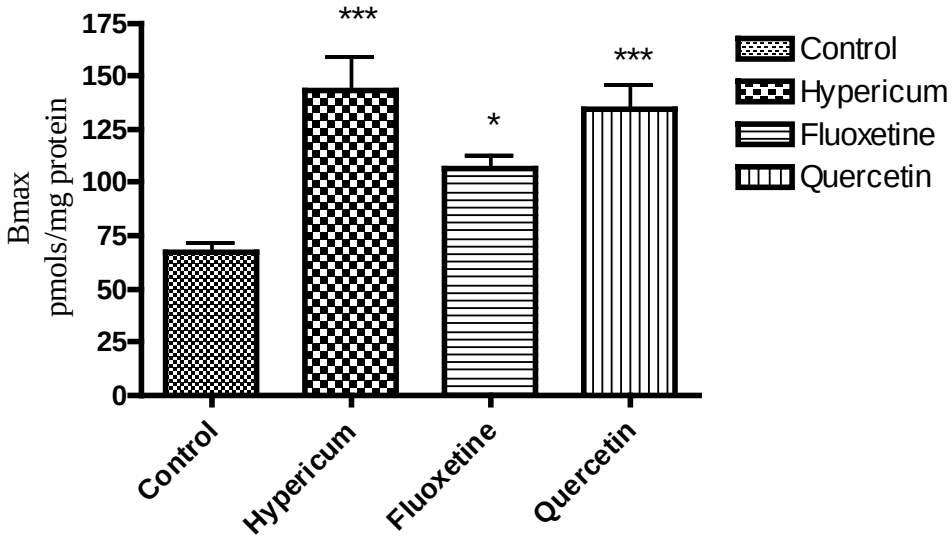


Figure 5.2.2 Effect of *Hypericum perforatum*, fluoxetine and quercetin on the density of 5-HT_2 receptors.
*P<0.05; ***P<0.001, means ± SEM, n = 5.

5.2.1.4 Discussion

The results of the present study indicate that three weeks treatment with *Hypericum perforatum* (300mg/kg), fluoxetine (1.25mg/kg) and quercetin (5mg/kg) induce adaptive changes to 5-HT_2 receptors. The significant increase in the density of 5-HT_2 receptors, after treatment with the HP extract, is in agreement with the findings of other authors (Muller W.E. 1997 &1998, Teufel-Mayer R. and Gleitz J. 1997). This, coupled with the novel finding of a significant increase in 5-HT_2 receptor density after three weeks treatment with quercetin (5mg/kg), suggests that quercetin is the constituent of HP responsible for these effects (See Figure 5.2.2). Previous research has only focused on the hyperforin enriched (38%) HP extract where down-regulation of 5-HT_2 receptors was found to occur (Muller W.E. *et al.* 1998).

The results of previous research into the effect of treatments with fluoxetine on 5-HT_2 receptor density is more complicated. A recent review of 5-HT_{2A} and 5-HT_{2C} receptor regulation by Van Oekenlen D. *et al.* (2003) reported decreased 5-HT_{2A} receptor densities, no changes and sometimes increased 5-HT_{2A} receptor densities, while there are consistent increased receptor densities reported for the 5-HT_{2C} receptors. A possible explanation for lack of change in [^{3}H]-ketanserine binding to 5-HT_{2A} receptors after treatment with fluoxetine is the suggestion that increased G-protein coupling occurred (Li Q. *et al.* 1997). The results of the present study indicate a significant increase in 5-HT_2 cortical receptor density after three weeks treatment with fluoxetine (1.25mg/kg) (See Figure 5.2.2).

Increased K_D values, indicating a decrease in the affinity of the 5-HT_2 receptor were observed after three weeks treatment with HP and fluoxetine. Similar results are reported by Goodnough D.B. and Baker G.B. (1994) after treatment with desipramine and fluoxetine. These authors suggest that this might be the result of residual drug being in the brain when the rats were killed.

The following study investigates the effect of different doses of quercetin on the 5-HT_2 receptors of rat brain.

5.2.2 EFFECT OF DIFFERENT CONCENTRATIONS OF QUERCETIN ON THE DENSITY OF SEROTONIN 2 RECEPTORS.

5.2.2.1 Introduction

The previous study has shown that three weeks treatment with *Hypericum perforatum* (HP) results in a significant increase in serotonin 2, (5-HT_2) receptor density. It was also shown that the effect of HP's constituent, quercetin, was to significantly increase the density of 5-HT_2 receptors. Changes in the regulation of 5-HT_2 receptors has been suggested to be a common biochemical marker of antidepressant efficacy (Van Oekelen D. *et al.* 2003). Previous studies with HP have reported similar findings for HP (Muller W.E. *et al.* 1997 &1998, Teufel-Mayer R. and Gleitz J. 1997). However, the constituent of HP responsible for these effects is still a matter of debate. The results of previous research into different constituents of HP affecting the density of 5-HT_2 receptor such as the study by Muller W.E. *et al.* 1998, showed that down-regulation of these receptors occurr after administration of a lipophilic HP extract with 38.8% hyperforin content. The results of this experiment suggest that hyperforin may be one, but not the only active compound in HP in terms of affecting 5-HT_2 receptors.

Quercetin is one of the flavonoids found in HP at a relatively high concentration and the results of the findings in the previous study show a significant effect on the 5-HT_2 receptors by this compound. In the present study, it was decided to investigate the effect of different doses of quercetin on the density of these receptors.

5.2.2.2 Materials and Methods

5.2.2.2.1 Chemicals and Reagents

The chemicals and reagents are the same as described in Section 5.1.1.2.1 and Section 5.2.1.2.1.

5.2.2.2.2 Animals

Adult male Wistar rats weighing between 200 and 250g were the same as described in section 2.3. The animals were housed and cared for as described in Appendix 1. The rats were dosed as described in Section 2.2.2.2.

5.2.2.2.3 Tissue preparation

The tissue preparation was the same as described in Section 5.1.1.2.3.

5.2.2.2.4 Receptor binding assay

The receptor binding assay was the same as described in Section 5.2.1.2.4.

5.2.2.2.5 Data analysis and statistics

The data analysis and statistics were the same as described in Section 5.1.1.2.5.

5.2.2.3 Results

Figures 5.2.3 and 5.2.4 show the effects of treatment with different dosages of quercetin (1mg/kg vs 2.5 mg/kg) on 5-HT_2 receptors in rat brain after three weeks treatment. A significant decrease in the K_D value, indicating a change in the affinity of the 5-HT_2 receptor, was observed for the rats treated with 2.5mg/kg (See Figure 5.2.3). A significant decrease in the density of the 5-HT_2 receptors was also observed for the rats treated with 2.5mg/kg quercetin (See Figure 5.2.4).

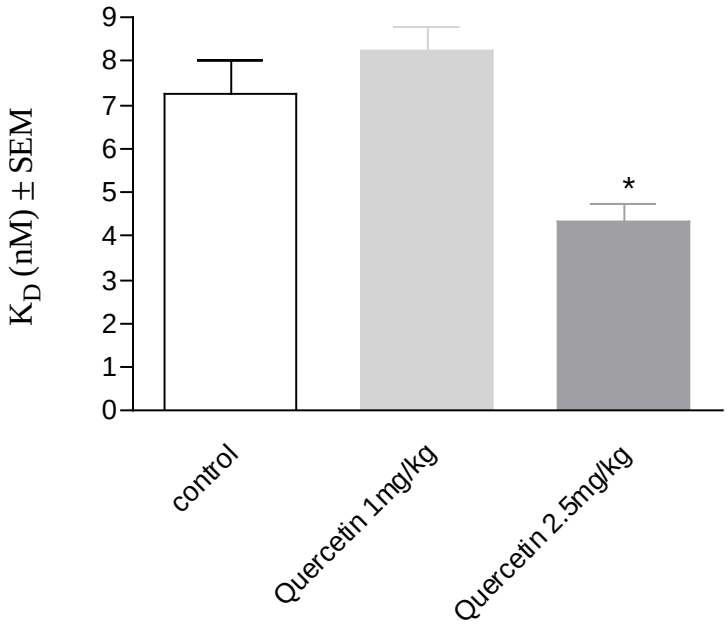


Figure 5.2.3 Effect of different dosages of quercetin (1 mg/kg vs 2.5mg/kg) on the affinity of 5-HT_2 receptors in rat brain. *P<0.05, means ± SEM, n = 5.

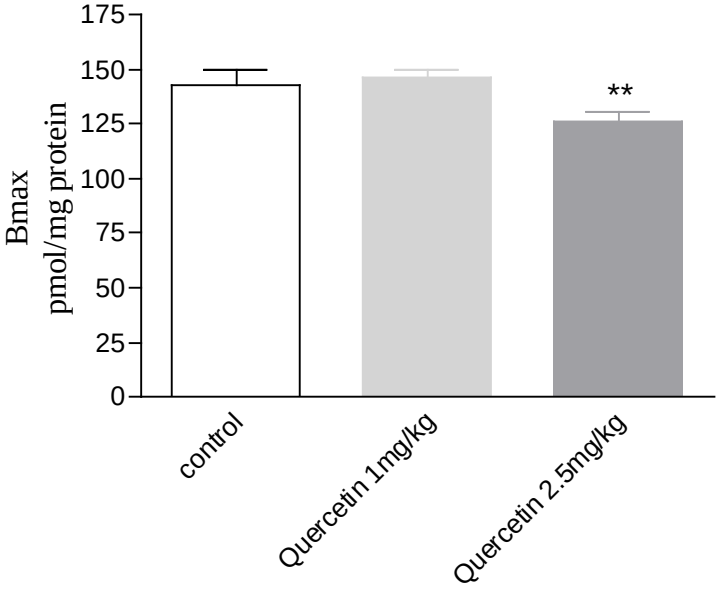


Figure 5.2 4 Effect of different dosages of quercetin (1mg/kg vs 2.5mg/kg) on the density(Bmax) of 5-HT_2 receptors in rat brain.**P< 0.01, means ± SEM, n=5.

5.2.2.4 Discussion

In the present study, the results show that three weeks treatment with quercetin at 1mg/kg induced no change to 5-HT_2 receptor density, while at 2.5mg/kg a significant decrease in the 5-HT_2 receptor density was observed (See Figure 5.2.3). These results are different from the results observed in the previous study where a dosage of 5mg/kg of quercetin induced an increase in 5-HT_2 receptor density. This suggests that the adaptive changes observed in 5-HT_2 receptors of rat brain, are dose sensitive. The results of the serotonin ELISA study reported in the previous chapter show increased 5-HT levels in the whole brain which is not unexpected with an increase in 5-HT receptor density.

Similar differences were observed in the K_D values. An increase in the affinity of the radioligand for the 5-HT_2 receptor was observed after three weeks treatment at the higher dose of 2.5mg/kg. It is unlikely that changes in affinity obscured any changes in Bmax in this study, as treatment with a higher dose in the previous study did not produce any significant changes in the K_D value and an increase in Bmax.

5.3 *N*-METHYL-D-ASPARTATE RECEPTOR BINDING

5.3.1 EFFECT OF *Hypericum perforatum*, QUERCETIN AND FLUOXETINE ON THE DENSITY OF *N*-METHYL-D-ASPARTATE RECEPTORS.

5.3.1.1 Introduction

The role of the *N*-methyl-D-aspartate (NMDA) receptor in the pathophysiology of psychiatric syndromes was reviewed by Heresco-Levy U. and Javitt D.C. (1998). The authors cite recent findings which indicate that dysfunction of central nervous system (CNS) pathways may represent one of the mechanisms involved in depression. This article reports of several studies using a variety of preclinical models for depression, independently confirming that compounds that reduce activity at NMDA receptors mimic the effects of clinically active antidepressants. Furthermore, it has been shown that adaptive changes occur in the NMDA receptor complex after chronic treatment with established antidepressant drugs. It is suggested that these adaptive changes could be a common feature of treatment with structurally unrelated compounds.

The NMDA receptor complex consists of several distinct recognition sites:

1. A cation channel that permits the conductance of calcium (Ca^{2+}) and sodium (Na^{+}).
2. A voltage dependent site that binds magnesium (Mg^{2+}) and at resting potential blocks channel conductance.
3. A co-agonist site that binds glycine (GLY).
4. An inhibitory site that binds phencyclidine (PCP), ketamine, dizocilpine (MK-801) and other non-competitive antagonists.
5. An allosteric modulatory site that recognizes polyamines.

The NMDA receptor and its channel have several exceptional properties. In particular, pronounced Ca^{2+} influx is allowed to occur and under pathological conditions this may mediate a

process known as excitotoxicity. This toxicity is not fully understood but is considered to involve excess inflow of Ca^{2+} through NMDA activated channels. The potential of NMDA receptors to induce neuronal damage has led to the hypothesised role of these receptors, in the pathogenesis of a wide variety of neuropsychiatric disorders such as depression, post traumatic stress disorder and alcoholism (Heresco-Levy U and Javitt D.C. 1998).

The ability of antidepressants to induce adaptive changes in the GLY regulatory sites of the NMDA receptor, is consistent with the hypothesis that the ligand gated ion channel associated with this receptor complex may serve as a final common pathway of antidepressant action. Support for this concept is the finding of NMDA receptor alterations in the frontal cortex of human suicide victims (Nowak G. *et al.,* 1995).

The highest densities of NMDA receptors are found in the cortex and hippocampus. The non-competitive antagonist, MK-801 cation channel blocker, has been shown to reduce immobility in the forced swim test with an efficacy similar to that of imipramine (Trullas R. and Skolnick P. 1990). The following studies investigate the effect of HP, quercetin and fluoxetine on the NMDA receptor density using the radioligand MK-801.

5.3.1.1.1 Materials and Methods

5.3.1.1.2 Chemicals and Reagents

Tritiated MK-801 (^{3}H-MK) (specific activity 28.9Ci/mmol) was purchased from NEN™ Life Science Products, Inc. (Boston, MA, USA). Ketamine was purchased locally and quercetin was from Sigma Aldrich (Switzerland). All other chemicals were obtained locally and were of the highest quality available.

5.3.1.1.3 Animals

Adult male Wistar rats weighing between 200 and 250g were used in this study. The animals were housed and cared for as described in Appendix 1. The rats were dosed as described in Section 5.1.1.2.2.

5.3.1.1.4 Tissue preparation

The tissue preparation was carried out as described in Section 5.1.1.2.3.

5.3.1.1.5 Receptor binding assay

The receptor binding assay was carried out as described in Section 5.1.1.2.4 except that the radioligand was MK-801 (0.2 – 50nM) and ketamine (10μM) was used as the cold ligand. The method is summarised in Table 5.3 below.

Table 5.3 Method for the [^{3}H]-MK-801 receptor binding assay.

Reagents	**Total Binding**	**Non-specific Binding**
Homogenate	300μl	300μl
[^{3}H]-MK-801 (0.25-25nM)	15μl	15μl
ketamine (10μM)	---	35μl
Buffer	35μl	---
Incubate at 25^{0}C for 1 hour		
Buffer	4ml	4ml
Filter rapidly under pressure. **Wash filters with 2 x 4ml ice cold buffer.**		
Scintillation Fluid	5ml	5ml
Vortex and leave overnight. **Count radioactivity.**		

5.3.1.1.6 Data analysis and statistics

The data analysis and statistics were carried out as described in Section 5.1.1.2.5.

5.3.1.2 Results

Figures 5.3.1 and 5.3.2 show the effects of treatment with *Hypericum perforatum*, fluoxetine and quercetin on [^{3}H]-MK-801 binding to the *N*-methyl-D-aspartate receptors (NMDA). Fluoxetine induced a significant decrease in the affinity (K_D) of the NMDA receptor (See Figure 5.3.1). There was a significant decrease in the density of the NMDA receptors for the rats treated with HP while fluoxetine and quercetin did not induce any significant changes in the density (Bmax) of the NMDA receptors (See Figure 5.3.2).

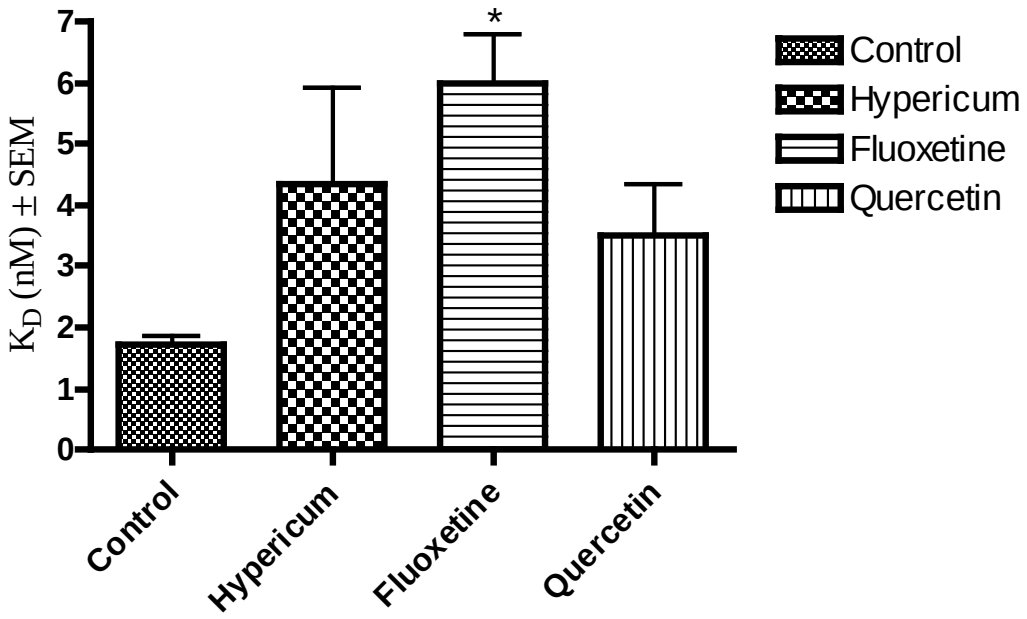


Figure 5.3.1 Effect of *Hypericum perforatum*, fluoxetine and quercetin on the affinity (K_D) of the NMDA receptor. *P<0.05, means ± SEM, n = 5.

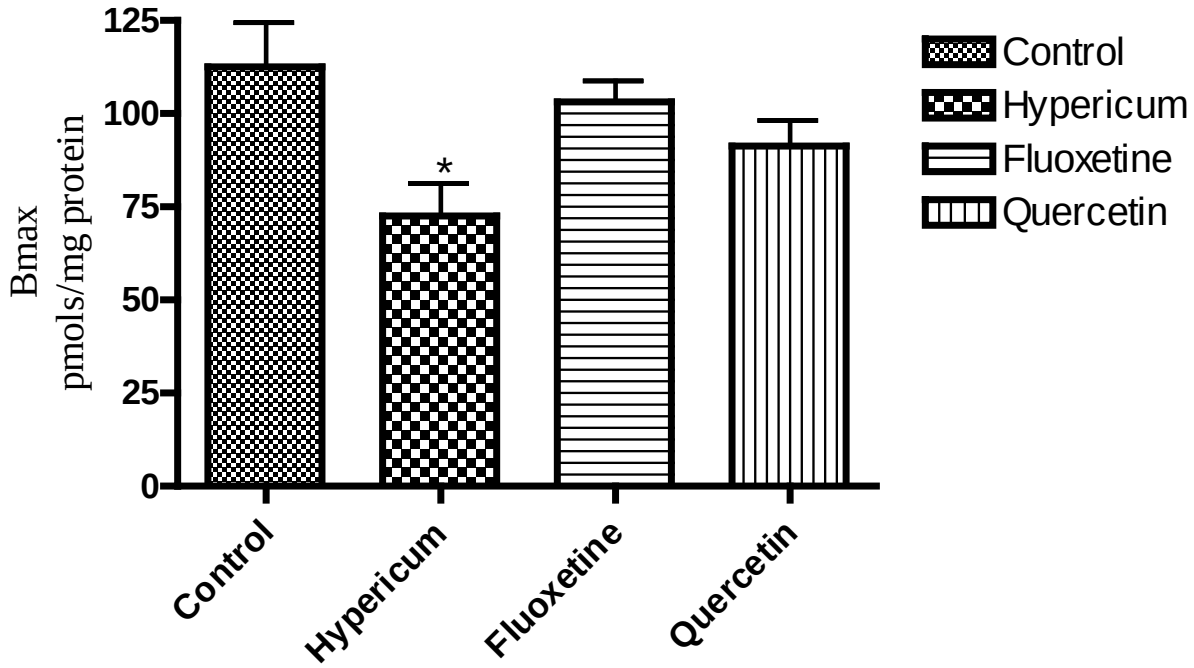


Figure 5.3.2 Effect of *Hypericum perforatum*, fluoxetine and quercetin on NMDA receptor density (Bmax). *P<0.05, means ± SEM, n = 5.

5.3.1.2.1 Discussion

The results of three weeks treatment with *Hypericum perforatum*, fluoxetine and quercetin show small adaptive changes to the NMDA receptor complex. There was a significant decrease in the density of the NMDA receptors for the group treated with HP (300mg/kg). There was a small increase in the K_D value for the rats treated with fluoxetine (1.25mg/kg) and no significant changes to the binding affinity induced by treatments with HP and quercetin (5mg/kg).

Previous studies have shown that chronic treatment of mice with clinically active antidepressants, including the selective serotonin re-uptake inhibitor fluoxetine, induces adaptive changes to the NMDA receptor complex and in particular, to ligand binding properties (Paul I.A. *et al.,* 1994; Nowak G. *et al.,* 1993 and 1996). These studies differ from the present study in the choice of radioligand and therefore its mode of binding to the NMDA receptor channel. The former studies focus on the co-agonist site that binds glycine, while the present study focuses on the inhibitory site that binds non-competitive antagonists such as MK-801. There have not been any other

studies reported in the literature on the effect of HP nor any of its constituents on the NMDA receptor complex. This first study suggests that HP is able to induce adaptive changes in this receptor complex, especially with regard to the binding of antagonists to the inhibitory site.

There was a small, though not significant, decrease in Bmax for the group treated with quercetin. It was decided to investigate different dosages of this compound on the binding of [^{3}H]-MK-801 to the inhibitory site of the NMDA complex in the following study.

5.3.2 EFFECT OF DIFFERENT CONCENTRATIONS OF QUERCETIN ON THE DENSITY OF *N*-METHYL-D-ASPARTATE RECEPTORS.

5.3.2.1 Introduction

The previous study has shown that three weeks treatment with *Hypericum perforatum* (HP) results in a significant decrease in NMDA receptor density. Adaptive changes that are brought about by treatment with antidepressants in the NMDA receptor complex have been suggested to be a common biochemical marker of antidepressant efficacy (Paul I. A. *et al.*, 1994). There are no previous studies investigating whether treatment with HP affects the NMDA complex. The constituent of HP responsible for the effects reported in the previous study are unknown.

Quercetin is one of the flavonoids found in HP at relatively high concentration and while the results of the findings in the previous study do not show a significant down-regulation of the NMDA receptors by this compound, as was shown by HP, it was decided to investigate the effect of different dosages of quercetin on the density of these receptors.

5.3.2.2 Materials and Methods

5.3.2.2.1 Chemicals and Reagents

The chemicals and reagents are the same as described in Section 5.1.2.2.1 and Section 5.3.1.2.1.

5.3.2.2.2 Animals

Adult male Wistar rats weighing between 200 and 250g as described in section 2.3 were used in this study. The animals were housed and cared for as described in Appendix 1. The rats were dosed as described in Section 2.3.2.2

5.3.2.2.3 Tissue preparation

The tissue preparation was carried out as described in Section 5.1.1.2.3.

5.3.2.2.4 Receptor binding assay

The receptor binding assay was carried out as described in Section 5.3.1.2.4.

5.3.2.2.5 Data analysis and statistics

The data analysis and statistics were performed as described in Section 5.1.2.2.5.

5.3.2.3 Results

Figures 5.3.3 and 5.3.4 show the effects of treatment with different dosages of quercetin (1mg/kg vs 2.5 mg/kg) on NMDA receptor status in the rat brain after three weeks treatment. There were no significant changes in the K_D value, indicating there is no change in the affinity of the NMDA receptor for the rats treated with 1mg/kg and 2.5mg/kg (See Figure 5.3.3). However, a significant decrease in the density of the NMDA receptors was also observed for the rats treated with both 1mg/kg and 2.5mg/kg quercetin (See Figure 5.3.4).

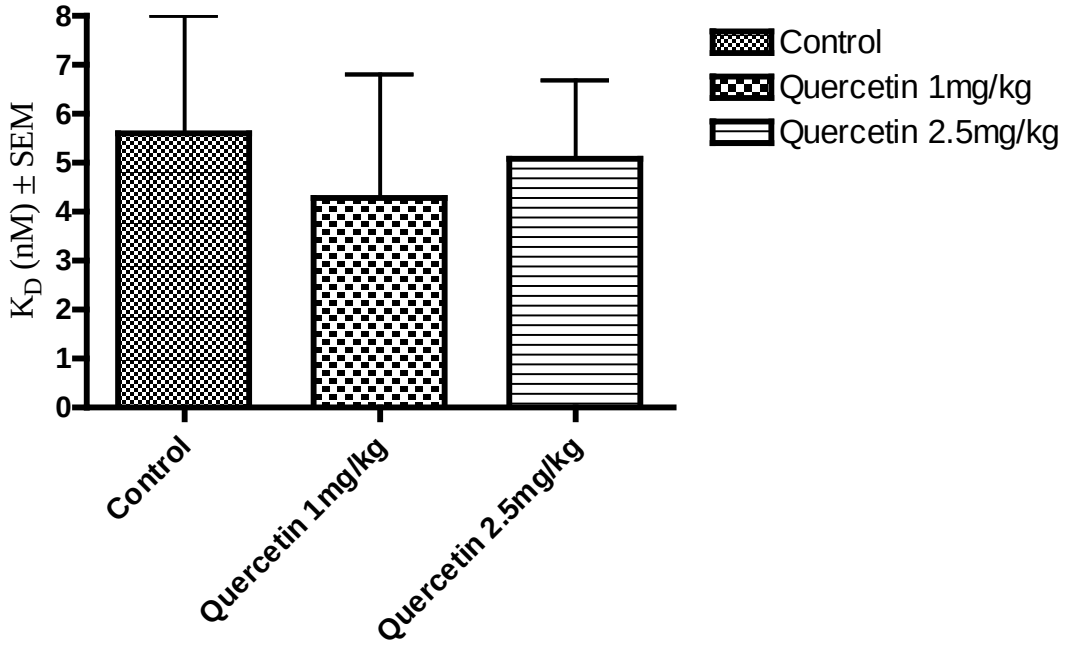


Figure 5.3.3 Effect of different concentrations of quercetin (1mg/kg vs 2.5mg/kg) on the affinity (Kd) for the NMDA receptor. Means ± SEM, n = 5.

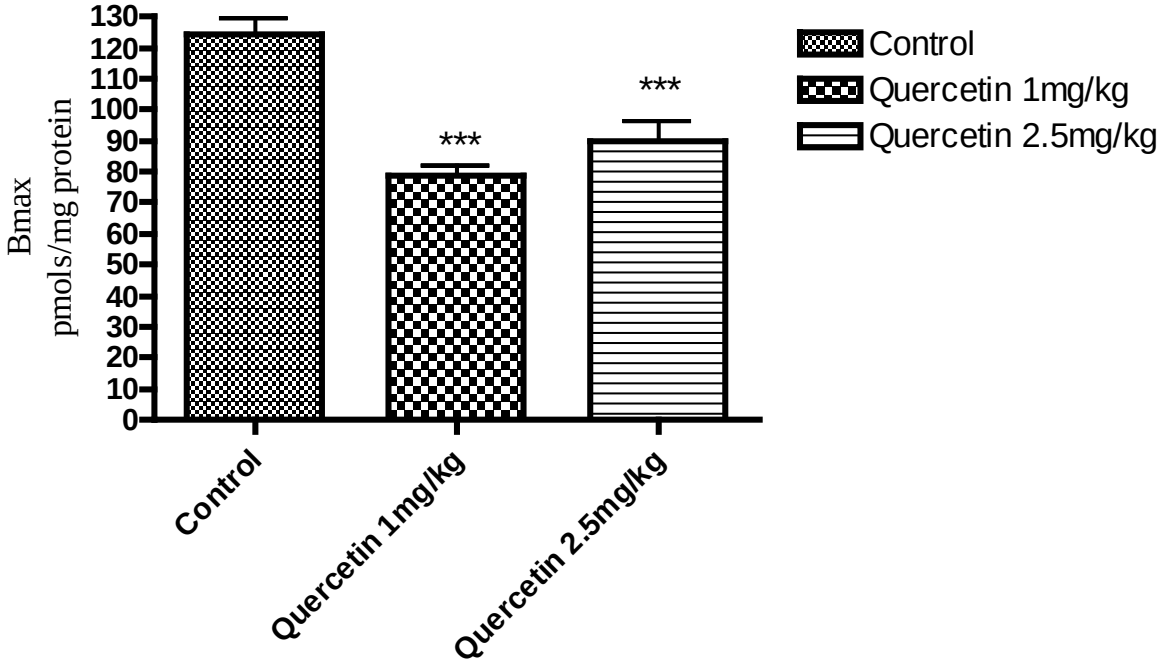


Figure 5.3.4 Effect of different concentrations of quercetin (1mg/kg vs 2.5mg/kg) on the NMDA receptor density. ***P<0.001, means ± SEM, n = 5.

5.3.2.4 Discussion

In the present study, the results show that three weeks treatment with quercetin at both 1mg/kg and 2.5mg/kg induced a significant decrease in NMDA receptor density (See Figure 5.3.4). The results observed in the previous study with a dose of 5mg/kg of quercetin induced no significant decrease in NMDA receptor density. This suggests that the adaptive changes observed in the NMDA receptors in rat brain, are more profound at a lower dose than the 5mg/kg used in the previous study.

This novel finding, taken with the significant down-regulation of the NMDA receptor complex reported in the previous study by HP, suggests that adaptations to the ligand-gated ion channel of the NMDA receptor complex may form part of the antidepressant activity of this natural remedy. Furthermore, the significant decrease in the NMDA receptor density reported in the present study after three weeks treatment with quercetin, suggests that this compound is one of the constituents of HP contributing to its antidepressant activity.

There were no significant differences observed in the K_D values, indicating that there was no change in the affinity of the NMDA receptor after three weeks treatment at the dosages of 1mg/kg and 2.5mg/kg.

CONCLUSIONS

The results of the studies reported here on the effect of three weeks of treatment of rats with *Hypericum perforatum,* quercetin and fluoxetine, show that these compounds have different effects on the regulation of β-adrenergic, 5-HT_2 and NMDA receptors. The significant decrease in the β-AR density shown for the rats treated with HP in previous studies reported in the literature was not found in this study. However, the significant decrease in these receptors in the rats treated with quercetin (5mg/kg) is a novel finding, suggesting that this compound at a high concentration, could be one of the constituents in HP contributing in this way, to its antidepressant effects.

The results of the studies on the effects of the different compounds, (HP, quercetin and fluoxetine), on the serotonin 2 receptors have shown quite diverse effects on their regulation. A significant change in the affinity of these receptors was observed for the rats treated with HP and fluoxetine. However, the significant increase in the densities of these receptors observed in the rats treated with HP, quercetin and fluoxetine, is supported by the majority of findings reported in the literature. Surprisingly, a lower dosage of quercetin (2.5mg/kg) produced the opposite effect of down-regulation of these receptors suggesting that the effects observed are dose sensitive.

The possible role of the NMDA receptors in the etiology of depression has been reviewed. The emerging body of data suggests that dysfunction of the NMDA receptor mediated transmission may have an important implication for the pathophysiology of depression (Heresco-Levy U. and Javitt D.C. 1998). The results of our studies on the effects of the different compounds (HP, quercetin and fluoxetine) on the NMDA receptor complex, reveal that the rats treated with fluoxetine showed a significant decrease in the affinity of the NMDA receptors. There was significant down-regulation of these receptors observed in the rats treated with HP and quercetin, and the effect was observed to be even greater at the lower dose of quercetin suggesting that the effects may be dose sensitive. These findings are novel as there have not been any previous reports in the literature of the effects of treatments with *Hypericum perforatum* nor any of its constituents, on the NMDA receptors. The implication of these findings is that the changes effected on the NMDA receptor complex by HP and/or its constituent, quercetin, may provide some insight into the mode of action of this natural products' antidepressant activity.

CHAPTER 6

LIQUID CHROMATOGRAPHY

INTRODUCTION

Monoamines such as dopamine (DA), serotonin (5-HT) and the metabolites are important neurotransmitters released into extracellular fluids by neurons in the brain. There have been several studies showing that dopamine might be important in mediating the effects of hypericum extract. In the "forced swimming" or "Porsolt test" hypericum induces a decrease in immobility similar to imipramine on the behaviour of mice (Butterweck V. *et al.* 1997, De Vry J. *et al.* 1999, Panocka I. *et al.* 2000). This effect is blocked on exposure to dopamine antagonists, haloperodol and sulpiride. An increase in the ratio of homovanillinic acid to dopamine indicates an increased turnover of dopamine, and lowered serum-prolactin levels occur after treatment with hypericum. The lowering of prolactin is an indirect indicator of elevated dopamine levels because an increase in dopamine generally leads to a decrease in prolactin (Winterhof H. *et al.* 1995). A small clinical study showed neuroendocrine evidence for dopaminergic actions of *Hypericum perforatum* extract (Li 160) in healthy volunteers (Franklin M. *et al.* 1999). HP has been shown to increase DA release and causes a delayed decrease of DOPAC in awake, freely moving rats (Qin Zhou *et al.* 2003). The results of a study by Butterweck *et al.,* (1998) suggest that it is the naphthodianthrone constituents of *Hypericum perforatum* which affect the dopaminergic system involved in antidepressant activity of HP.

Ethanolic hypericum extracts have been shown to dose-dependently inhibit serotonin uptake in rat peritoneal cells (Chatterjee S.S. *et al.*, 1998) as well as potentiate dopaminergic behavioural responses (Bhattacharya S.K. *et al.*, 1998). Calapai G. *et al.*, (1999) studied the effects of two different extracts of *Hypericum perforatum* (Li 160 containing 6% flavonoids and Ph-50 containing 50% flavonoids) on serotonin turnover in the cortex, diencephalon and brainstem of the rat. These results show that both extracts increase serotonin turnover in the cortex, but only the Ph-50 increases 5-HT levels in the diencephalon and brainstem.

6.1 DETERMINATION OF MONOAMINE NEUROTRANSMITTERS USING LIQUID CHROMATOGRAPHY WITH ELECTROCHEMICAL DETECTION.

6.1.1 Introduction

Hypericum perforatum consists of more than twenty four constituents, of which several, including the flavonoids, have been thought to contribute pharmacologically to its antidepressant activity. Quercetin is the simplest of the flavonoids occurring in relatively high concentration in HP (Butterweck V. *et al.* 2001). Studies based on the pharmacological properties of depression suggest that increases in the levels of 5-HT, noradrenaline and or DA in the cortex (Calapai G. *et al.* 1999) and subsequent inhibition of reuptake of these biogenic amines in the synapses, could form the basis of depression (Richelson E. 1996).

An isocratic liquid chromatographic system with electrochemical detection was used for the determination of dopamine (DA), 5-hydroxytryptamine (5-HT) and their metabolites 3,4 dihydroxyphenylacetic acid (DOPAC), 5-hydroxyindoleacetic acid (5-HIAA) and homovanillic acid (HVA) levels in rat brain. The rats were treated daily p.o. as described previously in chapter 2 (section 2.2) with *Hypericum perforatum* (300mg/kg), quercetin (5mg/kg) and fluoxetine (1.25mg/kg).

In the present study, the effects of *Hypericum perforatum* and quercetin on brain concentrations of monoamine neurotransmitters and turnover of dopamine and serotonin in the rat are reported.

6.1.2 Materials and Methods

6.1.2.1 Chemicals and Reagents

HPLC-grade methanol CHROMASOL® was obtained from Sigma-Aldrich (Switzerland). Disodium ethylenediaminetetraacetate (EDTA) from Micor (Johannesburg, SA). 1-Pentasulphonic acid sodium salt (Pic B5) from BDH Laboratory Supplies (England). Citric

Acid, Sodium Acetate, and Di-n-butylamine from UNILAB (SAARCHEM (Pty) Ltd, South Africa). 5-Hydroxytryptamine (serotonin) (5-HT) hydrochloride, 5-HT creatinine sulphate complex, 4-hydroxy-3-methoxy-phenyl acetic acid (homovanillic acid) (HVA) and 5-methoxy-N-acetyltryptamine (melatonin or aMT) were purchased from Sigma St Louis, MO, USA. 3,4-Dihydroxyphenylacetic acid from Sigma-Aldrich (Steinheim, Germany), 3-hydroxy-tryptamine hydrochloride (dopamine) (DA) from Fluka (Belgium) and 5-hydroxy-indole-3-acetic acid from Polysciences Inc (USA).

6.1.2.2 Animals

Experiments were conducted on adult male Wistar rats (200-250g), housed five per cage in a 12 hour light-dark cycle, with lights off at 18h00, at a constant temperature of 25^0C and free access to standard laboratory chow and tap water. The animals were randomly assigned to different experimental groups (n = 5/group) and the protocols for the experiments were approved by the Rhodes University Animal Ethics Committee. The same dosing procedure was followed as described in section 2.2.

6.1.2.3 Sample preparation

The animals were sacrificed by decapitation and the brains dissected on ice. The cerebellum was removed and the remainder of the brain was homogenised (10%w/v) in 50mM Tris acetate buffer (pH 7.4). The homogenate was centrifuged at 30 000 rpms at 4^0C for 20 minutes. The supernatant containing the endogenous monoamine neurotransmitters were frozen at -70^0C until the day of the assay.

6.1.2.4 Apparatus and chromatographic conditions

Liquid chromatographic experiments were conducted on a Waters model 510 pump connected to a Waters Rheodyne injector fitted to a 20µl loop and a Waters model 460 electrochemical detector (ECD) (MILLIPORE Waters Chromatography Division, Milford, MA 01757 USA). The ECD had the following settings: operating potential +0.700V, filter 1, range 5 and time constant 5

seconds. The flow rate was constant at 0.8ml/min. Injection volume was 70µl. The working electrode was a glassy carbon electrode and the reference electrode was a modified Ag/AgCl cell. Chromatographic separations were performed on a Waters Nova-Pak® C18 (3.9mm x 15cm) (Waters, South Africa) column. The mobile phase consisted of 97% aqueous buffer (50mM sodium acetate, 20mM Citric acid, 3.75mM Pic B5, EDTA (20mg in 300ml), 1mM di-n-butylamine) and 3% methanol. All experiments were performed at room temperature.

Tissue levels were determined by means of external standards and expressed in terms of pmoles per 20µl injection. Correlation coefficients and calibration curves ($y = mx + c$) based on peak height ratios were established by means of linear regression analysis: 5-HT: 0.9972, $1.48x + 0.3$; 5-HIAA: 0.9999, $x + 0.45$; DA: 0.9999, $0.27x + 0.048$; DOPAC: 0.9999, $0.22x + 0.15$; HVA: 0.9999, $0.14x + 0.3$.

All statistical procedures were performed using the GRAPHPAD PRISM software package, version 4.0, USA. Data analysis was performed by analysis of variance (ANOVA) with the Student-Newman-Keuls post hoc test for multiple comparisons. Data are expressed as means±S.E.M. Statistical significance was set at $P<0.05$.

6.1.3 Results

6.1.3.1 Effects of Hypericum perforatum and quercetin on 5-HT and 5-HIAA levels and on serotonin turnover in rat brain.

The content of the indoleamines (5-HT, 5-HIAA, DA, DOPAC and HVA) in the brain after three weeks treatment, expressed as ng/g brain tissue (means ± SEM), is shown in Table 6.1 below.

Table 6.1: Effect of *Hypericum perforatum* (300mg/kg) and quercetin (5mg/kg) on indoleamines in rat brain expressed as ng/g brain tissue (mean±SEM).

	Control	***Hypericum perforatum***	**Quercetin**
5-HT	144 ± 2.4	171 ± 7.2**	202 ± 2.7***
5-HIAA	178 ± 6.1	154 ± 20	156 ± 29
DA	918 ± 7.1	759 ± 61*	1084 ± 60*
DOPAC	307 ± 25	784 ± 95***	365 ± 16
HVA	247 ± 14	347 ± 88	419 ± 12

Values are mean±S.E.M. expressed as ng/g brain concentration of 5 rats per group. *P<0.05; **P<0.01; ***P<0.001. Data analysis was performed by analysis of variance followed by the Student-Newman-Keuls test for multiple comparisons.

Hypericum perforatum (300mg/kg p.o.) and quercetin (5mg/kg p.o.) significantly increased 5-HT levels (P<0.01 and P<0.001 respectively) as shown in figure 6.1 while there were no significant changes in 5-HIAA levels.

The 5-HT turnover, calculated as the ratio [5-HIAA/5-HT] was significantly reduced (P<0.05) as shown in figure 6.2.

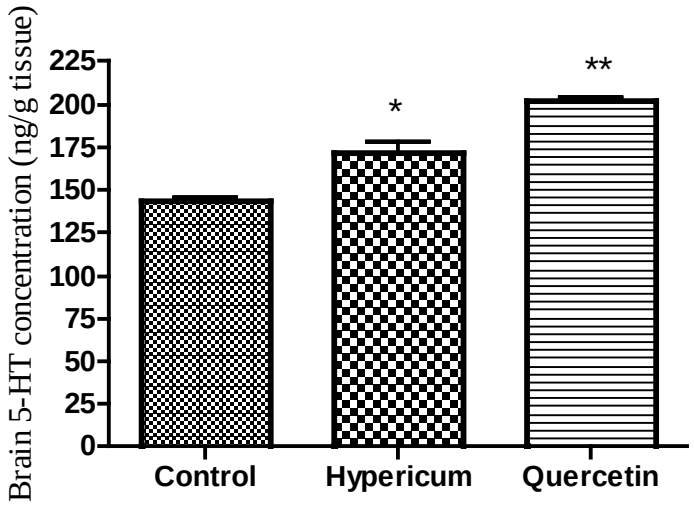


Figure 6.1 Comparison of 5-HT levels in rat brain.*P<0.01and **P<0.001 when compared with vehicle treated control rats using the Student -Newman-Keuls multiple comparison test. Means ± SEM

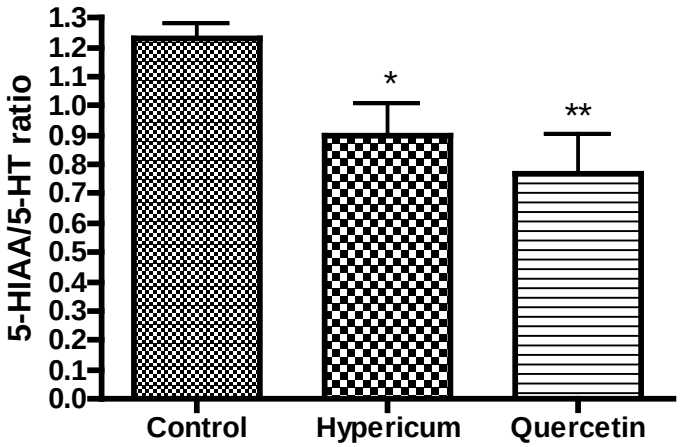


Figure 6.2 Effective treatments of *Hypericum* extract (300mg/kg p.o.) and quercetin (5mg/kg p.o.) on serotonin turnover in rat brain.
Data are expressed as mean ± SEM. Actual levels of 5-HT and 5-HIAA are shown in Table 6.1;*P<0.05;**P<0.01 when compared with vehicle treated control rats using Student-Newman-Keuls multiple comparison test.

6.1.3.2 Effects of Hypericum perforatum and quercetin on DA, DOPAC and HVA levels and on dopamine turnover in rat brain.

DA levels were significantly increased after treatment with quercetin ($P<0.05$) while there was no significant change in the rats treated with *Hypericum perforatum* as can be seen in figure 6.3. There was a pronounced change in DOPAC levels in the rats treated with *Hypericum perforatum* and no significant change in the rats treated with quercetin as can be seen in figure 6.4. There were no significant changes in the HVA levels.

Dopamine turnover as measured by [DOPAC+HVA]/DA was significantly increased in the brain by *Hypericum perforatum* ($P<0.01$) and only slightly but not significantly by quercetin as shown in Figure 6.5.

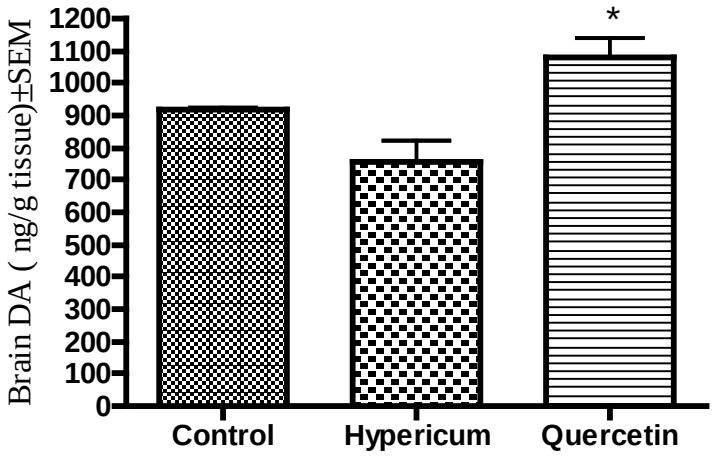


Figure 6.3 Comparison of DA levels in rat brain.*$P<0.05$ when compared with the vehicle treated control. Data are expressed as mean±S.E.M.using the Student-Newman-Keuls multiple comparison test.

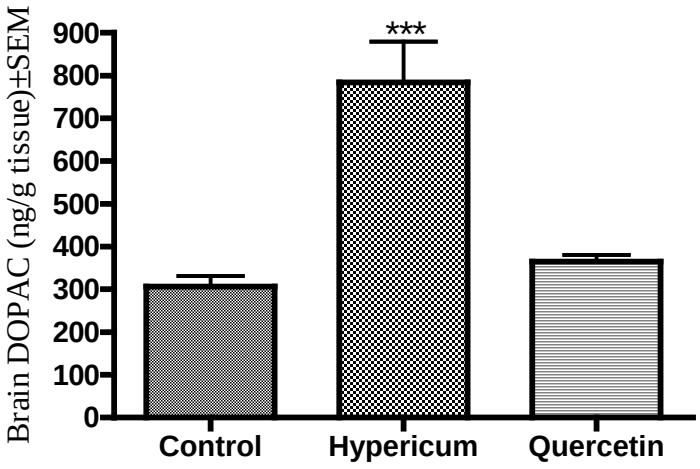


Figure 6.4 Comparison of DOPAC levels, ***P<0.001 when comparing with the vehicle treated control using the Student-Newman-Keuls multiple comparison test.Data are expressed as mean±SEM and n = 5

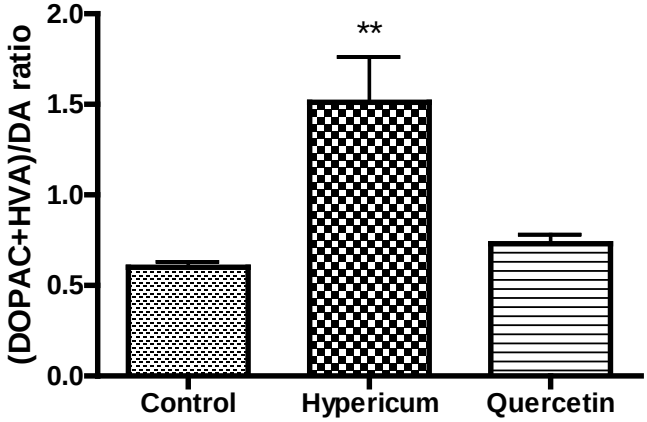


Figure 6.5 Effects of treatment with *Hypericum* extract (300mg/kg p.o.) and Quercetin (5mg/kg p.o.) on dopamine turnover in rat brain.
Data expressed as mean ±S.E.M. Actual levels of DA, DOPAC and HVA are shown in Table 6.1, **P<0.01 when compared with the corresponding control treated rats using the Student-Newman-Keuls multiple comparison test.

6.1.4 Discussion

The results of this study show that both *Hypericum perforatum* and quercetin significantly increase the serotonin levels and decrease serotonin turnover ([5-HIAA]/[5-HT] ratio) in rat brain. Changes in 5-HT turnover have been reported in other studies as a means of measuring antidepressant activity. A reduced 5-HT turnover rate has been shown to occur after chronic treatment with tricyclic antidepressants such as imipramine, desipramine and also after 8 weeks of treatment with 500mg/kg p.o. *Hypericum perforatum* (Butterweck V. *et al.*, 2002)

The results of this study show that only quercetin significantly increases dopamine levels in rat brain. This is in accordance with results reported by Calapai G. *et al.* (1999) where significantly increased levels of DA were only found in the diencephalon after acute administration of *Hypericum perforatum* extracts Li 160 or Ph-50.

A novel finding of this study reveals that the rats treated with *Hypericum perforatum* show a very significant increase in the level of DOPAC in the brain. This, coupled with the significant increase in dopamine turnover ([DOPAC+HVA]/DA), suggests an elevation of dopamine neurotransmission and degradation of DA to DOPAC.

6.2 DETERMINATION OF DOPAMINE AND MELATONIN LEVELS IN RAT BRAIN BY LIQUID CHROMATOGRAPHY-MASS SPECTROMETRY

6.2.1 Introduction

Dopamine and melatonin are neurohormones that have been neglected in the mechanism of action of antidepressant drugs. Significantly reduced dopamine turnover in depressed suicide victims has been reported (Bowden C. *et al.* 1997) and the effects of acute as well as chronic treatment with various *Hypericum perforatum* extracts suggest the involvement of the dopaminergic system on mood disorders (Calapai G. *et al.* 1999; Winterhof H. *et al.* 1995; Butterweck V. *et al.* 2002; Qin Zhou *et al.* 2003).

Melatonin is secreted by the pineal gland with circadian rhythmicity. Depression is characterized by variation in mood that could result from disruption in circadian rhythmicity. This suggests that differences in melatonin levels could be implicated in the biochemical basis of depression (Arendt J. 1979; Wetterberg L. 1979). Melatonin has specific binding sites for which it has a high affinity in different areas of the brain (Banoo S. 1991).

In this study, the levels of dopamine and melatonin in rat brain were quantified using the more selective and rapid liquid chromatography-mass spectrometry (LC-MS) technique. LC-MS is less sensitive than ECD and because it can be coupled to a gradient pump, which cannot be used with ECD, the retention time of the less polar melatonin could be significantly reduced.

6.2.2 Materials And Methods

6.2.2.1 Chemicals and Reagents

HPLC-grade methanol CHROMASOL® was obtained from Sigma-Aldrich (Switzerland). Ammonium acetate (NH_4OAc) from Saarchem (Merck Laboratory Supplies(Pty)Ltd and Formic

Acid from UNILAB (SAARCHEM(Pty)Ltd). Melatonin from Sigma (St Louis, MO, USA) and dopamine from Fluka (Belgium).

6.2.2.2 Animals

Adult male Wistar rats were cared for as described in section 6.1.2.2.

6.2.2.3 Sample Preparation

As described in section 6.1.2.3.

6.2.2.4 Apparatus and chromatographic conditions

Aliquots of 20µl of the filtered supernatants were injected on an LC-MS-(Finnigan Mat LCQ) through a C_{18} µ-Bondapak® Cartridge Column (250 x 4.6mm i.d.) fitted with a guard column using an autosampler fitted with a Rheodyne injector. A gradient elution was employed from 10% to 100% methanol over 17 minutes at a flow rate of 0.8ml/min. The mobile phase consisted of MeOH:50mM NH_4Oac adjusted to pH 3 with Formic Acid. The effluent from the LC system was routed to the MS electrospray interface set in positive mode and nitrogen was used as the nebulising gas. The MS parameters were as follows:

MS total run time(min): 23.00

A Divert Valve was used during run:

Divert Time(min)	Valve State
0.0	To Waste
2.0	To Source
16.50	To Waste

MS Detector Settings:

Two segments were employed to detect the dopamine (segment 1) and melatonin (segment 2) contents in the sample.

Segment 1 Information (Dopamine)

Duration (min): 7.03

Positive ion Single Ion Monitoring (*m/z* range: 153.7 – 154.7) mode was used to detect dopamine. Samples were tuned to obtain maximum sensitivity and the tune method employed included the following parameters:

Capillary Temp (^{0}C): 220.00
Sheath Gas Flow (arbitrary): 80.00
Aux Gas Flow (arbitrary): 0.00
Source voltage (kV): 4.50
Source current (uA): 80.00
Capillary Voltage (V): 9.00
Tube Lens Offset (V): 10.00
Multipole 1 Offset (V): -3.50
Multipole 2 Offset (V): -8.00
InterMultipole Lens Voltage (V): -72.00
Trap DC Offset Voltage (V): -10.00
SIM Micro Scans: 5
SIM Max Ion Time (ms): 200.00

Segment 2 Information (Melatonin)

Duration (min): 9.97

Positive ion mode with an *m/z* range of 60.0 – 240.0 was used. MS/MS was used to detect the daughter ion (*m/z* 174.1) of melatonin, parent mass *m/z* 233.2(M+1). Samples were once again tuned to obtain maximum sensitivity with the tune method employing the following parameters:

Capillary temp (C): 220.00
AGC Off Ion Time (ms): 5.00
Sheath Gas Flow (): 80.00
Aux Gas Flow (): 0.00
Source Voltage (kV): 4.50
Source Current: (uA): 80.00
Capillary Voltage(V): 46.00

Tube Lens Offset (V): 10.00
Multipole 1 Offset (V): -7.25
Multipole 2 Offset (V): -9.00
InterMultipole Lens Voltage (V): -54.00
Trap DC Offset Voltage (V): -10.00
MSn Micro Scans: 3
MSn Max Ion Time (ms): 200.00

The MS/MS parameters were:

Collision energy employed: 21.5%
Activation Q: 0.250
Activation time: 50.000 msec
Isolation width: 2.0 amu

Segment 3 Information
In this segment the flow was diverted to waste.
Duration (min): 6.00

Tissue levels were determined by means of external standards and expressed in terms of ng/g brain per 20µl injection. Correlation coefficients and calibration curves (y = mx + c) based on peak area ratios were established by means of linear regression analysis: DA: 0.9993, 23.63x + 0.9; aMT: 0.9990, 5.4x + 0.048

6.2.3 Results

The dopamine (DA) and melatonin (aMT) content in the brain after three weeks treatment is shown in Table 6.2 below.

Table 6.2 Effect of *Hypericum perforatum* (300mg/kg), fluoxetine (1.25mg/kg) and quercetin (5mg/kg) on dopamine and melatonin content in rat brain expressed as ng/g brain tissue. (Mean ± SEM)

	Control	*Hypericum perforatum*	Fluoxetine	Quercetin
DA	2482 ± 172	2557 ± 129	3156 ± 54	3737 ± 351*
aMT	125 ± 23	121 ± 3.1	102 ± 5.4	112 ± 23

Values are mean±S.E.M. expressed as ng/g brain concentration of 5 rats per group. *P<0.05 Data analysis was performed by analysis of variance with Student-Newman-Keuls test for multiple comparisons.

Quercetin significantly increased dopamine levels (*P<0.05) as shown in Figure 6.6 below while there were no other significant changes.

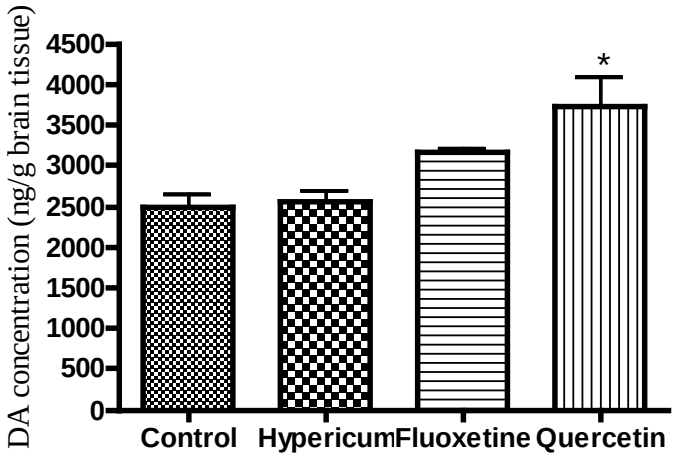


Figure 6.6 Effect of treatments of *Hypericum perforatum* (300mg/kg p.o.), fluoxetine (1.25mg/kg p.o.) and quercetin (5mg/kg p.o.) on dopamine levels in rat brain. Data are expressed as mean ± S.E.M. Actual levels of DA are shown in Table 6.2; *P<0.05 when compared with vehicle treated control rats using Student-Newman-Keuls multiple comparison test.

6.2.4 Discussion

The results of this study show that only quercetin causes a significant increase in the dopamine levels in rat brain, which is in accordance with the results of the HPLC-EC analysis (see Figure 6.3). This is a novel finding as quercetin is one of several flavonoids found in *Hypericum perforatum*. Previously reported studies with the flavonoid enhanced extracts (Ph-50) did not affect the levels of dopamine in the cortex but only in the diencephalon after acute administration (Calapai G. *et al.* 1999). The selective serotonin reuptake inhibitor, fluoxetine, did not significantly increase in dopamine levels.

There were no significant changes in the melatonin levels in the rat brain. This is in contrast to the results from the pineal study reported in chapter 2 (see figure 2.2.1) where quercetin increases melatonin levels secreted by the pineal gland. A possible reason for this discrepancy is that the pineal gland is the primary organ responsible for the secretion of melatonin and the sections of the brain used in the LC-MS study had much lower concentrations of melatonin present. However, these results suggest that fluctuations in the secretion of melatonin are not implicated in the antidepressant activity of HP, quercetin and fluoxetine.

CONCLUSIONS

Changes in the concentration of monoamine neurotransmitters and their metabolites occur after three weeks treatment with either *Hypericum perforatum* or quercetin. Based on these findings, serotonergic and dopaminergic neurotransmission may be involved in the expression of effects of HP and quercetin. The significant changes caused by quercetin suggest that this flavonoid is a major active constituent of HP that may contribute to the antidepressant effects of HP. These effects of the plant extract cannot be explained by the flavonoid quercetin alone and most likely occur as the result of several constituents of HP working synergistically. In conclusion, the findings of these studies suggest that HP as well as quercetin are both effective in the serotonergic and dopaminergic systems relevant for antidepressant activity.

CHAPTER 7

APOPTOSIS STUDY

7.1 MODULATION OF NEURONAL APOPTOSIS IN RATS TREATED WITH *Hypericum perforatum*, QUERCETIN AND FLUOXETINE.

7.1.1 Introduction

Apoptosis is a natural physiological suicide mechanism programmed into eukaryotic cells that occurs during normal tissue turnover. Apoptosis differs from necrosis in that the latter is characterised by rapid cell swelling and lysis resulting in leakage of cytoplasmic contents and the induction of an inflammatory response, while apoptosis is characterized by structural changes in the nucleus and cytoplasm that includes extensive damage to chromatin and degradation of the DNA into fragments without the induction of an inflammatory response (Thomson C., 1995). The DNA strand breaks can be identified by labelling the free 3'-OH terminals with modified nucleotides such as Terminal deoxynucleotidal transferase (TdT) in an enzymatic reaction (TUNEL-reaction). Flourescein labels incorporated in the nucleotide polymers can be detected and quantified by fluorescence microscopy.

Neuronal cells have a limited capacity for self-renewal and most neurons survive for the life of an organism. Apoptosis can be triggered by a variety of extrinsic and intrinsic signals that include oxidative stress, calcium toxicity and excitatory toxicity through physiologic activators such as the neurotransmitters glutamate, dopamine and N- methyl –D-aspartate (Thompson C. 1995). Previous studies have shown that some herbal medications including HP have a protective effect on neuronal cell death caused by oxidative stress (Yang M-H. *et al*. 2002) that HP modulates apoptosis in mice splenic lymphocytes and that this action could be mediated in part by a decrease in Fas-Ag expression and in part by an increase in Bcl-2 expression (Di Carlo G. *et al.* 2003). HP extracts contain a variety of active compounds that may exert different effects on immunopharmacological activity (von Eggelkraut-Gottanka S.G. *et al*. 2002). Several major

compounds of HP, hypericin, pseudohypericin and hyperforin, have been shown to induce cytotoxic effects and apoptosis upon photoactivation (Miccoli L. et al. 1998; Schempp C.M. *et al*. 2002a and 2002b). Quercetin, another major component of HP, has been shown to protect against hydrogen peroxide-induced apoptosis by inhibiting mitochondrial dysfunction in H9c2 cardiomyoblast cells (Park C. *et al.*, 2003) and to protect mouse thymocytes from glucose oxidase-mediated apoptosis (Lee J-C. *et al*. 2003).

Breakdown in the regulation of apoptosis is implicated in degenerative diseases of the CNS and a wide variety of neurodegenerative disorders may result from an accelerated rate of cell death. It can therefore be argued that a drug therapy which increases a cell's resistance to apoptosis in the brain can be seen as being neuroprotective. In this experiment, an investigation was made to determine whether HP, quercetin or fluoxetine were able to reduce the amount of apoptosis occurring in the brain after 3 weeks treatment in an *in vivo* situation.

7.1.2 Materials and Methods

7.1.2.1 Animals

Adult male Wistar rats were cared for as described in Appendix 1 and dosed with fluoxetine (1.25mg/kg p.o.), HP (300mg/kg p.o.) and quercetin (5mg/kg p.o.) for 3 weeks in the manner described in Section 2.2.2.2.

7.1.2.2 Chemicals and Reagents

Paraffin wax was obtained from Lasec (South Africa). SHUR/MOUNT was purchased from Triangle Biomedical Sciences Inc., USA. Aminopropyltriethoxysilane (APES) was purchased from NT lab, Fluka. The *in situ* cell death detection kit, fluorescein, proteinase K (nuclease free) and DNase 1, grade 1 (positive control) were purchased from Roche Diagnostics, Nonnewald, Penzberg. Formaldehyde, glacial acetic acid, xylene, absolute ethanol and chloroform were purchased from Saarchem, Gauteng, South Africa.

7.1.2.3 Tissue Preparation

The rats were sacrificed, the brains were removed and dissected on ice as described in section 2.1.2 and then processing of the tissue for paraffin embedding was started. The method described as Davidson's Alcohol Formalin Acetic Acid Fixative (Davidson's AFA) for fixation of the brains was used to maintain the morphology and prevent necrosis of the tissue. The method followed for the processing of the tissue for embedding is a modification of the technique described by Geiger K.D. *et al*. 1997. The process of embedding requires several steps, where the water contained in the tissues is progressively replaced, first by ethanol, then by chloroform and finally by paraffin.

Initially the brains were fixed in Davidson's solution comprising of 330ml 95% ethyl alcohol, 220ml 100% formalin (37-39%), 115ml glacial acetic acid and 335ml distilled water for 48 hours. After fixation, the cerebellum was removed and brains were dissected in half longitudinally and allowed to fix for a further 24 hours before being transferred to a 50% ethanol solution for 2 hours followed by a 70% ethanol solution in which these were stored until the embedding process, as outlined in the following table, could be completed.

Table 7.1 Procedure for embedding brain in paraffin wax.

Step	Processing Agent	Time (Hours)
1	80% Ethanol	2
2	90% Ethanol	2
3	96% Ethanol	2 x 2
4	100% Ethanol	3 x 2
5	50:50 Ethanol:Chloroform	2
6	100% Chloroform ($CHCl_3$)	2
7	50:50 $CHCL_3$: Paraffin wax	1
8	Paraffin Wax (60^o C)	1
9	As in Step 8, under pressure	¼

The molten paraffin wax containing the half brain was then poured into a mould formed from two

L-shaped metal bars and left to solidify with the flat medial sagittal section facing downwards. The solid wax block was then trimmed, the top heated and mounted onto a wooden block so that the flat medial sagittal side faced upwards.

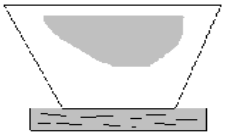


Figure 7.1: Diagram showing trimmed wax block with half of rat brain embedded in paraffin wax, fixed to a wooden block, ready for sectioning.

Sections, 5μm thick of the brain, were sliced using a RMC MT-7 rotary microtome. The sections formed long ribbons and these were placed in a warm water bath (40^0C) to smooth out the wrinkles before being placed onto microscope slides (Sail Brand, China). Three sections were placed on each slide that had been pretreated with APES according to the TESPA method as described by Kerrigan L.A. and Zack D.J. 1997. The slides were incubated overnight in an oven at 60^0C.

The following day the slides were dewaxed and rehydrated as set out in Table 7.2 below. Removal of the paraffin should be as complete as possible because any remaining paraffin adversely affects the TUNEL reaction. The sections were heated at 60^0C for 20 minutes and then hydrated through several baths of xylene and decreasing concentrations of ethanol. Care was taken to ensure that the slides did not dry out at any time and after the procedure was complete, the slides were washed with PBS (pH 7.4) for 30 seconds.

Table 7.2 Procedure for dewaxing and rehydrating brain sections (Kerrigan L.A. and Zack D.J. 1997).

Step	Processing Agent	Time (minutes)
1	Xylene (dewaxing)	5
2	Xylene	5
3	100% Ethanol	3
4	100% Ethanol	3
5	90% Ethanol	3
6	80% Ethanol	3

7.1.2.4 Preparation of brain sections for TUNEL reaction and detection of apoptosis

The TUNEL assay was performed using the Roche® Applied Science *In situ* death detection kit, Fluourescein (Rohm & Haas, Philadelphia, USA) according to the manufacturer's protocol with a few modifications based on techniques described in Neuromethods volume 29 (Geiger K.D. *et al.* 1997, Kerrigan L.A. &Zack D.J. 1997) outlined in the steps below. From the control group, one slide was used for the positive control and two slides for the negative control. The slides were then observed under a fluorescence microscope (Olympus BX-61, Wirsam Scientific, South Africa). The equipment used in the analysis of the slides comprised of a Colorview camera (SiS) (Systems GmbH, Munster, Germany) and sections were examined under epifluorescence using 10, 20 and 40 X objective. Excitation was effected using a Y-UFP cube (excitation 513nm; emission 527nm; Chroma Corporation, Battlebro, USA).

Step by step procedure:

1. The slides were rinsed with distilled water followed by PBS solution for 8 minutes.
2. All the sections were incubated with Proteinase K (20µg/ml in 10mM Tris-HCl, pH 7.4) for 15 minutes at 37^0C. This caused the tissue sections to be partially deproteinised because Proteinase K digests cross-links between proteins thereby increasing cell permeability and access to DNA so that strand breaks can be labelled later.

3. The positive control was then treated with DNase 1 for 10 minutes at 25^0C to induce DNA strand breaks. DNase 1 is an endonuclease that introduces DNA breaks by hydrolysis of double/single stranded DNA, usually at sites next to pyrimidine nucleotides. The concentration of DNase 1 used was 3000U/ml prepared in 50mM Tris-HCl, pH 7.4 containing 1mg/ml BSA.
4. All the slides were then washed twice with PBS to reduce residual DNase background activity and carefully blotted around the tissue with a paper towel.
5. 50µl Label Solution from vial 2 containing nucleotide mixture in reaction buffer that did not contain terminal transferase, from the *in situ* cell detection kit was added to each of the negative controls.
6. 50µl TUNEL reaction mixture was added to the slides including the positive control and excluding the negative control. The TUNEL reaction mixture was prepared by adding 50µl label solution (vial 2) to 450µl enzyme solution (vial 1). Vial 1 enzyme solution consisted of TdT from calf thymus (EC 2.7.7.31) in storage buffer. This procedure was carried out in darkness since the TUNEL reaction mixture is sensitive to light.
7. All the slides were placed in a zip-lock bag to prevent drying out and to keep an even layer of reaction mixture over the tissue while incubating in the dark humidified atmosphere for 1 hour at 37^0C. TdT is heat sensitive and temperatures above 40^0C inactivate the enzyme.
8. The reaction was terminated by washing the slides 3 times, separately in PBS solution, then blotted dry with tissue paper and mounted in aqueous SHUR/MOUNT solution. SHUR/MOUNT is an aqueous mountant that helps in preserving the fluorescence in the tissue.
9. Coverslips were placed on the sections which were kept in darkness and allowed to dry until directly analyzed by fluorescence microscopy using an excitation wavelength in the range of 515-565nm (green).

7.1.3 Results

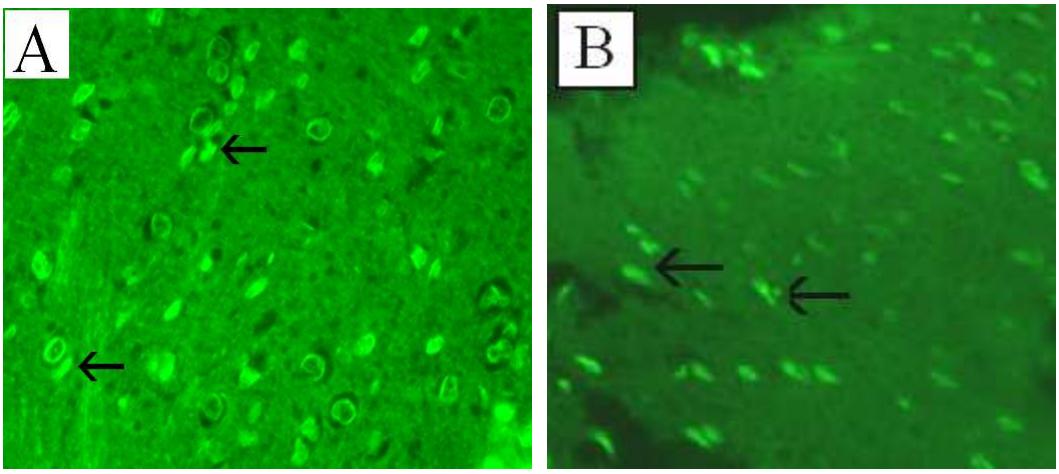


Figure 7.2 Neuronal apoptotic cells can be observed as green spots (see arrows) using fluorescence microscopy. Labelling of nuclei with fragmented DNA strands in rat brain with TUNEL from the *in situ* cell death detection kit, fluourescein.

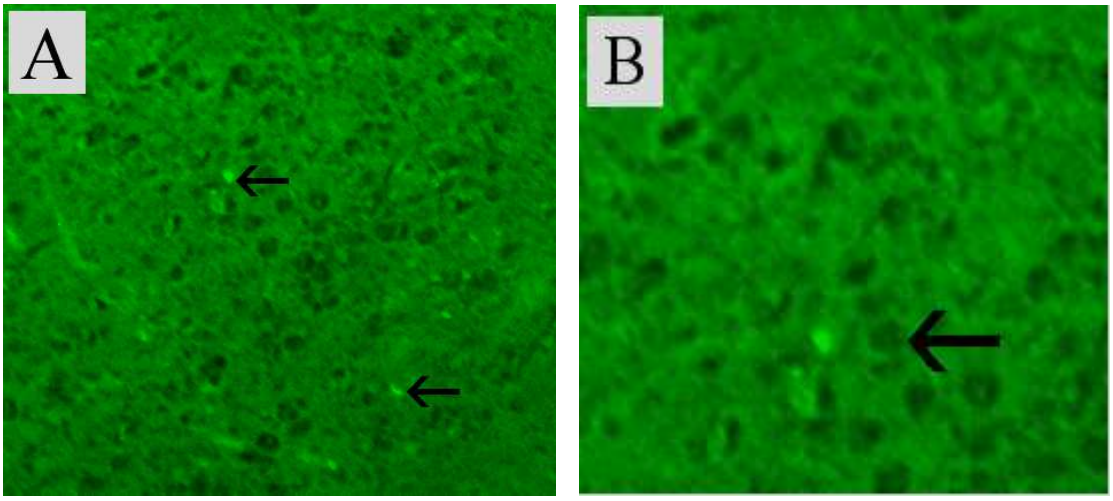


Figure 7.3 Neuronal cells from the vehicle treated control rat brains. Apoptosis evident by fluorescence (green spots) of DNA fragments as indicated in A. Apoptosis is not evident in most cells as seen in enlargement B.

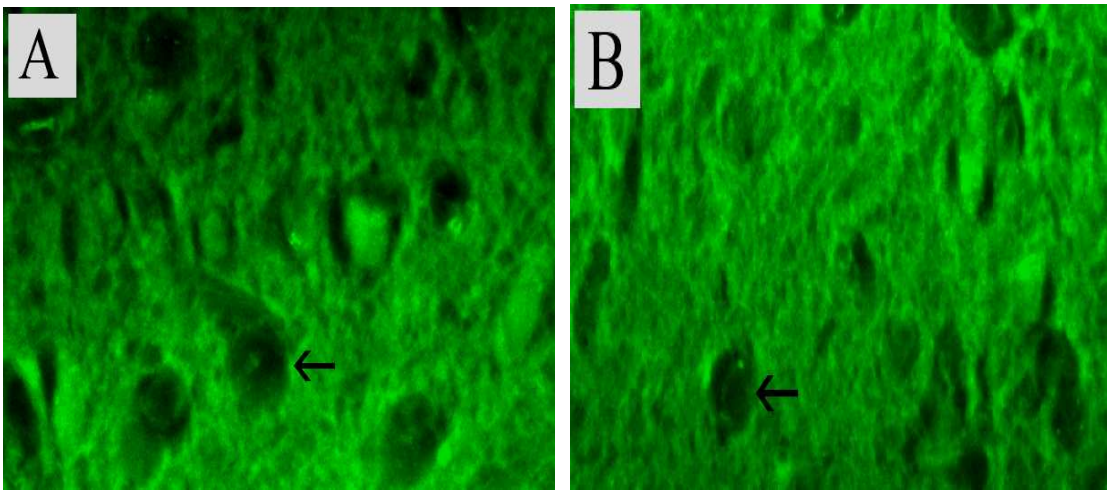


Figure 7.4 Neuronal cells from brain cortex of rats treated with *Hypericum perforatum.* Arrows indicating pyramidal neurons lacking green fluorescence stained nuclei

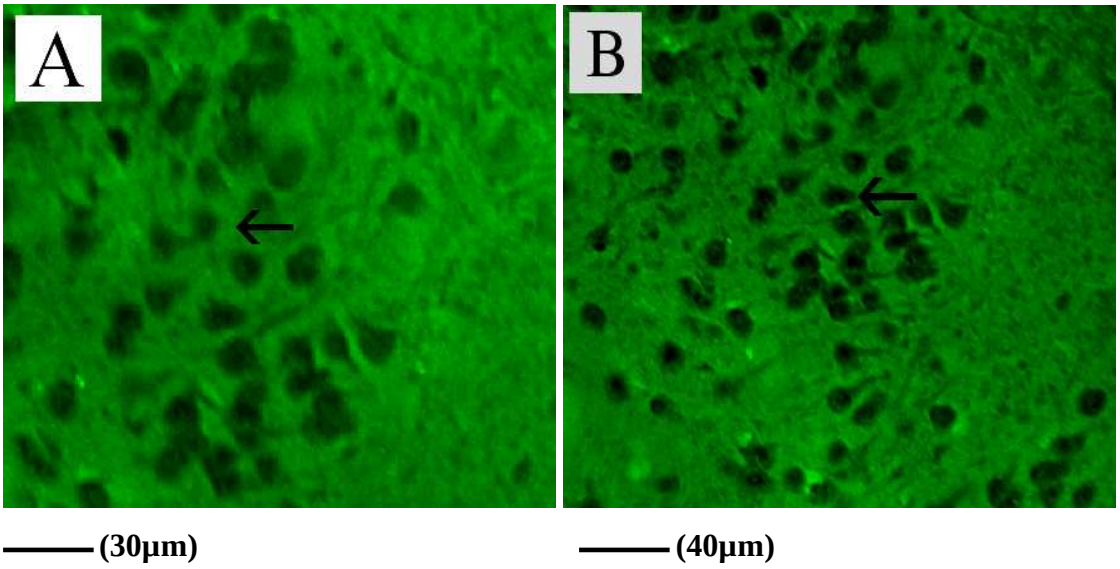
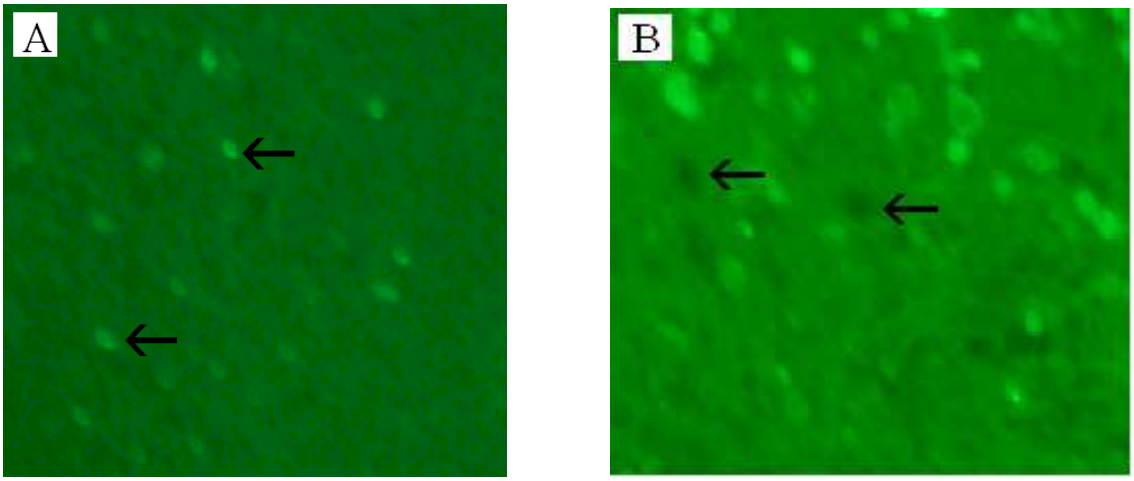


Figure 7.5 Neuronal cells from rats treated with quercetin. Dark pyramidal neurons seen with nuclei lacking green fluorescence.



_____ (45µm) **_____ (35µm)**

Figure 7.6 Neuronal tissue from rats treated with fluoxetine. Arrows in A indicating green fluorescence of neuronal cells undergoing apoptosis. Arrows in B showing neuronal cells without green fluorescence.

7.1.4 Discussion

The detection of apoptosis was based on the fluorescence observed from the labelling of the DNA strand breaks. These can be clearly observed in the positive control (Figure 7.2) where DNA strand breaks were induced by the addition of DNase enzyme. The neuronal tissue from the rats treated with HP (Figure 7.4) and quercetin (Figure 7.5) have significantly less DNA strand breaks than the control (Figure 7.3). These results support the findings of the anti-apoptotic effects of HP by other authors (Di Carlo G. *et al.* 2003; Yang M-H. *et al.* 2002) and suggest that quercetin may be one of the active components of HP involved in its anti-apoptotic effects. This is a novel finding because other authors have reported that *in vitro* both hypericin and hyperforin, two major compounds found in HP, show cytotoxic and apoptotic inducing effects (Miccoli L. *et al.* 1998; Schempp C.M. *et al.* 2002a; Schempp C.M. *et al.* 2002b). This finding supports other studies which report that quercetin protects against apoptosis in H9c2 cardiomyoblast cells (Park C. *et al.* 2003) and in mouse thymocytes (Lee J-C. *et al.* 2003).

The results from the rats treated with fluoxetine (Figure 7.6) are interesting in that the neuronal cells are not clear but there is significantly more fluorescence (green spots) that can be seen in the tissue than the vehicle treated control (Figure 7.3). This suggests that an increase in the amount of

apoptosis has occurred and that fluoxetine causes neuronal degeneration. This finding supports the opinion that most antidepressants possess significant toxicities (Baldessarini R.J. 1996). In another study supporting this view, the effects of pretreatment with various antioxidants reduced glutathione and combined ascorbate and Trolox exposure (24h) on the acute toxicities of different antidepressants including fluoxetine in the rat (C6) glioma and human (1321N1) astrocytoma cell lines using the neural red uptake assay. The authors also suggest that the cytotoxicity may be partly mediated through oxidative stress, with alterations in signal transduction pathways (Slamon N.D. and Pentreath V.W. 2000).

The image fluorescence was not as good as it should be as the correct filter cube was not available and the best available was used, which was only effective in the lower end of the range. The results can therefore only be taken as preliminary and could be improved on using the correct filter combination.

CONCLUSIONS

Apoptosis is a complex process characterised by cell shrinkage, chromatin condensation, internucleosomal DNA fragmentation and the formation of 'apoptotic bodies' (Chandra J. *et al.* 2000). DNA strand breaks are known to occur during apoptosis and these nicks in the DNA molecules can be labelled and detected via the TUNEL assay (Yang M-H. *et al.* 2002). The results of this study demonstrate a decrease in the amount of apoptosis occurring in brain tissue from rats treated with HP and quercetin and an increase in the rats treated with fluoxetine. While these results are preliminary, they add further support for the beneficial effects of HP's use as an antidepressant and suggest that it is the flavonoid, quercetin, mediating its anti-apoptotic effects.

CHAPTER 8

CONCLUSIONS

Extracts of *Hypericum perforatum* (HP) are commonly used for the treatment of mild to moderate depression. The efficacy of the HP extracts produced in the Northern hemisphere has been confirmed by several clinical trials (Linde K. *et al.*, 1999, Franklin M. *et al.* 1999) and by animal models of depression (Butterweck V. *et al.* 1997, Bhattacharya S.K. *et al.* 1998, Ozturk Y. 1997). There are several reports of the possible mechanism of antidepressant action of different standardized extracts of HP cultivated in the northern hemisphere cited in this thesis. Antidepressant activity has also been reported for several constituents of HP including hyperforin, hypericin, pseudohypericin and several of the flavonoids (Butterweck V. *et al.* 1998, 2001b, 2002, Chatterjee S.S. *et al.* 1998, Erdelmeier C.A.J., 1998, Müller W.E *et al.* 1998, 2001). The mode of action of HP and these compounds is still not completely clear, but HP has been shown to be a potent inhibitor of noradrenaline (NA), serotonin (5-HT), dopamine (DA), GABA and glutamate by a mechanism that is not directly related to an inhibition at the re-uptake sites (Wonnemann M. *et al.* 2000). It has been speculated that the antidepressant action of HP is the result of several of its constituents acting synergistically.

At present there are no previous studies evaluating the antidepressant efficacy of any of the HP extracts grown in the Western Cape, South Africa. The objective of this thesis was to gain further insight into the mode of antidepressant action of HP and some of its relevant constituents, quercetin and caffeic acid as well as to compare a HP extract cultivated in the Western Cape of Southern Africa with the results of similar studies which used a standardized product produced in the northern hemisphere. The studies reported in this thesis investigate the effect of a HP produced in the Western Cape of Southern Africa in different biochemical mechanisms that have been suggested to participate in of antidepressant activity.

The first study investigated the effect of HP, quercetin and caffeic acid on pineal metabolism. These results were compared with those of fluoxetine, a known selective re-uptake inhibitor (SSRI). The most significant effects on the pineal gland were brought about by quercetin and

fluoxetine. A noteworthy result of these studies was shown to be the increase in the amount of melatonin released by the pineal gland in the rats treated with these compounds. Changes in the synthesis of melatonin are implicated in depression. The results of the study on pineal metabolism suggest that fluoxetine and quercetin may mediate the antidepressant activity via such a mechanism. The results also reveal that neither the HP extract nor the different compounds tested produce antidepressant effects via the inhibition of the enzyme, monoamine oxidase. Furthermore, the effect of the treatment with quercetin was only observed with a minimum dosage of 5mg/kg as lower dosages were ineffective. The results of these studies on the pineal gland are novel in that there are no previous reports of the effect of HP nor it's constituents, quercetin and caffeic acid, on pineal metabolism. These results also provide further insight into HP possible mechanism of antidepressant action.

Results of previous studies reported in the literature, investigating the possible mode of action of drugs with antidepressant activity, indicate that changes in indoleamine metabolism could be related to the metabolism of the liver enzyme, tryptophan-2,3-dioxygenase. It has been shown that inhibition of this enzyme results in increased levels of plasma tryptophan being available for uptake to the brain. This could lead to increased levels of serotonin in the brain and since low levels of serotonin are implicated in depression, inhibition of TDO could be a possible mode of action of antidepressant drugs. The results of the studies on the effect of fluoxetine, HP, quercetin and caffeic acid on the activity of TDO reported in this thesis show significant inhibition of TDO by HP and caffeic acid. This suggests that this could be a possible mechanism of antidepressant action of HP and that caffeic acid may be one of the compounds contributing to its overall antidepressant effects. There are no previous reports investigating the effect of HP nor any of its constituents on TDO activity.

A primary location of serotonin metabolism is in the serotonergic neurons of the brain and modulation of serotonin concentrations in the forebrain have been suggested to be a core feature of depression. Quantification of serotonin levels was determined using enzyme-linked immunosorbent assay (ELISA) and the results of these studies show significant increases in serotonin levels in the rats treated with quercetin and caffeic acid suggesting that both these constituents of HP could be attributing to its overall antidepressant activity. This result is similar

to the results reported in the literature that used standardised HP produced in the Northern hemisphere.

Further quantification of 5-HT, dopamine (DA) and their metabolites 3,4 dihydroxyphenyl acetic acid (DOPAC), 5-hydroxyindole acetic acid (5-HIAA) and homovallinic acid (HVA) using high performance liquid chromatography (HPLC) with electrochemical detection (ECD) revealed significant increases in 5-HT levels and a decrease in 5-HT turnover in the rats treated with HP as well as the rats treated with quercetin. DA turnover was only significantly increased in the rats treated with HP. These results are similar to the results from experiments conducted in the northern hemisphere using standardized HP extracts produced there.

A novel result from the HPLC-ECD study, which was confirmed in the study using liquid chromatography-mass spectrometry (LC-MS), was the significant increase in DA levels in the rats treated with quercetin. There are no previous studies that have investigated the effect of quercetin on DA levels in rat brain. The results of these studies on the quantification of 5-HT and DA levels in rat brain suggest that both HP and quercetin express their antidepressant effects through serotonergic and dopaminergic neurotransmission.

Adaptive changes in the regulation of different receptor complexes in the brain have been implicated as playing a major role in the etiology of depression. The results of the receptor binding studies revealed that three weeks treatment of rats with HP, quercetin and fluoxetine had different effects on the densities of β-adrenergic (β-AR), serotonin-2 (5-HT_2) and *N*-methyl-D-aspartate (NMDA) receptors. Treatment with HP resulted in significant down regulation of the β-AR and NMDA receptors without any significant effect on the affinity (K_D) of the receptor. In contrast, a significant decrease in affinity and increase in receptor density was shown for the $5HT_2$ receptors. Similar effects were observed in the case of the rats treated with quercetin suggesting that this compound is one of the constituents of HP responsible for these effects. The results for the HP studies on β-AR and 5-HT_2 receptors were also similar to those reported from studies done using HP produced in the northern hemisphere. There are no previous reports of the effect of HP on the regulation of the NMDA receptor complex so the results of these studies provide a novel insight into the mode of action of HP.

Apoptosis in neuronal cells can be triggered by a variety of factors such as oxidative stress, calcium and excitatory toxicity brought about through various neurotransmitter activators including glutamate, DA and NMDA. Detection of apoptosis, using fluorescent microscopy observed from the labeling of DNA strand breaks, revealed a decrease in the amount of apoptosis in the rats treated with HP and quercetin. These results add further support for the beneficial effects of HP's use as an antidepressant since breakdown in the regulation of apoptosis has been implicated in neuro-degenerative and depressive disorders. Similar neuro-protective effects have been reported for standardized HP extracts produced in the Northern hemisphere.

In conclusion, the results of the studies undertaken in this thesis using a HP extract produced in the Western Cape, South Africa have shown several similarities to the studies conducted in the Northern hemisphere. These suggest that the quality of this locally produced tincture is similar to the standardized products of the northern hemisphere. The novel findings reported in this thesis provide further insight into the mechanism of antidepressant action of HP and suggest that quercetin is one of the constituents mediating some of HP's antidepressant activity.

APPENDIX 1

HOUSING AND CARING FOR ANIMALS

All the animals used in the studies were male Wistar rats purchased from the South African Institute for Medical Research (Johannesburg, South Africa). The rats weighed between 200 and 250g and were housed, five per cage, in opaque plastic cages with metal grid floors. The animal room was windowless with automatic ventilation, temperature and lighting. An extractor fan ensured constant removal of stale air, the temperature was maintained at 25^0C ± 3^0C and the lighting cycle was 12 hours light and 12 hours dark (lights on at 6 am). The intensity of the light illumination during the light phase was approximately 300μ Watts/cm^2. The rats were fed commercial cubes and water *ad libitum.* The cages were cleaned daily and all studies involving the animals were approved by the Rhodes University animal ethics committee.

APPENDIX 2

PROTEIN DETERMINATION

The concentration of the protein required in each of the studies was determined by the method set out by Lowry O.H. *et al.* (1951), using bovine serum albumin (300µg/ml) as the standard. The summary of the method used is outlined in Table 1A below. A typical standard curve obtained from the data is shown in Figure 1A.

Table 1A Method for standard curve used in determination of protein concentration.

Conc. (µg/ml)	**blank**	**60**	**120**	**180**	**240**	**300**
Standard (ml)	**0**	**0.2**	**0.4**	**0.6**	**0.8**	**1.0**
H_2O (ml)	**1.0**	**0.8**	**0.6**	**0.4**	**0.2**	**0.0**
Total ml	**1.0**	**1.0**	**1.0**	**1.0**	**1.0**	**1.0**
STAND FOR 5 MINUTES AT ROOM TEMPERATURE (25^0C)						
Reagent A (ml)	**5**	**5**	**5**	**5**	**5**	**5**
STAND FOR 10 MINUTES AT ROOM TEMPERATURE (25^0C)						
Reagent B (ml)	**0.3**	**0.3**	**0.3**	**0.3**	**0.3**	**0.3**
STAND IN THE DARK AT 25^0C FOR 30 MINUTES READ AT 500nm						

Reagent A: Alkaline Copper Reagent

1ml 1% $CuSO_4$

1 ml 2% Na tartrate

98 ml 2% Na_2CO_3 in 0.1M NaOH

Reagent B: Folin-Ciocalteu phenol reagent.

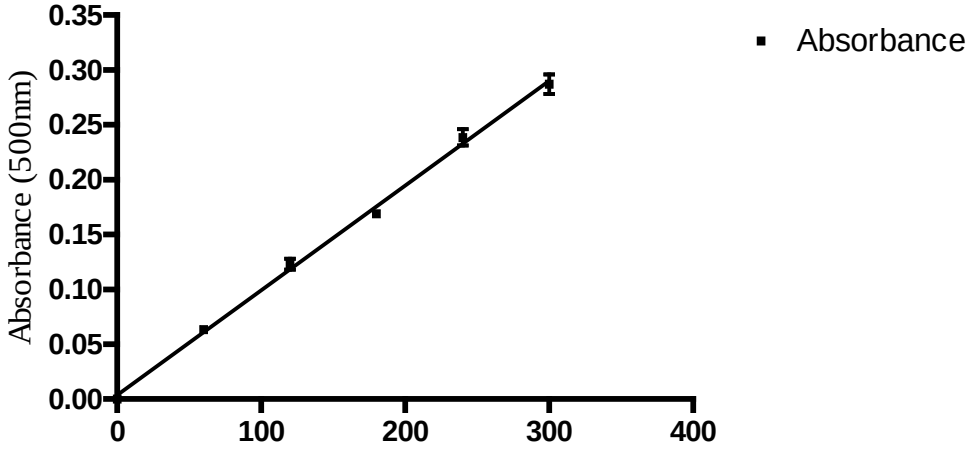


Figure 1A Protein standard curve

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