ANTIOXIDANT AND ANTICHOLINESTERASE ACTIVITIES OF *Ipomoea aquatica* Forssk. AND *Ipomoea reptans* Poir

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ANTIOXIDANT AND ANTICHOLINESTERASE ACTIVITIES OF *Ipomoea aquatica* Forssk. AND *Ipomoea reptans* Poir

ABSTRACT

Ipomoea is the largest genus from the Convolvulaceae family and known to possess various phytochemical compounds. In this study, the antioxidant and anticholineseterase activities of *I. aquatica* and *I. reptans* were investigated. *I. aquatica* and *I. reptans* are green leafy vegetables and have been utilized for centuries across cultures in managing diseases including in treating neuronal disease such as Alzheimer's disease (AD). In AD, improper cognitive function has been linked to irregular neuron transmission such as the aberrant activity of acetylcholinesterase enzyme and oxidative stress. Based on the experiments conducted, both plant extracts showed the presence of alkaloids, terpenoids, phenolic compounds and flavonoids. The DPPH scavenging assay showed the highest activity in *I. aquatica* leaf in methanolic extract (IA-LM) and *I. aquatica* stem in methanolic extract (IA-SM), indicating *I. aquatica* has greater scavenging and hydrogen donating ability compared to *I. reptans*. There was no correlation ($R^2 = -0.523$, $P < 0.05$) found between DPPH assay and TPC assay, infering that the phenolic compounds present in both plant extracts did not contribute to the scavenging activity observed. The ferric reducing antioxidant power (FRAP) and ferrous ion, Fe^{2+} chelating assays showed a correlation with the TPC assay suggesting that the phenolic compounds have iron chelating abilities. However, both assays observed low values in all extract indicating that the phenolic compounds present have low reducing power and metal binding ability. The hydrogen peroxide, H_2O_2 scavenging assay displayed activities < 60 %. In the anticholinesterase assay, acetylcholinesterase (AChE) inhibitory activity was the highest in the chloroform extracts for both plant species, followed by methanol, distilled water and hexane extracts. A correlation with the TPC assay ($R^2 = 0.636$, $P < 0.05$) showed that the phenolic compounds present in *I. aquatica* and *I. reptans* have anticholinesterase

activities. Therefore, it can be deduced that both *I. aquatica* and *I. reptans* have significant antioxidant and anticholinesterase activities.

Keywords: antioxidant, anticholinesterase, *I. aquatica*, *I. reptans*, phenolic compounds

AKTIVITI ANTIOKSIDA DAN ANTIKOLINESTERASE BAGI *Ipomoea aquatica* Forssk. DAN *Ipomoea reptans* Poir

ABSTRAK

Ipomoea adalah merupakan genus terbesar daripada famili Convolvulaceae yang mempunyai banyak sebatian fotokimia yang berfaedah. Dalam kajian ini, kedua-dua aktiviti antioksida dan antikolinesterase bagi *I. aquatica* dan *I. reptans* dikenalpasti. *I. aquatica* dan *I. reptans* adalah merupakan sejenis sayuran berdaun hijau yang digunakan sebagai rawatan untuk pelbagai penyakit sejak dahulu lagi di seluruh dunia. Ini termasuk dalam mengubati penyakit saraf seperti penyakit Alzheimer. Penyakit Alzheimer berpunca daripada ketidakseimbangan dalam aktiviti enzim asetilkolinesterase and tekanan oksidatif yang mengakibatkan gangguan dalam daya kognitif pesakit. Berdasarkan eksperimen yang telah dilakukan, kesemua ekstrak *I. aquatica* dan *I. reptans* dilihat mempunyai alkaloid, terpenoid, sebatian fenol dan flavonoid. Ujian DPPH menunjukkan aktiviti yang paling tinggi dalam ekstrak daun *I. aquatica* di dalam metanol (IA-LM) and ekstrak batang *I. aquatica* di dalam metanol (IA-SM). Ini bermaksud *I. aquatica* adalah lebih baik dalam meneutralkan radikal DPPH dan dalam kebolehan menderma hidrogen berbanding *I. reptans*. Tiada korelasi antara ujian DPPH dan TPC ($R^2 = -0.523$, $P < 0.05$) menunjukkan sebatian fenol di dalam ekstrak bagi kedua-dua jenis tumbuhan tidak menyumbang kepada aktiviti yang dilihat dalam ujian DPPH. Korelasi antara ujian FRAP dan pengkelat Fe^{2+} bersama TPC mencadangkan bahawa sebatian fenol menyumbang kepada aktiviti FRAP dan daya pengkelat Fe^{2+}. Namun, aktiviti kesemua ekstrak *I. aquatica* dan *I. reptans* untuk ujian-ujian ini didapati rendah. Ini menunjukkan sebatian fenol dalam *I. aquatica* dan *I. reptans* mempunyai daya pengekelat logam yang rendah. Ujian hidrogen peroksida, H_2O_2 menyaksikan aktiviti < 60 % bagi semua ekstrak *I. aquatica* dan *I. reptans*. Ujian antikolinesterase mendedahkan aktiviti yang tertinggi dalam ekstrak kloroform bagi kedua-dua jenis tumbuhan, diikuti

oleh metanol, air suling dan heksana. Korelasi antara ujian tersebut dengan ujian TPC (R^2 = 0.636, $P < 0.05$) menunjukkan sebatian fenol dalam kedua-dua jenis tumbuhan mempunyai aktiviti antikolinesterase. Oleh itu, berdasarkan keputusan yang diperoleh, dapat disimpulkan bahawa kedua-dua *I. aquatica* dan *I. reptans* mempunyai aktiviti antioksida dan antikolinesterase.

Kata kunci: antioksida, antikolinesterase, *I. aquatica*, *I. reptans*, sebatian fenol

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TABLE OF CONTENTS

ABSTRACT ..... **iii**

ABSTRAK ..... **v**

ACKNOWLEDGEMENTS ..... **vii**

TABLE OF CONTENTS ..... **viii**

LIST OF FIGURES ..... **xi**

LIST OF TABLES ..... **xii**

LIST OF SYMBOLS AND ABBREVIATIONS ..... **xv**

LIST OF APPENDICES ..... **xviii**

CHAPTER 1 : INTRODUCTION **1**

CHAPTER 2 : LITERATURE REVIEW **3**

2.1 *Ipomoea* 3

2.1.1 *Ipomoea aquatica* Forssk. and *Ipomea reptans* Poir 3

2.1.2 Phytochemical contents 6

2.1.3 Applications 7

2.2 Alzheimer's disease (AD) 8

2.2.1 The Free Radical Hypothesis 10

2.2.2 The Cholinergic Hypothesis 11

2.2.3 Treatments 13

2.2.3.1 Antioxidants 13

2.2.3.2 Acetylcholinesterase Inhibitor (AChEI) 14

CHAPTER 3 : MATERIALS AND METHODS **17**

3.1 Sample Collection 17

3.2 Chemical and Reagents 17

3.3 Apparatus 18

3.4 Plant Extracts Preparation....18
3.5 Phytochemical Compound Identification....19
3.5.1 Thin Layer Chromatography (TLC)....19
3.5.2 Mass Spectrophotometry....21
3.5.2.1 Liquid Chromatography-Mass Spectrometry (LC-MS)....21
3.5.2.2 Gas Chromatography-Mass Spectrometry (GC-MS)....22
3.6 Quantification of Phytochemical Compounds....23
3.6.1 Total Phenol Contents (TPC)....23
3.6.2 Total Flavonoid Contents (TFC)....23
3.7 Antioxidant Assays....24
3.7.1 DPPH Radical Scavenging Activity Assay....24
3.7.2 Ferric Reducing Antioxidant Power (FRAP) Assay....25
3.7.3 Ferrous Ion (Fe^{2+}) Chelating Activity Assay....25
3.7.4 Hydrogen Peroxide (H_2O_2) Scavenging Assay....26
3.8 Acetylcholinesterase Inhibitory Assay....26
3.9 Statistical Analysis....27
CHAPTER 4 : RESULTS....29
4.1 Yield....29
4.2 Thin Layer Chromatography....30
4.3 Mass Spectrometry....59
4.3.1 Liquid Chromatography-Mass Spectrometry (LC-MS)....59
4.3.2 Gas Chromatography-Mass Spectrometry (GC-MS)....60
4.4 Total Phenol Content (TPC) and Total Flavonoid Content (TFC)....64
4.5 Antioxidant Assays....65
4.5.1 2,2-Diphenyl-1-picrylhydrazyl (DPPH) Radical Scavenging Assay....65
4.5.2 Ferric Reducing Antioxidant Power (FRAP) Assay....67
4.5.3 Ferrous Ion (Fe^{2+}) Chelating Activity Assay....68

4.5.4 Hydrogen peroxide, H_2O_2 scavenging assay 68

4.6 Acetylcholinesterase Inhibitory Assay 70

4.7 Pearson Correlation Coefficient (R^2) Analysis 72

CHAPTER 5 : DISCUSSION 74

5.1 Extraction of Plant Materials 74

5.2 Thin Layer Chromatography (TLC) Analysis 75

5.3 Liquid Chromatography-Mass Spectrometry (LC-MS) and Gas Chromatography-Mass Spectrometry (GC-MS) Analyses 76

5.4 Total Phenol Content (TPC) and Total Flavanoid Content (TFC) Assays Analyses 78

5.5 Antioxidant Analysis of Plant Extracts 79

5.5.1 2,2-Diphenyl-1-Picrylhydrazyl (DPPH) Assay Analysis 79

5.5.2 Ferric Reducing Antioxidant Power (FRAP) Assay Analysis 80

5.5.3 Fe^{2+} Chelating Assay Analysis 81

5.5.4 Hydrogen Peroxide Assay Analysis 83

5.6 Anticholinesterase Assay Analysis 83

CHAPTER 6 : CONCLUSION 86

CHAPTER 7 : REFERENCES 87

CHAPTER 8 : APPENDIX 106

LIST OF FIGURES

Figure 2.1	:	Taxonomic hierarchy of *I. aquatica* and *I. reptans* ……..	3
Figure 2.2	:	The geographical distribution of *I. aquatica*…….	4
Figure 2.3	:	*I. aquatica*…….....	5
Figure 2.4	:	*I. aquatica* seeds……	5
Figure 2.5	:	*I. reptans*…….....	5
Figure 2.6	:	*I. reptans* white flower……..……	5
Figure 2.7	:	Alzheimer's disease prevalence worldwide…….....	9
Figure 2.8	:	The hallmarks of AD…..…….....	10
Figure 2.9	:	Projection of cholinergic transmission in the brain of normal and Alzheimer's brain……...……..…	12
Figure 2.10	:	The factors contributing to the abnormalities in the cholinergic transport system in the brain……..	12
Figure 2.11	:	The cholinergic transmission at a neuronal junction...……...	14
Figure 3.1	:	Calculation of the retention factor, R_f from the TLC plate…….	21
Figure 3.2	:	The summary on the methodology of the study	28
Figure 4.1	:	The TPC for *I. aquatica* and *I. reptans* extracts……..	64
Figure 4.2	:	The TFC for *I. aquatica* and *I. reptans* extracts……..	65
Figure 4.3	:	The DPPH scavenging assay for the extracts of *I. aquatica* and *I. reptans* at 800 µg/ml at 517 nm……...	66
Figure 4.4	:	The inhibition of $FeCl_2$ by different extracts of *I. aquatica* and *I. reptans* at 100 µg/ml at 562 nm……..	68
Figure 4.5	:	The H_2O_2 scavenging activity by different extracts of *I. aquatica* and *I. reptans* at 100 µg/ml at 230 nm……	69
Figure 4.6	:	The inhibition of *I. aquatica* and *I. reptans* hexane extracts on the activity of acetylcholinesterase measured at 410 nm……..	70
Figure 4.7	:	The inhibition of *I. aquatica* and *I. reptans* chloroform extracts on the activity of acetylcholinesterase measured at 410 nm…...	71
Figure 4.8	:	The inhibition of *I. aquatica* and *I. reptans* methanolic extracts on the activity of acetylcholinesterase measured at 410 nm…...	71
Figure 4.9	:	The inhibition of *I. aquatica* and *I. reptans* distilled water extracts on the activity of acetylcholinesterase measured at 410 nm…….....	72

LIST OF TABLES

Table 2.1	:	Summary on the AChEI drugs……....	15
Table 3.1	:	The *I. aquatica* and *I. reptans* extracts prepared ……..	19
Table 4.1	:	The yield (mg/g) for *I. aquatica* and *I. reptans* extract……..	29
Table 4.2	:	Thin layer chromatography (TLC) for *I. aquatica* leaf hexane (IA-LH) extract with chloroform solvent……...	31
Table 4.3	:	Thin layer chromatography (TLC) profile for *I. aquatica* stem hexane (IA-SH) extract in chloroform solvent……...	32
Table 4.4	:	Thin layer chromatography (TLC) profile for *I. reptans* leaf hexane extract (IR-LH) in chloroform solvent……...	33
Table 4.5	:	Thin layer chromatography (TLC) profile for *I. reptans* stem hexane (IR-SH) extract in chloroform solvent……...	34
Table 4.6	:	Thin layer chromatograhy (TLC) profile for *I. aquatica* leaf chloroform (IA-LC) extract in chloroform solvent…….....	35
Table 4.7	:	Thin layer chromatography (TLC) profile for *I. aquatica* stem chloroform (IA-SC) extract in chloroform solvent……..…..	36
Table 4.8	:	Thin layer chromatography (TLC) profile for *I. reptans* leaf chloroform (IR-LC) extract in chloroform solvent……..……..	37
Table 4.9	:	Thin layer chromatography (TLC) profile for *I. reptans* stem chloroform (IR-SC) extract in chloroform solvent…..……..	38
Table 4.10	:	Thin layer chromatography (TLC) profile for *I. aquatica* leaf methanol (IA-LM) extract in chloroform solvent……..……	39
Table 4.11	:	Thin layer chromatography (TLC) profile for *I. aquatica* stem methanol (IA-SM) extract in chloroform solvent……..…	40
Table 4.12	:	Thin layer chromatography (TLC) profile for *I. reptans* leaf methanol (IR-LM) extract in chloroform solvent..……	41
Table 4.13	:	Thin layer chromatography (TLC) profile for *I. reptans* stem methanol (IR-SM) extract in chloroform solvent……..…….....	42
Table 4.14	:	Thin layer chromatography (TLC) profile for *I. aquatica* leaf distilled water (IA-LD) extract in chloroform solvent……...	43
Table 4.15	:	Thin layer chromatography (TLC) profile for *I. aquatica* stem distilled water (IA-SD) extract in chloroform solvent……...	43
Table 4.16	:	Thin layer chromatography (TLC) profile for *I. reptans* leaf distilled water (IR-LD) extract in chloroform solvent……...	44

Table 4.17 : Thin layer chromatography (TLC) profile for *I. reptans* stem distilled water (IR-SD) extract in chloroform solvent…… 44

Table 4.18 : Thin layer chromatography (TLC) profile for *I. aquatica* leaf hexane (IA-LH) extract in 10 % methanol solvent…… 45

Table 4.19 : Thin layer chromatography (TLC) profile for *I. aquatica* stem hexane (IA-SH) extract in 10 % methanol solvent……. 46

Table 4.20 : Thin layer chromatography (TLC) profile for *I. reptans* leaf hexane (IR-LH) extract in 10 % methanol solvent……. 47

Table 4.21 : Thin layer chromatography (TLC) profile for *I. reptans* stem hexane (IR-SH) extract in 10 % methanol solvent……. 48

Table 4.22 : Thin layer chromatography (TLC) profile for *I. aquatica* leaf chloroform (IA-LC) extract in 10 % methanol solvent…….. 49

Table 4.23 : Thin layer chromatography (TLC) profile for *I. aquatica* stem chloroform (IA-SC) extract in 10 % methanol solvent…….. 50

Table 4.24 : Thin layer chromatography (TLC) profile for *I. reptans* leaf chloroform (IR-LC) extract in 10 % methanol solvent…….. 51

Table 4.25 : Thin layer chromatography (TLC) profile for *I. reptans* stem chloroform (IR-SC) extract in 10 % methanol solvent……..… 52

Table 4.26 : Thin layer chromatography (TLC) profile for *I. aquatica* leaf methanol (IA-LM) extract in 10 % methanol solvent……..…. 53

Table 4.27 : Thin layer chromatography (TLC) profile for *I. aquatica* stem methanol (IA-SM) extract in 10 % methanol solvent……..…. 54

Table 4.28 : Thin layer chromatography (TLC) profile for *I. reptans* leaf methanol (IR-LM) extract in 10 % methanol solvent……..…. 55

Table 4.29 : Thin layer chromatography (TLC) profile for *I. reptans* stem methanol (IR-SM) extract in 10 % methanol solvent……. 56

Table 4.30 : Thin layer chromatography (TLC) profile for *I. aquatica* leaf distilled water (IA-LD) extract in 10 % methanol solvent……. 57

Table 4.31 : Thin layer chromatography (TLC) profile for *I. aquatica* stem distilled water (IA-SD) extract in 10 % methanol solvent……. 57

Table 4.32 : Thin layer chromatography (TLC) profile for *I. reptans* leaf distilled water (IR-LD) extract in 10 % methanol solvent……..... 58

Table 4.33 : Thin layer chromatography (TLC) profile for *I. reptans* stem distilled water (IR-SD) extract in 10 % methanol solvent…..….. 58

Table 4.34 : The compounds detected in the LC-MS for the selected *I. aquatica* and *I. reptans* extracts…… 60

Table 4.35 : The suggested GC-MS result for *I. aquatica* leaf in methanolic (IA-LM) extract.....61

Table 4.36 : The suggested GC-MS result for *I. reptans* leaf in methanolic (IR-LM) extract.....61

Table 4.37 : The suggested GC-MS result for *I. aquatica* leaf in distilled water (IA-LD) extract.....62

Table 4.38 : The suggested GC-MS results for *I. aquatica* stem in distilled water (IA-SD) extract.....63

Table 4.39 : The suggested GC-MS result for *I. reptans* leaf in distilled water (IR-LD) extract.....63

Table 4.40 : The FRAP value for *I. aquatica* and *I. reptans* extracts.....67

Table 4.41 : The Pearson correlation coefficient test for all seven assays carried out. 73

LIST OF SYMBOLS AND ABBREVIATIONS

≤	:	Less than and equal
<	:	Less than
Aβ plagues	:	β-amyloid plagues
ACh	:	Acetylcholine
AChE	:	Acetylcholinesterase
AChEI	:	Acetylcholinesterase inhibitor
AD	:	Alzheimer's disease
Ca	:	Calcium
Cd	:	Cadmium
Cu	:	Copper
DPPH	:	2,2- diphenyl-1-picrylhydrazyl
DTNB	:	5,5′-Dithiobis (2-nitrobenzoic acid)
EDTA.Na_2	:	Disodium ethylenediaminetetraacetate dihydrate disodium salt
Fe	:	Iron
Fe^{2+}	:	Ferrous ion
Fe^{3+}	:	Ferric ion
$FeCl_2$	:	Iron (II) chloride
$FeCl_3$	:	Iron (III) chloride
FRAP	:	Ferric reducing antioxidant power
GAE	:	Gallic acid equivalent
GC-MS	:	Gas chromatography-mass spectrophotometry
H_2O_2	:	Hydrogen peroxide
Hb	:	Mercury
HCl	:	Hydrochloric acid
HPLC	:	High performance liquid chromatography

IA-L	:	*I. aquatica* leaf
IA-LC	:	*I. aquatica* leaf in chloroform extract
IA-LD	:	*I. aquatica* leaf in distilled water extract
IA-LH	:	*I. aquatica* leaf in hexane extract
IA-LM	:	*I. aquatica* leaf in methanolic extract
IA-S	:	*I. aquatica* stem
IA-SC	:	*I. aquatica* stem in chloroform extract
IA-SD	:	*I. aquatica* stem in distilled water extract
IA-SH	:	*I. aquatica* stem in hexane extract
IA-SM	:	*I. aquatica* stem in methanolic extract
IR-L	:	*I. reptans* leaf
IR-LC	:	*I. reptans* leaf in chloroform extract
IR-LD	:	*I. reptans* leaf in distilled water extract
IR-LH	:	*I. reptans* leaf in hexane extract
IR-LM	:	*I. reptans* leaf in methanolic extract
IR-S	:	*I. reptans* stem
IR-SC	:	*I. reptans* stem in chloroform extract
IR-SD	:	*I. reptans* stem in distilled water extract
IR-SH	:	*I. reptans* stem in hexane extract
IR-SM	:	*I. reptans* stem in methanolic extract
K	:	potassium
LC-MS	:	Liquid chromatography-mass spectrophotometry
Mg	:	Magnesium
$MgCl_2.6H_20$	:	Magnesium chloride hexahydrate
Mn	:	Manganese
MS	:	Mass spectrometer
NaCl	:	Sodium chloride

Ni	:	Nickel
NMDA	:	N-methyl-D-aspartate
P	:	Phosphorus
P	:	Probability
Pb	:	Lead
QE	:	Quercetin equivalent
R^2	:	Pearson correlation coefficient
R_f	:	Retention factor
ROS	:	Reactive oxygen species
TFC	:	Total flavonoid contents
TLC	:	Thin layer chromatography
TPC	:	Total phenol content
TPTZ	:	2,4,6-Tris(2-pyridyl)-s-triazine
UV	:	Ultraviolet
Zn	:	Zinc

LIST OF APPENDICES

Appendix A	:	Thin layer chromatography……	106
Appendix B	:	Total phenolic content (TPC)……	111
Appendix C	:	Total flavonoid content (TFC)……..	111
Appendix D	:	Liquid chromatography – mass spectrometry (LC-MS)……	112
Appendix E	:	Gas chromatography – mass spectrometry (GC-MS) spectra…	117
Appendix F	:	DPPH scavenging assay…….	122
Appendix G	:	FRAP assay…….....	122
Appendix H	:	Fe^{2+} chelating assay …….....	123
Appendix I	:	Hydrogen peroxide scavenging assay ……...	123
Appendix J	:	Acetylcholinesterase assay…….	124
Appendix K	:	Reprint permissions for figures……..	124

CHAPTER 1 : INTRODUCTION

Ipomoea aquatica or commonly known as water spinach and *Ipomoea reptans* are green leafy plants belong to the Convolvulaceae family (Prasad *et al.*, 2008). They can be found distributed across the world, primarily in the tropical and subtropical regions (Umar *et al.*, 2007; Prasad *et al.*, 2008). Having both plants rich in nutrients such as carbohydrates, amino acids, antioxidants and vitamins, *I. aquatica* especially, has been utilized for various purposes since thousands of years ago (Prasad *et al.*, 2008). These include as food, in traditional medicine and recently in bioremediation. In some traditional medicine practices, *I. aquatica* has been used to treat jaundice, nervous debility, liver and eye diseases, high blood pressure and constipation (Alkiyumi *et al.,* 2012; Lawal *et al.*, 2015; Malakar & Choudhury 2015; Dewanjee *et al.,* 2015; The National Institutes of Health (NIH), 2016). Apart from that, recent studies have also discovered important bioactivity in *I. aquatica* which includes acetylcholinesterase inhibitory activity in the treatment of Alzheimer's disease (AD) (Dzoyem & Eloff 2015; Raghunath *et al.,* 2018; Chen *et al.,* 2018). However, a limited knowledge is known about the bioactivity and the utilization of *I. reptans* especially in treating diseases compared to *I. aquatica. I. aquatica* and *I. reptans* are also relatively cheap and easy to grow, making them suitable candidates to be utilized for the development of natural drugs to treat diseases.

AD is a disease associated with nervous debility, cognitive decline and memory impairment (Alzheimer's Association, 2015). Multiple causes have been implicated in the development of AD including oxidative stress and the deficiency in the cholinergic transmission in the brain (Dumont & Beal 2011; Hamulakova *et al.,* 2016). Till date, the primary treatment for the disease relies heavily on the use of drugs such as acetylcholinesterase inhibitors (AChEIs). However, these drugs have been linked to numerous adverse side effects in patients (Mukherjee *et al.,* 2007; Hansen *et al.,* 2008;

Ali *et al.,* 2015; Gawad *et al.,* 2015; Greig 2015; Rang *et al.,* 2016; Zhang *et al.,* 2016). This urge for an immediate attention into searching for alternatives in developing a safe and viable drug in mediating the disease.

Consequently, these have brought the attention on looking into natural products such as medicinal plants and herbs for the development of new drugs. Besides that, the low output of combinatorial chemistry and rational drug design and lack of access to conventional drugs have also pushed the interest in utilizing natural product for drug development (David *et al.,* 2015).

Provided the pronouced benefits and bioactivities in these plants and lack of research on the plants usage as drug candidates for the disease, this research can provide an additional insight and a better understanding on utilizing the plants for future prospects.

Therefore, the research objectives of this study are:

i. To determine the phytochemical constituents in *I. aquatica* and *I. reptans.*
ii. To determine the total phenol and flavonoid contents in *I. aquatica* and *I. reptans,* and
iii. To evaluate the antioxidant and anticholinesterase activities of *I. aquatica* and *I. reptans*

CHAPTER 2 : LITERATURE REVIEW

2.1 *Ipomoea*

Ipomoea is the largest genus within the Convolvulaceae (morning glory) family with over 600 – 700 identified species including *Ipomoea aquatica* Forssk. *and I. reptans* Poir. (Austin & Huáman, 1996; Prasad *et al.*, 2008). The plant members of the species are twining or climbing woody or herbaceous plants, with usually heart-shaped leaves and large, showy, trumpet-shaped flowers of various colours including white, purple, blue, pink and red. (Galetto & Bernardello, 2004; Meira *et al.*, 2012). They are known to be rich in nutrients and minerals such as carotenoids, chlorophylls, essential amino acids, alkaloids, calcium, Ca, iron, Fe and magnesium, Mg (Meira *et al.*, 2012). Traditionally, this group of plant species has been used for nutritional, medicinal, ritual and also agricultural purposes (Meira *et al.*, 2012).

2.1.1 *Ipomoea aquatica* Forssk. and *Ipomea reptans* Poir

I. aquatica and I. reptans are a close relative within the genus. Figure 2.1 describes the taxonomic hierarchy between both plants.

Kingdom	Plantae	Plantae
Phylum	Tracheophyta	Tracheophyta
Class	Magnoliopsida	Magnoliopsida
Order	Solanales	Solanales
Family	Convulvulaceae	Convulvulaceae
Genus	*Ipomoea*	*Ipomoea*
Species	*Ipomoea aquatica*	*Ipomoea reptans*

Figure 2.1 : Taxonomic hierarchy of *I. aquatica and I. reptans* (Knees & Patzelt, 2013)

They can be found grown worldwide, usually in the tropical and subtropical regions including in countries such as Malaysia, Hongkong, Singapore, Indonesia, Cambodia, Laos, Vietnam, Africa, the United States of America, (USA) Central and South America

and Australia (Umar *et al.*, 2007; Prasad *et al.*, 2008; Dewanjee *et al.*, 2017; CAB International, 2018). Figure 2.2 shows the worldwide distribution for *I. aquatica*.

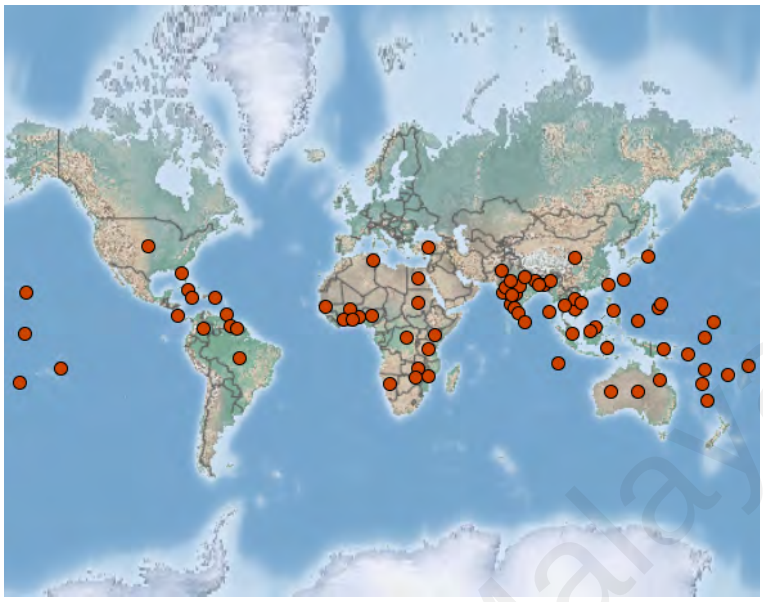

Figure 2.2 : The geographical distribution of *I. aquatica* (CAB International, 2019). The red dots represent the distribution of *I. aquatica* in the world. Reprinted permission from www.cabi.org./isc.

I. aquatica and *I. reptans*, are aquatic or semi-aquatic, annual or perennial plants, grown wildly, on water surfaces such as rivers, freshwater lakes or ponds or on moist soils such as muddy banks (Prasad *et al.*, 2008; Saha *et al.*, 2008; Thi & Hwang, 2015; Lawal *et al.*, 2015; Gad *et al.*, 2017; Michigan State University, 2017). They are also commercially cultivated for consumption and can be eaten raw as salads, boiled, fried, steamed or pickled or made into fodder for animal consumptions (Malalavidhane *et al.*, 2000; Kim *et al.*, 2008; Thi & Hwang, 2015).

Morphologically, they differ slightly especially in the shape of the leaves and the colour of the flower. Figure 2.3 – 2.6 show the structure of *I. aquatica* and *I. reptans* including the flowers, leaves and seeds. *I. aquatica* has elliptic leaves, funnelform purple flower and trails above water surfaces (Austin, 2007; Prasad *et al.*, 2008). On the contrary, *I. reptans* has ovate-oblong leaves with white flower and grows upright on moist soils (UMass Center for Agriculture, Food and the Environment, 2016).

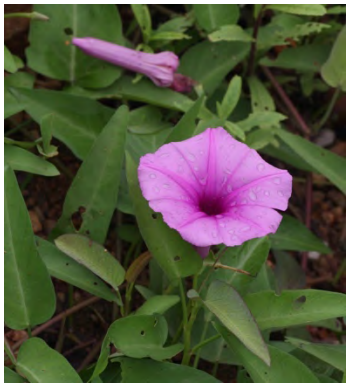

Figure 2.3 : *I. aquatica* (Bingham, *et al.*, 2019). Reprinted permission from www.zimbabweflora.co.zw.

Figure 2.4 : *I. aquatica* seeds (Scher , 2018). Reprinted permission from idtools.org.

Figure 2.5 : *I. reptans.*

Figure 2.6 : *I. reptans* white flower.

To accommodate the plants aquatic to semi-aquatic nature, both plants have hollow and smooth stems with rooting nodes, 'labyrinth seeds' and hairy fruit. *I. aquatica* can grow up to 9 to 70 feet long at 4 inches per day which results in its hollow stems forming networks, producing long stem and less branches to support its structure (UMass Center for Agriculture, Food and the Environment, 2016; Michigan State University, 2017). The labyrinth seed provides buoyancy for the plant, aids in supporting the structure and enables them to float on water surfaces (Austin, 2007). The fruit is protected inside a spherical capsule which turns woody upon maturation (Michigan State University, 2017). Both plants can be cultivated through direct seed germination or easily propagated from the cuttings of the plant (Prasad *et al.*, 2008; UMass Center for Agriculture, Food and the Environment, 2016). The optimal growth temperature for the cultivation is between 24°C

to 29 °C and requires more water compared to other crops (UMass Center for Agriculture, Food and the Environment, 2016).

However, in the USA, *I. aquatica* is considered as a noxious weed as it competes with the existent native plants. *I. aquatica* may form networks which cover water surfaces thereby shading the underneath submerged plant (UF / IFAS Center for Aquatic and Invasive Plants, 2018). This poses threats to the vulnerable native plants and affect the ecosystem.

2.1.2 Phytochemical contents

I. aquatica and *I. reptans* contain numerous nutrients. Shim (2012) recorded that *I.aquatica* contain significant amounts of phytochemical compounds such as phytosterol, phenolic compounds, vitamin E, amino acids, sugar, sugar alcohol and fatty acids. Phenolic compounds include tannins, flavonoids, saponins, carotenes, organic acids such as malic acid and citric acid, quercetin, luteolin and alkaloids (Prasad *et al.*, 2008; Vasu *et al.*, 2009; Yadav & Agarwala, 2011; Lawal *et al.*, 2017). Umar *et al.* (2007) reported a substantial level of mineral elements in *I. aquatica* leaves such as potassium, K, sodium, Na, magnesium, Mg, calcium, Ca, iron, Fe and phosphorus, P. Nonetheless, *I. aquatica* has considerably low amount of copper, Cu, manganese, Mn and zinc, Zn (Umar *et al.*, 2007).

Similarly, Febriyono *et al.* (2017) and Jumaryatno *et al.* (2018) stated the presence of protein, carbohydrates, fatty acids, phenolic compounds, Ca, P, Fe, Na, vitamin A, vitamin B, vitamin C, 3-methoxy quercetin, 4-methoxy quercetin polyphenol, and anthocyanin in *I. reptans*. Another finding suggested the presence of α-tocopherol, β-carotene and ferulic acid in *I. reptans* (Ismail *et al.*, 2004).

Nonetheless, it should also be noted that, different developmental stages of the plants, cutting frequencies, growth conditions and processing methods such as ways of cooking

and drying procedures, might affect the plants level of phytochemical contents and minerals (Chitsa *et al.*, 2014, Sarkar *et al.*, 2014; Lawal *et al.*, 2015, Thi & Hwang, 2015).

2.1.3 Applications

The utilization of *I. aquatica* as traditional medicine has been documented back to 3000 years ago across different cultures. This includes in treating jaundice and nervous debility (Ayurveda), constipation, liver diseases, mental problems, abscesses and diabetes (Tanzania) and intestinal problem (Somalia) (Saha *et al.*, 2008; Malakar & Choudhury, 2015). Current researches on the plant have also revealed numerous bioactivities such as hypoglycaemic ability in reducing blood glucose level, good diuretic and cytoprotective abilities against heavy metal liver poisoning, antiproliferative, antimutagen and antitumour, antimicrobial properties, nootropic ability and antidepressant and antiepileptic activities (Malalavidhane *et al.*, 2000; 2001; Malalavidhane *et al.*, 2003; Saha *et al.*, 2008; Hamid *et al.*, 2011; Alkiyumi *et al.*, 2012; Dewanjee *et al.*, 2015; Dua *et al.*, 2015; Lawal *et al.*, 2015; 2017; Malakar & Choudhury, 2015; Dewanjee *et al.*, 2017; El-Sawi *et al.*, 2017).

However, the scientific research on the application of *I. reptans* in treating diseases is limited compared to *I. aquatica.* Nevertheless, Hayati *et al.* (2017) and Jumaryatno *et al.* (2018) had showed that *I. reptans* has antidiabetic properties. *I. reptans* has also been shown to be able to sequester heavy metals, similar to *I. aquatica* (Herliana *et al.*, 2018).

Due to the plants capability to take up heavy metals and toxic compounds, *I. aquatica* and *I. reptans* have been considered for the use in phytoremediation. The plants are used as sequestering agents to take up heavy metals such as lead, Pb, Zn, and Cu from contaminated environment and as a biomonitoring agent to evaluate the safety of waterbodies (Chanu & Gupta, 2014; 2016; 2018; Ng *et al.*, 2016; Rane *et al.*, 2016; Herliana *et al.*, 2018).

However, as the plants are widely consumed, cautions should be given especially to those intended for human consumption. A proper management on the plants' farms and cultivation centres needs to be given to ensure the water used for irrigation is not contaminated with harmful substances. According to Saidin *et al.* (2018), *I. aquatica* and *I. reptans* collected from local market in Singapore showed pronounced level of heavy metals including Zn, Cu, Mn, Fe and nickel, Ni. In Bangkok, Thailand, seven *I. aquatica* cultivation sites intended for local food market consumption sampled were found to contain comparable amounts of Pb, methylmercury, total mercury, Hb, and cadmium, Cd (Göthberg *et al.*, 2009). This poses a serious risk especially to infants and unborn babies as they are more susceptible to methylmercury poisoning (Göthberg *et al.*, 2009). The effects could be detrimental such as central nervous system damage, cerebral palsy, blindness and growth problems (Heller, 2017)

I. aquatica and *I. reptans* grown for phytoremediation in contaminated water such as wastewater should be handled with care to avoid human consumption. Control measures on the application of pesticides and fertilizers should be implemented to reduce the potential health risk and ensure safety upon human consumption of the plant (Saidin *et al.*, 2018).

2.2 Alzheimer's disease (AD)

Alzheimer's disease is an age-related, irreversible, progressive developing neurodegenerative disease associated with the deficits in cognitive skills among older generation (Niedowicz *et al.*, 2011; Begum *et al.*, 2015; Huang *et al.*, 2016). Affecting people with the age of 65 years of age and above, the disease is putting a high burden on the health sector and economically, as the older population expands (Niedowicz *et al.*, 2011). It is estimated that between 2018 and 2050, the cost for caring AD and dementia patients will be US$ 20.2 trillion (Alzheimer's Association, 2018). In 2016, about 47

million people live with dementia and is estimated to rise to >131 million by 2050 (Prince *et al.*, 2016). East Asia has the highest prevalence of AD followed by Western Europe, South Asia and North America (Alzheimer's Research UK, 2018). Figure 2.7 shows the AD prevalence worldwide.

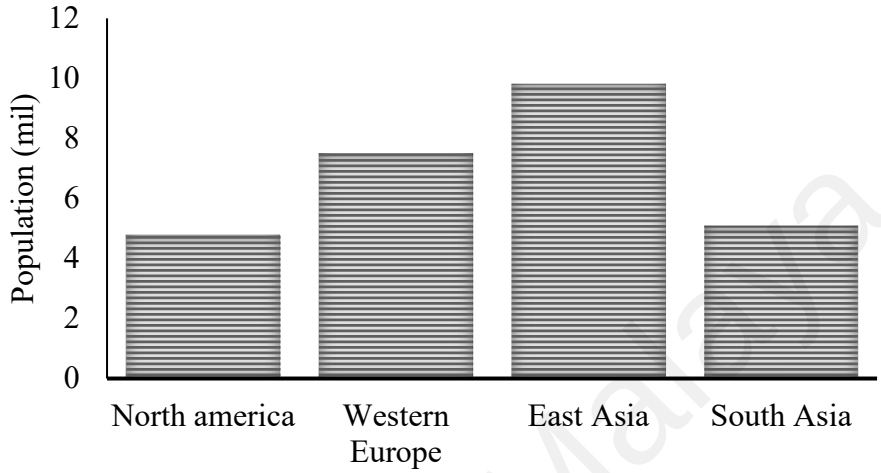

Figure 2.7 : Alzheimer's disease prevalence worldwide (Alzheimer's Research UK, 2018). Data adapted from Alzheimer's Research UK.

In AD, the areas of the brain that are involved in memory and cognitive learning such as the hippocampus, basal forebrain and cortex are primarily affected (Duan *et al.*, 2014). AD patients suffer from deficits in memory, language, problem solving and other cognitive skills which ultimately affect their ability to perform normal daily routines (Alzheimer's Association, 2015). AD is multifaceted and complex. Figure 2.8 characterized the underlying pathologies in AD patients' brain.

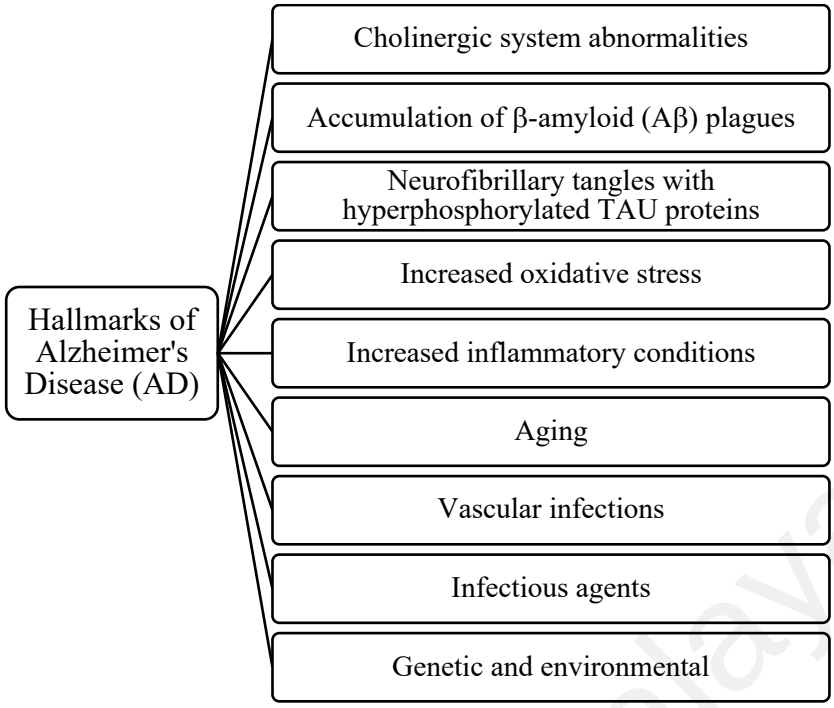

Figure 2.8 : The hallmarks of AD (Crews & Masliah, 2010; Ahmad *et al.*, 2015; Strac *et al.*, 2015). Data collected from multiple resources.

2.2.1 The Free Radical Hypothesis

Age is a key risk factor in the onset of AD (Practicò, 2008). As the age progresses, free radicals build up and accumulate in the body. Free radical hypothesis of aging states that the accumulation of reactive oxygen species (ROS) with age can cause irreversible damage and death to cell components such as the nucleus, mitochondrial DNA, membranes and cytoplasmic proteins (Christen, 2000). Neuron is prone to oxidative damage as it is rich in polyunsaturated fatty acids which makes it susceptible to free radical attack and due to the highly oxygen-rich environment in the brain (Christen, 2000; Aliev *et al.*, 2008). Besides that, certain areas of the brain which release neurotransmitters such as catecholamines are known to be predisposed to radical attack due to auto-oxidation (Aliev *et al.*, 2008). Fe, an essential component in carrying out metabolic processes, is also known to contribute to the formation of ROS through Fe-catalyzed formation of ROS (Aliev *et al.*, 2008)

Subsequently, accumulation of ROS leads to oxidative stress (Jiang *et al.*, 2016). Oxidative stress occurs due to the overproduction and uncontrolled production of ROS as a result from the inability of the body's natural protective mechanism to neutralize these reactive metabolites (Ahmad *et al.*, 2015; Jiang *et al.*, 2016; Rang *et al.*, 2016).

Multiple researches conducted at cellular level, on animal AD models and AD patients have suggested the occurrence of oxidative modifications such as oxidation of nucleic acid, proteins, lipid and macromolecules (Nunomura *et al.,* 2006; Feng & Wang, 2012). Consequently, this leads to the dysregulation and malfunctioning of mitochondria and increased in the ROS in the brain (Swerdlow & Kish, 2002; Wang *et al.*, 2014; Cardoso *et al.*, 2016; Gibson & Thakkar, 2017). Another hallmarks of AD, which is the deposition of Aβ plague has also been found to induce inflammation which increased the ROS production and causing neuronal death (Smith *et al.*, 2002; De Felice *et al.*, 2007).

2.2.2 The Cholinergic Hypothesis

Another cause underlying the development of AD is due to the depletion of cholinergic transmission in the brain. The brain cholinergic system comprises of complicated network of neurons with series of projections into different parts of the brain (Pepeu & Giovannini, 2017). They release acetylcholine (ACh) as neurotransmitter which may induce or alter the excitability of neurons and synaptic plasticity and transmission (Picciotto *et al.*, 2012).

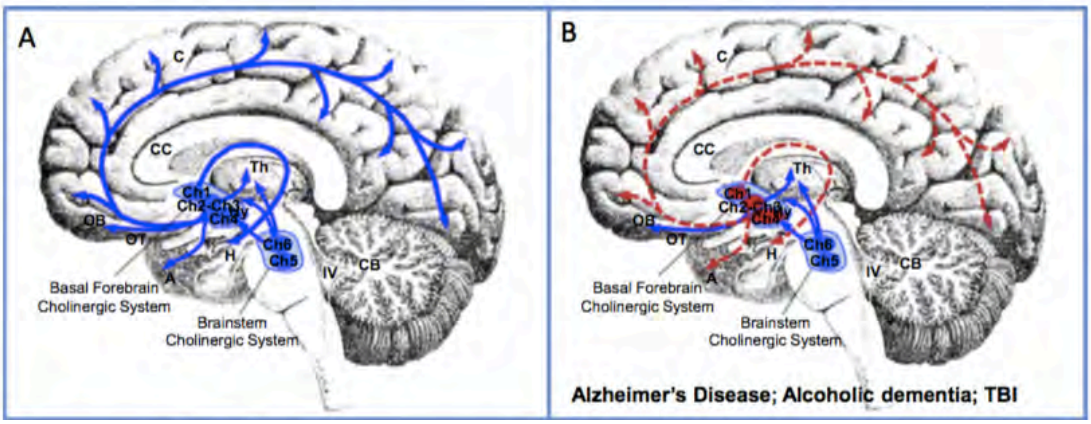

Figure 2.9 : Projection of cholinergic transmission in the brain of normal and Alzheimer's brain. Blue line indicates normal projection of cholinergic transmission in parts of the brain. Red line indicates the degeneration in the cholinergic transmission in parts of the brain (Pepeu & Giovannini, 2017). Reprint permission from Elsevier.

The cholinergic hypothesis of AD proposed that the cognitive impairment observed in AD patients is linked to the degeneration in the cholinergic neurons and depletion in the neurotransmission in the cerebral cortex and other brain areas (Francis *et al.*, 1999). Figure 2.9 shows the areas of cholinergic transmission degeneration in the brain of AD patients. As ACh is involved in regulating memory processing and learning, abnormalities in the cholinergic transmission system have been associated with the onset of AD (Beninger *et al.*, 1989; Hasselmo & Bower, 1993; Francis, 2005). Figure 2.10 states the various factors that contribute to the impairment in the cholinergic transmission system in the brain.

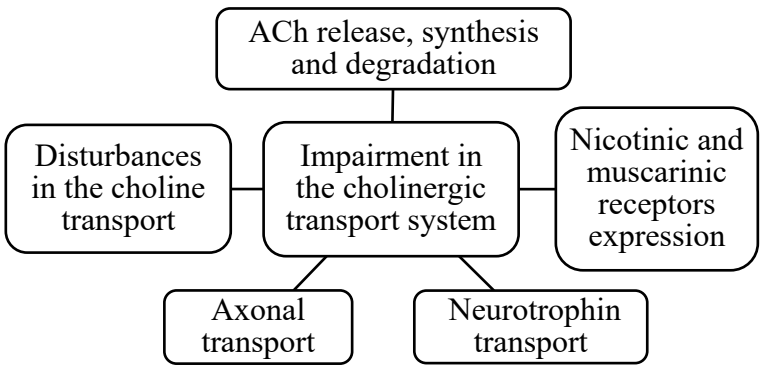

Figure 2.10 : The factors contributing to the abnormalities in the cholinergic transport system in the brain (Terry & Buccafusco, 2003; Racchi *et al.*, 2004). Data collected from multiple resources.

2.2.3 Treatments

In treating AD, only two classes of drug have been approved by the Food, Drugs and Therapeutics (FDA) which are the acetylcholinesterase inhibitors (AChEIs) and N-methyl-D-aspartate (NMDA) receptor antagonists (Casey *et al.*, 2010). However, these drugs do not treat the underlying pathologies surrounding the progression of AD but rather, serve as a palliative treatment in alleviating the symptoms in AD patients (Kumar *et al.*, 2015). Hence, efforts have been done into looking for other potential therapies importantly, in utilizing natural sources as drugs.

2.2.3.1 Antioxidants

Provided the complex etiologies underlying the development of AD, it can be assumed that an antioxidant treatment alone does not suffice for the alleviation of the disease (Williams *et al.*, 2011). However, it was revealed based on several epidemiological studies that diet rich in antioxidants could delay the cognitive deficits suffered by AD patients (Williams *et al.*, 2011; Valls-Pedret *et al.*, 2015). Williams *et al.* (2011) suggested that a multi-targeted approach involving the combination of antioxidants and drugs or other phytochemical compounds could be employed in treating AD patients.

A few plants with high antioxidant activites have been shown to be potential sources for the treatment of AD. Fu *et al.* (2011) showed that three carotenoid; violaxanthine, lutein and β-carotene identified in *I. aquatica* showed high antioxidant activities. Sivaraman *et al.* (2016), revealed that the extract of *I. aquatica* improved cognitive behaviour, memory and neurotransmitter levels in Alzheimer's mice.

Besides that, *Curcuma longa* (turmeric) which contains curcumin has been shown to have anticancer, antioxidant, antiamyloid and anti-inflammatory properties (Obulesu & Rao, 2011). Similarly, *C. longa* has also been utilized in Ayurveda and widely studied for

its application in the treatment of AD (Lim *et al.*, 2001; Obulesu & Rao, 2011; Zhou *et al.*, 2011; Wang *et al.*, 2013).

2.2.3.2 Acetylcholinesterase Inhibitor (AChEI)

Acetylcholinesterase (AChE) is a type of cholinesterase enzyme involved in the termination of nerve impulse at cholinergic terminal in the central and peripheral nervous system (Colovic *et al.*, 2013). It hydrolyses ACh into its constituents; acetate and choline, is highly specific and has a rapid catalytic activity (Dvir *et al.*, 2010; Colovic *et al.*, 2013). Figure 2.11 shows the cholinergic transport system at a neuronal junction.

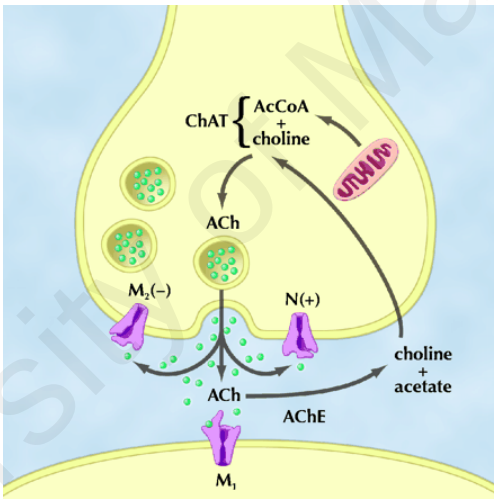

Figure 2.11 : The cholinergic transmission at a neuronal junction (Gauthier, 2002). Reprint permission from Joule Inc.

Acetylcholinesterase inhibitor (AChEI) functions to increase the level of ACh in the cholinergic terminal by inhibiting the action of AChE. Consequently, neurotransmission in the nerve terminal is improved and cognitive functions are restored.

Although AChEIs are widely used in treating AD, they are highly associated with adverse side effects among AD patients (Mukherjee *et al.*, 2007). Table 2.1 describes the side effects associated with AChEI drugs.

Table 2.1 : Summary on the AChEI drugs. Data collected from multiple resources.

Drugs	Properties	Side effects	Types of AD	Notes	References
Tacrine	Non-selective. Pseudo-irreversible	Nausea, diarrhoea, hepatotoxicity, abdominal pain, sweating, convulsion	Mild, moderate	Withdrawn from the US market	(Rang *et al.*, 2016; DrugBank, 2018)
Donepezil	Noncompetitive, reversible, selective to CNS and AChE	Diarrhoea, dizziness, vomiting, weight loss	Mild, moderate, severe	-	(Hansen *et al.*, 2008; Jelic & Darreh-Shori, 2010; Greig, 2015; Rang *et al.*, 2016)
Rivastigmine	Noncompet-itive, pseudo-irreversible, selective to CNS	Acute cholinergic syndrome upon excessive exposure, death, falling, diarrhoea, dizziness, vomiting, weight loss, somnolence, hypertension, nausea, application site pruritus	Mild, moderate	-	(Hansen *et al.*, 2008; Ali *et al.*, 2015; Gawad *et al.*, 2015; Zhang *et al.*, 2016; Rang *et al.*, 2016; Suzuki *et al.*, 2017)
Galantamine	Competitive, reversible, non-selective, can bind to the allosteric sites of nicotinic receptors	Diarrhoea, dizziness, vomiting, weight loss	Mild, moderate	-	(Raskind *et al.*, 2000; Hansen *et al.*, 2008; Rang *et al.*, 2016; Drugbank, 2018)

Consequently, these have raised concerns and shifted the focus into searching for alternatives such as bioactive compounds from natural resources in developing a viable

and safe drug. Multiple studies have shown several bioactive compounds found in plants that have anticholinesterase activity. These include phytochemical compounds such as alkaloids, terpenoids, flavonoids, rosmarinic acid, huperzine A, caffeic acid, tiliroside, phloroglucinol, curcumin and resveratrol (Williams *et al.*, 2011; Roseiro *et al.*, 2012; Gülçin *et al.*, 2016; Garcia *et al.*, 2017). Moreover, some of these plants have already been employed in traditional medicine to alleviate neurological diseases including *I. aquatica*. Dhanasekaran *et al.* (2015) displayed that *I. aquatica* in hydroalcholic extract exerted significant anticholinesterase activity. Through ligand docking studies, compounds such quercetin and chlorogenic acid present in *I. aquatica* were found to have a good affinity and binding score with AChE (Sivaraman *et al.*, 2014). Following another research conducted, a trial on animal model suggested that *I. aquatica* lowered AChE levels in the brain and mediated Aβ-induced cognitive and memory impairment (Sivaraman *et al.*, 2016). Curcumin and resveratrol have also been seen to have anti-amyloidogenic properties in which they could lower the deposition of toxic Aβ plagues (Kim *et al.*, 2010).

CHAPTER 3 : MATERIALS AND METHODS

3.1 Sample Collection

Two species from the *Ipomoea* genus; *I. aquatica* and *I. reptans* were collected. Wild mature *I. aquatica* was collected at a clearing area next to the Kundang Lakes Country Club, Rawang, Selangor, Malaysia on February 2018 and mature *I. reptans* was bought from the Pasar Tani MAEPS Serdang, Seri Kembangan, Selangor. Both plants were collected and bought only once and were authenticated by Mr. Ghazali bin Sabda of Rimba Ilmu Botanical Garden, University of Malaya, Kuala Lumpur. The voucher specimens of *I. aquatica* and *I. reptans* plant samples were deposited in the Herbarium of University of Malaya, Kuala Lumpur with a voucher number of KLU 49890 and KLU 49891 respectively.

3.2 Chemical and Reagents

2,2- diphenyl-1-picrylhydrazyl (DPPH), 2,4,6-Tris(2-pyridyl)-s-triazine (TPTZ), 5,5′-Dithiobis(2-nitrobenzoic acid) (DTNB), acetylcholinesterase, acetylthiocholine iodide, anisaldehyde, berberine, bovine serum albumin V, ferrozine, folin-ciocalteu's reagent, gallic acid, phenylalanine, potassium iodide, potassium phosphate monobasic, quercetin, sodium acetate, tannic acid, tris and vanillin were purchased from Sigma-Aldrich, USA. Ascorbic acid, aluminum chloride, disodium ethylenediaminetetraacetate dihydrate (EDTA) disodium salt, hexane, hydrogen peroxide, magnesium chloride, potassium phosphate dibasic, sodium carbonate and sodium hydroxide were bought from Systerm Chemicals, Malaysia. Iron (III) chloride, methanol and sodium chloride were obtained from R & M Chemicals, Malaysia. 95% ethanol, bismuth nitrate hydrochloric acid and sodium nitrite were purchased from Merck, Germany. Chloroform and glacial acetic acid were bought from Fisher Scientific, USA. Ferrous sulphate was obtained from

Friendemann Schmidt, Australia and sulphuric acid was purchased from HmbG GmbH, Germany.

3.3 Apparatus

UV-Vis Spectrophotometer-1700 (Shimadzu, Japan), microplate reader (Tecan Sunrise, Austria), Agilent 6200 series TOF/ 6500 series (Agilent, USA), Agilent's Gas Chromatography with an MSD detector (Agilent, USA)

3.4 Plant Extracts Preparation

Both *I. aquatica* and *I. reptans* collected were washed and the leaves, stems, fruits, flowers and roots were separated. The plant parts were air dried at room temperature, under the shade for a week. After fully dried, the leaves and stems for both plants were ground using a blender into fine powder. The powdered plant parts were then subjected to chemical extraction with hexane, chloroform, methanol and distilled water in the order of increasing polarity consecutively. During each extraction, the extract was incubated in a water bath at 37 °C for 7 hours. Upon extraction, the filtrates were collected and concentrated up to 15 ml using rotary vacuum evaporator at 40 °C. Distilled water extracts were freeze dried at −100 °C. The extracts were then stored in air tight containers at 2 °C until further usage. A total of 16 different extracts were prepared as summarized in Table 3.1.

Table 3.1 : The *I. aquatica* and *I. reptans* extracts prepared.

Hexane extracts			Chloroform extracts		
IA-LH	:	*I. aquatica* leaf	IA-LC	:	*I. aquatica* leaf
IA-SH	:	*I. aquatica* stem	IA-SC	:	*I. aquatica* stem
IR-LH	:	*I. reptans* leaf	IR-LC	:	*I. reptans* leaf
IR-SH	:	*I. reptans* stem	IR-SC	:	*I. reptans* stem

Methanolic extracts			Distilled water extracts		
IA-LM	:	*I. aquatica* leaf	IA-LD	:	*I. aquatica* leaf
IA-SM	:	*I. aquatica* stem	IA-SD	:	*I. aquatica* stem
IR-LM	:	*I. reptans* leaf	IR-LD	:	*I. reptans* leaf
IR-SM	:	*I. reptans* stem	IR-SD	:	*I. reptans* stem

After chemical extraction was done, yield of each extract was determined. Extracts were dried thoroughly to remove excess moisture and solvent to obtain its dry weight. Yield for the extracts were calculated based on the following formula and expressed in mg/g of plant.

$$Yield\ (mg/g) = \frac{Weight\ of\ dry\ extract\ (mg)}{Weight\ of\ plant\ (g)} \quad (3.1)$$

3.5 Phytochemical Compound Identification

3.5.1 Thin Layer Chromatography (TLC)

All extract was subjected to thin layer chromatography (TLC) for the identification of the phytochemical compounds present. The experiment was done according to the protocol described by Kagan and Flythe (2014) with some modifications. Two solvents were used; chloroform and 10 % methanol. 10 % methanol was prepared by mixing

methanol and chloroform in 1:9 by volume. The solvent was poured 0.5 cm deep into a tank. On the TLC plate (20 cm × 20 cm, thickness 1.5 mm, TLC Aluminium Silica Gel $60F_{254}$ sheets), a baseline was drawn where each extract was spotted. The TLC plate was left inside the tank for a few minutes to allow the absorption of the solvent. Once the solvent had been absorbed by the TLC plate, the solvent front developed and observed bands were marked. The developed plates were subjected to UV light at 254 nm and sprayed with different reagents to allow the visualization of phytochemical compounds.

(a) Folin-Ciocalteu's Reagent Test

Folin-Ciocalteu's reagent was sprayed onto the developed plate to detect the presence of phenol compounds. Blue coloured bands formed indicated the presence of phenol.

(b) Vanillin-Sulphuric Acid Reagent Test

Vanillin-sulphuric acid reagent was prepared according to Matysik *et al.* (2016) with several modifications. 1 g of vanillin, 100 ml of ethanol and 10 ml of concentrated sulphuric acid were mixed to make the reagent. The developed plate was then sprayed with the reagent and heated at 120 °C for 3-5 minutes. Pink coloured bands formed showed the presence of terpenoid.

(c) Dragendorff's Reagent Test

Dragendorff's reagent was prepared by according to Mehrotra *et al.* (2011) with slight modifications. 1.7 g bismuth nitrate mixed 100 ml of distilled water and 16 g of potassium iodide dissolved in 40 ml of distilled water were prepared. 5 ml of bismuth nitrate solution, 5 ml of potassium iodide solution, 20 ml of acetic acid and 70 ml of distilled water were then mixed. The resulting reagent was then sprayed onto the developed plate to allow the observation of orange/ brown coloured bands for the presence of alkaloid.

Each band was then analyzed to determine their retention factor value, R_f. R_f allows the characterization of a compound based on the distance travelled by the spot compared to the distance travelled by the solvent. Determination of the R_f was done using the following formula (Nichols, 2018):

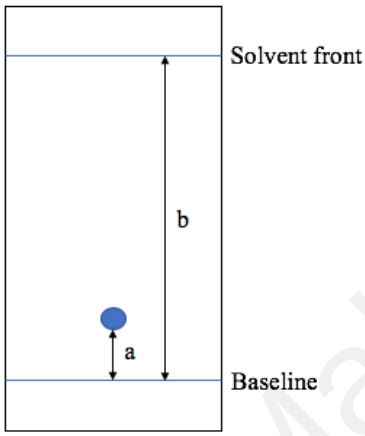

Figure 3.1 : Calculation of the retention factor, R_f from the TLC plate.

$$\text{Retention factor, } R_f = \frac{\text{Distance travelled by the spot (a)}}{\text{Distance travelled by the solvent (b)}} \quad (3.2)$$

3.5.2 Mass Spectrophotometry

Both liquid chromatography-mass spectrometry (LC-MS) and gas chromatography-mass spectrometry (GC-MS) were conducted to allow identification of the compounds present in *I. aquatica* and *I. reptans* extracts. Five extracts were selected for evaluation based on their performances in the antioxidant and anticholinesterase assays carried out. They were; (i) IA-LM, (ii) IR-LM, (iii) IA-LD, (iv) IA-SD and (v) IR-LD.

3.5.2.1 Liquid Chromatography-Mass Spectrometry (LC-MS)

Liquid chromatography-mass spectrometry (LC-MS) was carried based on the procedure described by Mauri *et al.* (2006) with minor modifications. Samples were sent to Centre for Natural Products Research and Drug Discovery (CENAR), University

Malaya, Kuala Lumpur for processing. Analysis of the samples were performed by using Agilent 6200 series TOF/ 6500 series in a C18 Hypersil column (100 mm × 3mm, 5 μm). The eluents used were methanol and water. The gradients were established at 0-1 min at 30 % methanol, followed by 1-7 mins at 30 % to 45 % methanol and 7-5 mins at 45 % methanol. The flow rate was set at 0.55 ml/min with a volume of 50 μl of extract per injection. Gallic acid, tannic acid, quercetin and phenylalanine were used as standards. A negative ion scan mode at m/z 200-700 was used to detect the presence of compounds.

3.5.2.2 Gas Chromatography-Mass Spectrometry (GC-MS)

Gas chromatography-mass spectrometry (GC-MS) was performed according to Prasad *et al.* (2005). The separation was carried out at the Nanotechnology and Catalysis Research Centre (NANOCAT) University Malaya, Kuala Lumpur using Agilent's Gas Chromatography with a MSD detector with DB624 capillary column (60 m × 320 mm × 0.18 mm). Argon gas was used as a carrier gas at a flow rate of 2 ml/min . Temperature was set at 120 °C for 1 min, then increased and maintained up to 280 °C at a rate of 10 °C/min. The temperature for injection was 250 °C, detection at 260 °C, inlet at 300 °C, interface at 280 °C, ion source at 230 °C and quadrupole at 150 °C. Total running time was 40 mins. Injection was done at 1 μL with a split ratio of 1:10.

3.6 Quantification of Phytochemical Compounds

3.6.1 Total Phenol Contents (TPC)

The total phenol content (TPC) of the extracts were determined by using Folin-Ciocalteu's reagent based on Alhakmani *et al.* (2014) with some modifications. Gallic acid at various concentrations (100 – 1000 mg/l) were used to construct a standard curve. 20 µl of extract dissolved in ethanol was mixed with 100 µl of Folin-Ciocalteu's reagent (diluted 10-fold with distilled water) in a 96-well plate and incubated for 5 minutes. Then, 75 µl of sodium carbonate solution (75 mg/ml) was added to the mixtures. The mixtures were incubated in darkness at room temperature for 2 hours before being measured using a microplate reader (Tecan Sunrise, Austria) at 700 nm. All test was done in triplicates (n=3). The concentration for each extract was determined from the linear equation of the gallic acid standard curve. The TPC of the extract is calculated by using the following formula.

$$C = \frac{C_1 \times V}{m} \tag{3.3}$$

Where C is TPC (mg GAE/g), C_1 is the concentration of gallic acid (mg/ml), V is the volume of extract (ml) and m is the mass of plant extract (g). Results were expressed as mg gallic acid equivalent per g of dry extract (mg GAE/g).

3.6.2 Total Flavonoid Contents (TFC)

The total flavonoid content (TFC) was determined by using aluminum colorimetry method as described by Do *et al.* (2014) with minor modifications. Quercetin at various concentration (100 – 1000 mg/l) was used to generate the standard calibration curve. 50 µl of extract dissolved in ethanol, 15 µl of sodium nitrite solution (5 % w/v) and 70 µl of distilled water were mixed in a 96-well plate and incubated at room temperature for 5

mins. 15 µl of aluminium chloride solution (10 % w/v) was added to the mixtures and incubated further for 6 mins at room temperature. 100 µl of sodium hydroxide solution (1 M) was then added and the absorbance readings for the mixtures were taken at 510 nm using a microplate reader (Tecan Sunrise, Austria). All test was done in triplicates (n=3). The concentration for each extract was determined from the linear equation of the quercetin standard curve. The TFC of the extract is calculated by using the following formula.

$$C = \frac{C_1 \times V}{m} \quad (3.3)$$

Where C is TFC (mg QE/g), C_1 is the concentration of quercetin (mg/ml), V is the volume of extract (ml) and m is the mass of plant extract (g). Results were expressed as mg quercetin equivalent per g of dry extract (mg QE/ g).

3.7 Antioxidant Assays

3.7.1 DPPH Radical Scavenging Activity Assay

DPPH radical scavenging assay was carried out based on the protocol described by Do *et al.* (2014) with some modifications. A standard curve was constructed using ascorbic acid at different concentrations (6.25 – 800 µg/ml). Ascorbic acid was used as a positive control. 40 µl of extract dissolved in methanol at different concentrations (6.25 – 800 µg/ml) and 200 µl of DPPH solution in ethanol (50 µM) were added in a 96-well plate and incubated in darkness at room temperature for 15 mins. Absorbance readings of the mixtures were taken using a microplate reader (Tecan Sunrise, Austria) at 517 nm. All test was carried out in triplicates (n=3). The half maximal inhibitory concentration (IC_{50}) for the extracts were determined.

The percentage of radical inhibition of each extracts was determined by using the following formula:

$$\text{DPPH radical scavenging activity (\%)} = \frac{A_{control} - A_{sample/standard}}{A_{control}} \times 100 \quad (3.4)$$

3.7.2 Ferric Reducing Antioxidant Power (FRAP) Assay

Ferric reducing antioxidant power (FRAP) assay was done based on the methods described by Zengin *et al.* (2015) with slight changes. Ferrous sulfate at various concentrations (0.125 – 1.0 mM) were used to construct a standard curve. Ferrous sulfate was used as a positive control. FRAP reagent was prepared by mixing $FeCl_3$ (20mM), TPTZ (10mM), and acetate buffer (0.3M, pH 3.6) in a 1:1:10 by volume. 20 µl of extract dissolved in methanol was mixed with 200 µl of FRAP reagent in a 96-well plate and incubated for 8 minutes at room temperature. The absorbance reading was carried out at 595 nm using a microplate reader (Tecan Sunrise, Austria). All test was carried out in triplicates (n=3). FRAP value for each extract was determined from the linear equation of ferrous sulfate standard curve. Results were expressed as mmol of ferric ion, Fe^{3+} per g of dry extract (mmol Fe^{3+}/ g).

3.7.3 Ferrous Ion (Fe^{2+}) Chelating Activity Assay

The extracts ability to chelate Fe^{2+} was performed based on the methods described by Tohma *et al.* (2017) with some modifications. EDTA.Na_2 at different concentrations (12.5-100 µg/ml) were used to construct a standard calibration curve. EDTA.Na_2 was used as a positive control. 100 µl of extract dissolved in methanol at various concentrations (12.5-100 µg/ml), 10 µl of $FeCl_2$ (2 mM), 120 µl of distilled water and 100 µl of ferrozine (5 mM) were mixed in a 96-well plate and incubated for 20 minutes at room temperature. The absorbance reading was taken for each extract at 562 nm using

a microplate reader (Tecan Sunrise, Austria). All test was done in triplicates (n=3). The half maximal inhibitory concentration (IC_{50}) for each extract was determined. The chelating activity of each extract was calculated based on the following formula:

$$Fe^{2+} \text{ chelating activity } (\%) = \frac{A_{control} - A_{sample/standard}}{A_{control}} \times 100 \quad (3.5)$$

3.7.4 Hydrogen Peroxide (H_2O_2) Scavenging Assay

The ability of the extracts to scavenge hydrogen peroxide (H_2O_2) was carried out based on the protocol described by Al-Amiery *et al.* (2015) with some modifications. Ascorbic acid at different concentrations (6.25 – 100 µg/ml) were used to construct a standard calibration curve. Ascorbic acid was used as a positive control. 0.1 ml of extract dissolved in methanol at various concentrations (6.25 – 100 µg/ml), 0.3 ml of phosphate buffer (50 mM) and 0.6 ml of H_2O_2 (2 mM) were mixed in a glass cuvette. The mixture was incubated for 10 minutes at room temperature. The absorbance reading was made at 230 nm using UV-Vis spectrophotometer-1700 (Shimadzu, Japan). All test was done in triplicates (n=3). The half maximal inhibitory concentration (IC_{50}) for each extract was determined. The H_2O_2 scavenging activity was calculated using the following formula:

$$H_2O_2 \text{ scavenging activity } (\%) = \frac{A_{control} - A_{sample/standard}}{A_{control}} \times 100 \quad (3.6)$$

3.8 Acetylcholinesterase Inhibitory Assay

The ability for the extracts to inhibit the activity of cholinesterase enzyme was performed based on the method described by Machado *et al.* (2015) with slight modifications. Berberine at various concentration range (6.25 – 400 µg/ml) was used to construct a standard calibration curve. Berberine was used as a positive control. Two solutions were prepared. Solution A: Tris/ HCl buffer (50 mM, pH 8) with bovine albumin

fraction V (0.1%) and solution B: Tris/ HCl buffer (50 mM, pH 8) with NaCl (0.1 M) and $MgCl_2.6H_20$ (0.02 M). Ellman's reagent was prepared by mixing DTNB in solution B (3 μM). Extracts dissolved in methanol were diluted with Tris/ HCl buffer into various concentrations (6.25 – 400 μg/ml). To start the reaction, 25 μl of acetylthiocholine iodide (15 μM), 125 μl of Ellman's reagent, 50 μl of solution A, 25 μl of extract and 25 μl of acetylcholinesterase (AChE) (0.20 U/ml) were added into a 96-well plate. The mixtures were incubated for 20 minutes at room temperature. The absorbance reading was made at 405 nm. All test was done in triplicates (n=3). The half maximal inhibitory concentration (IC_{50}) for each extract was determined. The inhibitory activity of the extract determined by using the following formula:

$$\text{Anticholinesterase activity (\%)} = \frac{A_{control} - A_{sample/standard}}{A_{control}} \times 100 \qquad (3.7)$$

Figure 3.2 shows the summary of the methodology of the study.

3.9 Statistical Analysis

All result was expressed as the mean ± standard error (S.E.) for the seven independent experiments. Differences between extracts were analysed by one-way analysis of variance (ANOVA) followed by Duncan's post hoc multiple comparison test at 5 % ($P < 0.05$). SPSS 25.0 version, Chicago, IL, USA was used to perform the whole tests.

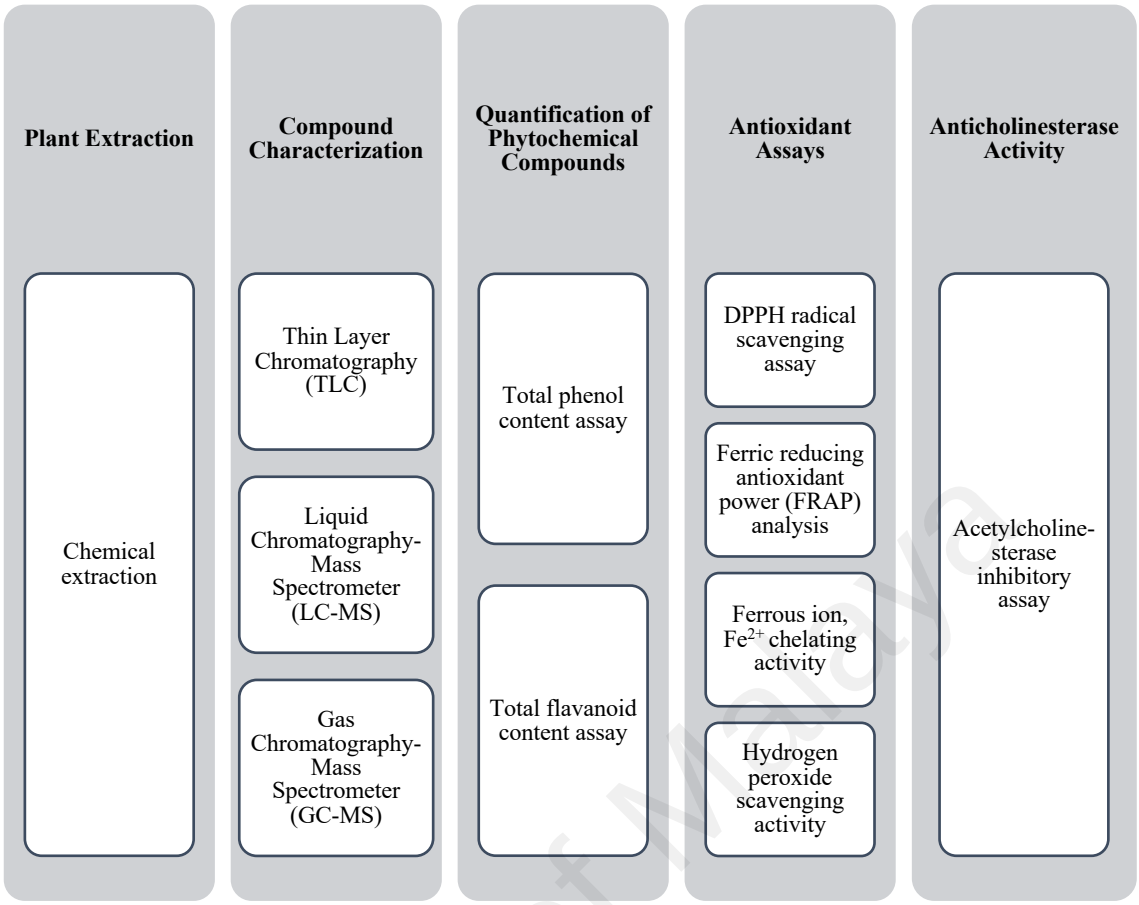

Figure 3.2 : The summary on the methodology of the study.

CHAPTER 4 : RESULTS

4.1 Yield

I. aquatica and *I. reptans* were first separated into leaves, stems, flowers and roots. These parts were dried and finely ground to allow easier extraction and separation of the phytochemical compounds present in the plant. The plants were extracted using various solvents of different polarity, gradually starting from a non-polar solvent to polar solvents. Hexane, chloroform, methanol and distilled water were used as solvents. Only the leaves and stems of both plant species were used for this study as they are the edible parts of the plants.

Table 4.1 describes the yield for each of the extracts prepared in mg/g. From the table, it can be observed that the highest yield was obtained in the methanolic extracts for both *I. aquatica* and *I. reptans*. IR-SM (30.5 mg/g) had the highest yield, followed by IA-LM (21.0 mg/g) and IR-LM (19.7 mg/g). As methanol is polar and is effective at isolating phenolic compounds, more phenolic compounds could have been extracted in the methanolic extracts of both plants. Other extracts had lower yields (≤ 10.0 mg/g). *I. aquatica* had a lower yield compared to *I. reptans*.

Table 4.1 : The yield (mg/g) for all *I. aquatica* and *I. reptans* extract.

Extract	Yield (mg/g) of extract in different solvent			
	Hexane	**Chloroform**	**Methanol**	**Distilled water**
IA-L	3.1	9.1	21.0	7.6
IA-S	2.6	1.3	10.8	8.4
IR-L	5.1	3.8	19.7	10.2
IR-S	0.7	8.3	30.5	9.3

IA-L : *I. aquatica* leaf
IA-S : *I. aquatica* stem
IR-L : *I. reptans* leaf
IR-S : *I. reptans* stem

4.2 Thin Layer Chromatography

Thin layer chromatography was done as an early investigation to detect the presence of active natural compounds present in the extracts. Upon administration of different reagents, the presence of phytochemical compounds can be observed. Table 4.2 – 4.33 show the phytochemical compounds present in the *I. aquatica* and *I. reptans* extracts in both chloroform and 10 % methanol solvents.

Observation under visible light for the all of the extracts of *I. aquatica* and *I. reptans* displayed green and yellow bands on the TLC plates while fluorescent red and blue bands were observed under the UV light. Some green and yellow bands fluoresced under the UV light.

Staining with Folin-Ciocalteu's reagent revealed dark-blue bands on a yellow-greenish background indicating the presence of phenol. Vanillin-sulphuric acid reagent produced pink, orange/ brown and green bands on a yellow background showing the presence of terpenoid, phenol, alkaloid and alcohol. Dragendorff's reagent showed orange/brown and green bands on an orange background denoting the presence alkaloid and phenol.

10 % methanol was found to be better at separating the phytochemical compounds present in both *I. aquatica* and *I. reptans* extracts. This indicates that a polar solvent is more suitable for the separation of phytochemical compounds in *I. aquatica* and *I. reptans*. 10 % methanol also has a higher eluting strength compared to chloroform, making it more effective in separating phytochemical compounds of varying polarities. 10 % methanol was better at separating terpenoid and phenol.

More phytochemical compounds were detected in non-polar extracts compared to polar extracts for both plant species in both solvents. This shows that most of the phytochemical present in both plant extracts were non-polar, making it very soluble in non-polar extracts.

All reagents did not produce any visible stain in distilled water extracts.

Table 4.2 : Thin layer chromatography (TLC) profile for *I. aquatica* leaf hexane (IA-LH) extract with chloroform solvent.

Extract	Band	R_f	Observations					Remarks
			Visible light	UV light	Folin-Ciocalteu	Vanillin-sulphuric acid	Dragendorff's	
I. aquatica leaf hexane (IA-LH)	1	0.06	Green (+)	Red (+)	-	Pink (+++)	-	Terpenoids
	2	0.07	Yellow (+)	-	Blue (+)	-	-	Phenols
	3	0.13	Yellow (+)	Red (+)	Blue (+)	-	-	Phenols
	4	0.17	Yellow (++)	-	Blue (++)	-	-	Phenols
	5	0.28	Green (+)	Red (+++)	-	Pink (++)	-	Terpenoids
	6	0.34	Green (+)	Red (++)	-	-	Brown (+)	Alkaloids
	7	0.42	-	Red (++)	-	Pink (+)	-	Terpenoids
	8	0.43	Green (+)	-	Blue (+)	-	-	Phenols
	9	0.46	Yellow (+)	Red (++)	-	Brown (+++)	Brown (++)	Phenols
	10	0.48	Yellow (+)	Red (+++)	Blue (+)	-	-	Phenols
	11	0.50	-	Blue (+)	-	Orange (+)	-	Alkaloids
	12	0.58	-	Red (++)	-	Pink (+)	-	Terpenoids
	13	0.58	Green (++)	Red (+++)	Blue (++)	-	-	Phenols
	14	0.68	Yellow (+)	Red (++)	Blue (++)	-	-	Phenols
	15	0.87	-	-	-	Pink (++)	-	Terpenoids
	16	0.88	Yellow (+++)	Red (++)	Blue (+++)	Brown (+++)	-	Phenols

Colour intensity: (+) weak, (++) moderate, (+++) strong, (-) absent

Table 4.3 : Thin layer chromatography (TLC) profile for *I. aquatica* stem hexane (IA-SH) extract in chloroform solvent.

Extract	Band	R_f	Observations					Remarks
			Visible light	UV light	Folin-Ciocalteu	Vanillin-sulphuric acid	Dragendorff	
I. aquatica stem hexane (IA-SH)	1	0.13	Yellow (+)	Red (+)	-	Pink (+)	-	Terpenoids
	2	0.21	Green (+)	Red (++)	-	-	-	-
	3	0.24	-	Red (+)	-	-	-	-
	4	0.33	-	Red (+)	-	Brown (++)	-	Terpenoids
	5	0.34	-	Blue (++)	-	-	-	-
	6	0.44	-	Blue (+)	-	-	-	-
	7	0.56	Green (+)	Red (+++)	-	-	-	-
	8	0.58	-	Red (+)	-	-	-	-
	9	0.71	-	Red (+)	-	-	-	-
	10	0.77	-	Blue (+)	-	-	-	-
	11	0.85	-	Blue (+)	-	Pink (+)	-	Terpenoids
	12	0.90	Yellow (+)	-	-	Pink (+)	-	Terpenoids

Colour intensity: (+) weak, (++) moderate, (+++) strong, (-) absent

Table 4.4 : Thin layer chromatography (TLC) profile for *I. reptans* leaf hexane (IR-LH) extract in chloroform solvent.

Extract	Band	R_f	Observations					Remarks
			Visible light	UV light	Folin-Ciocalteu	Vanillin-sulphuric acid	Dragendorff	
I. reptans leaf hexane (IR-LH)	1	0.04	Green (++)	Red (++)	-	Pink (+)	-	Terpenoids
	2	0.08	Green (+)	-	-	-	-	Chlorophyll
	3	0.09	Yellow (++)	Red (+++)	-	Pink (+)	-	Terpenoids
	4	0.15	Green (+)	Red (+++)	-	Pink (+)	-	Terpenoids
	5	0.25	Yellow (+)	Red (+)	Blue (+)	-	-	Phenols
	6	0.25	Green (+)	-	-	-	-	Chlorophyll
	7	0.30	Green (+)	-	-	-	-	Chlorophyll
	8	0.33	-	-	-	Brown (++)	-	Terpenoids
	9	0.38	-	Blue (+)	-	Pink (+)	-	Terpenoids
	10	0.40	Yellow (+)	Red (+)	-	Pink (+)	-	Terpenoids
	11	0.48	Yellow (+)	-	-	Pink (+)	-	Terpenoids
	12	0.53	Yellow (+)	Blue (++)	-	-	-	-
	13	0.56	-	Red (+)	-	Pink (+)	-	Terpenoids
	14	0.69	-	Red (+)	-	Pink (+)	-	Terpenoids
	15	0.70	Yellow (+)	-	Blue (+)	-	-	Phenols
	16	0.79	-	Blue (+)	-	Pink (+)	-	Terpenoids
	17	0.89	Yellow (+++)	-	Blue (+++)	-	-	Phenols

Colour intensity: (+) weak, (++) moderate, (+++) strong, (-) absent

Table 4.5 : Thin layer chromatography (TLC) profile for *I. reptans* stem hexane (IR-SH) extract in chloroform solvent.

Extract	Band	R_f	Observations					Remarks
			Visible light	UV light	Folin-Ciocalteu	Vanillin-sulphuric acid	Dragendorff	
I. reptans stem hexane (IR-SH)	1	0.09	Green (+)	Red (+++)	-	Pink (+)	-	Terpenoids
	2	0.12	Yellow (+)	-	-	Pink (+)	-	Terpenoids
	3	0.15	-	Red (+)	-	Pink (+)	-	Terpenoids
	4	0.33	-	-	-	Brown (++)	-	Terpenoids
	5	0.42	-	Blue (++)	-	-	-	-
	6	0.90	Yellow (++)	-	-	-	Brown (++)	Alkaloids

Colour intensity: (+) weak, (++) moderate, (+++) strong, (-) absent

Table 4.6 : Thin layer chromatograhy (TLC) profile for *I. aquatica* leaf chloroform (IA-LC) extract in chloroform solvent.

Extract	Band	R_f	Observations					Remarks
			Visible light	UV light	Folin-Ciocalteu	Vanillin-sulphuric acid	Dragendorff	
I. aquatica leaf chloroform (IA-LC)	1	0.06	Green (+++)	Red (+++)	Blue (++)	Green (+++)	-	Alcohols
	2	0.11	Green (+++)	Red (+++)	Blue (++)	Green (+++)	Green (+)	Alcohols
	3	0.12	-	-	-	Orange (+++)	-	Terpenoids
	4	0.13	Yellow (++)	Red (++)	Blue (++)	Green (+++)	Green (+++)	Alcohols
	5	0.16	-	-	-	Pink (++)	-	Terpenoids
	6	0.18	Green (++)	Blue (++)	-	Green (+++)	-	Alcohols
	7	0.21	Green (+++)	Red (++)	Blue (++)	Green (+++)	-	Alcohols
	8	0.22	Yellow (+++)	Red (+++)	-	Pink (+)	Green (++)	Phenols
	9	0.26	Green (+)	Red (++)	Blue (+)	-	Brown (+)	Phenols
	10	0.42	Yellow (+)	Red (+)	-	-	-	-
	11	0.42	-	Blue (++)	-	-	-	-
	12	0.43	Green (++)	Red (+++)	Blue (+++)	-	Brown (+++)	Phenols
	13	0.48	Green (++)	Red (+++)	-	-	Green (+++)	Phenols
	14	0.52	Yellow (+)	-	-	-	-	Xanthophylls
	15	0.60	-	Red (++)	-	-	Green (+++)	Phenols
	16	0.71	-	Blue (++)	-	Pink (+)	-	Terpenoids
	17	0.72	Green (+++)	Red (+++)	-	-	Green (++)	Phenols
	18	0.82	Yellow (+)	Red (+)	-	Pink (+)	Brown (+)	Terpenoids
	19	0.83	Yellow (+++)	Blue (+)	Blue (++)	Green (+++)	-	Alcohols

Colour intensity: (+) weak, (++) moderate, (+++) strong, (-) absent

Table 4.7 : Thin layer chromatography (TLC) profile for *I. aquatica* stem chloroform (IA-SC) extract in chloroform solvent.

Extract	Band	R_f	Observations					Remarks
			Visible light	UV light	Folin-Ciocalteu	Vanillin-sulphuric acid	Dragendorff	
I. aquatica stem chloroform (IA-SC)	1	0.07	-	Blue (++)	Blue (+)	Pink (+)	-	Phenols
	2	0.09	Yellow (+)	Red (++)	-	Pink (+)	-	Terpenoids
	3	0.12	-	-	-	Orange (+)	-	Terpenoids
	4	0.12	Yellow (+)	Blue (+++)	Blue (+)	-	-	Phenols
	5	0.22	Yellow (+)	-	-	-	-	Xanthophylls
	6	0.25	-	Blue (+)	-	-	-	-
	7	0.30	Green (+)	Red (++)	Blue (+)	Green (+)	-	Alcohols
	8	0.50	Green (++)	Red (+++)	Blue (++)	-	Brown (+)	Phenols
	9	0.64	-	Red (+)	-	-	-	-
	10	0.79	-	Red (+)	-	-	-	-

Colour intensity: (+) weak, (++) moderate, (+++) strong, (-) absent

Table 4.8 : Thin layer chromatography (TLC) profile for *I. reptans* leaf chloroform (IR-LC) extract in chloroform solvent.

Extract	Band	R_f	Observations					Remarks
			Visible light	UV light	Folin-Ciocalteu	Vanillin-sulphuric acid	Dragendorff	
I. reptans leaf chloroform (IR-LC)	1	0.09	Yellow (+)	-	-	-	-	Xanthophyll
	2	0.11	-	Blue (+)	-	-	-	-
	3	0.11	Yellow (+++)	Red (+)	Blue (++)	Green (+++)	-	Alcohols
	4	0.12	-	-	-	Orange (+)	-	Terpenoids
	5	0.13	-	Red (+)	-	Green (+)	-	Alcohols
	6	0.14	Green (+)	Red (++)	Blue (+)	Green (+++)	-	Alcohols
	7	0.15	Yellow (++)	-	-	-	Green (++)	Alkaloids
	8	0.23	Green (+)	Blue (+)	-	-	-	-
	9	0.27	Green (+)	Red (+)	Blue (+)	-	-	Phenols
	10	0.29	Yellow (+)	-	-	-	-	Xanthophyll
	11	0.35	-	Red (+)	-	-	-	-
	12	0.35	Green (+)	Red (++)	-	-	-	-
	13	0.41	Green (+)	Red (++)	-	-	-	-
	14	0.46	Green (+)	Red (++)	Blue (+++)	-	-	Phenols
	15	0.49	-	Red (+)	-	-	-	-
	16	0.58	Green (+++)	Red (++)	-	-	Brown (++)	Alkaloids
	17	0.63	-	Red (+)	-	-	-	-
	18	0.73	-	Red (+)	-	-	-	-
	19	0.87	Yellow (+)	Blue (+)	-	-	-	-
	20	0.94	Yellow (+)	Red (+)	-	-	-	-

Colour intensity: (+) weak, (++) moderate, (+++) strong, (-) absent

Table 4.9 : Thin layer chromatography (TLC) profile for *I. reptans* stem chloroform (IR-SC) extract in chloroform solvent.

Extract	Band	R_f	Observations					Remarks
			Visible light	UV light	Folin-Ciocalteu	Vanillin-sulphuric acid	Dragendorff	
I. reptans stem chloroform (IR-SC)	1	0.03	-	Red (++)	-	Pink (+)	-	Terpenoids
	2	0.04	-	Blue (+)	-	Pink (+)	-	Terpenoids
	3	0.11	Yellow (+)	Red (+)	Blue (+)	Green (+)	-	Alcohols
	4	0.12	-	-	-	Orange (+)	-	Terpenoids
	5	0.14	-	Red (+)	-	-	-	-
	6	0.18	Green (+)	Red (+)	Blue (+)	-	-	Phenols
	7	0.19	Yellow (+)	Red (+)	-	-	-	-
	8	0.21	-	Red (++)	-	-	-	-
	9	0.35	Green (+)	Red (+)	-	Green (++)	-	Alcohols
	10	0.38	Green (+)	Red (+)	-	-	-	-
	11	0.43	Green (+)	Red (+)	Blue (++)	-	-	Phenols
	12	0.70	Green (++)	Red (+++)	-	-	Brown (+)	Alkaloids
	13	0.71	-	Red (+)	-	-	-	-
	14	0.77	-	Red (++)	-	-	-	-

Colour intensity: (+) weak, (++) moderate, (+++) strong, (-) absent

Table 4.10 : Thin layer chromatography (TLC) profile for *I. aquatica* leaf methanol (IA-LM) extract in chloroform solvent.

Extract	Band	R_f	Observations					Remarks
			Visible light	UV light	Folin-Ciocalteu	Vanillin-sulphuric acid	Dragendorff	
I. aquatica leaf methanol (IA-LM)	1	0.04	-	Red (+)	-	Brown (+)	-	Terpenoids
	2	0.06	-	Blue (+)	-	-	-	-
	3	0.10	-	Red (+)	-	Pink (+)	-	Terpenoids
	4	0.13	-	Red (++)	-	-	-	-
	5	0.14	-	Blue (+++)	-	-	-	-
	6	0.25	-	Red (+)	-	-	-	-
	7	0.30	Green (+)	Red (+++)	-	-	-	-
	8	0.40	-	Blue (+)	-	-	-	-
	9	0.43	-	Red (+)	-	-	-	-

Colour intensity: (+) weak, (++) moderate, (+++) strong, (-) absent

Table 4.11 : Thin layer chromatography (TLC) profile for *I. aquatica* stem methanol (IA-SM) extract in chloroform solvent.

Extract	Band	R_f	Observations					Remarks
			Visible light	UV light	Folin-Ciocalteu	Vanillin-sulphuric acid	Dragendorff	
I. aquatica stem methanol (IA-SM)	1	0.07	-	Blue (++)	-	-	-	-
	2	0.19	-	Blue (+++)	-	-	-	-
	3	0.21	-	Red (+)	-	-	-	-
	4	0.24	-	Red (+)	-	-	-	-
	5	0.25	-	Red (+)	-	-	-	-
	6	0.57	-	Red (++)	-	-	-	-

Colour intensity: (+) weak, (++) moderate, (+++) strong, (-) absent

Table 4.12 : Thin layer chromatography (TLC) profile for *I. reptans* leaf methanol (IR-LM) extract in chloroform solvent.

Extract	Band	R_f	Observations					Remarks
			Visible light	UV light	Folin-Ciocalteu	Vanillin-sulphuric acid	Dragendorff	
I. reptans leaf methanol (IR-LM)	1	0.03	-	Red (++)	-	-	-	-
	2	0.06	-	Blue (++)	-	-	-	-
	3	0.08	-	Red (+++)	-	-	-	-
	4	0.12	-	Red (+)	-	-	-	-
	5	0.13	Green (+)	Red (+++)	-	-	-	-
	6	0.14	-	Blue (+)	-	-	-	-
	7	0.15	-	Red (++)	-	-	-	-
	8	0.24	Yellow (+)	Red (+++)	-	-	-	-
	9	0.24	Green (++)	Red (+++)	-	-	Brown (++)	Alkaloids
	10	0.25	-	Red (+)	-	-	-	-
	11	0.33	-	Red (+)	-	-	-	-
	12	0.37	Green (++)	Red (+++)	-	-	-	-
	13	0.38	-	Red (+)	-	-	-	-
	14	0.47	-	Red (+)	-	-	-	-

Colour intensity: (+) weak, (++) moderate, (+++) strong, (-) absent

Table 4.13 : Thin layer chromatography (TLC) profile for *I. reptans* stem methanol (IR-SM) extract in chloroform solvent.

Extract	Band	R_f	Observations					Remarks
			Visible light	UV light	Folin-Ciocalteu	Vanillin-sulphuric acid	Dragendorff	
I. reptans stem methanol (IR-SM)	1	0.06	-	Blue (+)	-	-	-	-
	2	0.11	-	Red (+)	-	-	-	-
	3	0.14	-	Red (+)	-	-	-	-
	4	0.15	-	Red (+)	-	-	-	-

Colour intensity: (+) weak, (++) moderate, (+++) strong, (-) absent

Table 4.14 : Thin layer chromatography (TLC) profile for *I. aquatica* leaf distilled water (IA-LD) extract in chloroform solvent.

Extract	Band	R_f	Observations					Remarks
			Visible light	UV light	Folin-Ciocalteu	Vanillin-sulphuric acid	Dragendorff	
I. aquatica leaf distilled water (IA-LD)	1	0.07	-	Blue (+)	-	-	-	-
	2	0.21	-	Blue (+)	-	-	-	-

Table 4.15 : Thin layer chromatography (TLC) profile for *I. aquatica* stem distilled water (IA-SD) extract in chloroform solvent.

Extract	Band	R_f	Observations					Remarks
			Visible light	UV light	Folin-Ciocalteu	Vanillin-sulphuric acid	Dragendorff	
I. aquatica stem distilled water (IA-SD)	1	0.09	-	Blue (+)	-	-	-	-
	2	0.18	-	Blue (+++)	-	-	-	-

Colour intensity: (+) weak, (++) moderate, (+++) strong, (-) absent

Table 4.16 : Thin layer chromatography (TLC) profile for *I. reptans* leaf distilled water (IR-LD) extract in chloroform solvent.

Extract	Band	R_f	Observations					Remarks
			Visible light	UV light	Folin-Ciocalteu	Vanillin-sulphuric acid	Dragendorff	
I. reptans leaf distilled water (IR-LD)	1	0.07	-	Blue (+)	-	-	-	-

Table 4.17 : Thin layer chromatography (TLC) profile for *I. reptans* stem distilled water (IR-SD) extract in chloroform solvent.

Extract	Band	R_f	Observations					Remarks
			Visible light	UV light	Folin-Ciocalteu	Vanillin-sulphuric acid	Dragendorff	
I. reptans stem distilled water (IR-SD)	1	0.12	-	Blue (+)	-	-	-	-

Colour intensity: (+) weak, (++) moderate, (+++) strong, (-) absent

Table 4.18 : Thin layer chromatography (TLC) profile for *I. aquatica* leaf hexane (IA-LH) extract in 10 % methanol solvent.

Extract	Band	R_f	Observations					Remarks
			Visible light	UV light	Folin-Ciocalteu	Vanillin-sulphuric acid	Dragendorff	
I. aquatica leaf hexane (IA-LH)	1	0.10	-	Red (+)	-	Pink (++)	-	Terpenoids
	2	0.15	-	Blue (+)	-	Pink (++)	-	Terpenoids
	3	0.16	Green (+)	Blue (+)	-	-	-	-
	4	0.20	-	Red (+)	-	Pink (++)	-	Terpenoids
	5	0.27	-	Blue (+)	-	Orange (+)	-	Terpenoids
	6	0.28	Green (+)	Red (+++)	-	Orange (+)	-	Terpenoids
	7	0.33	Green (+)	Red (++)	-	Pink (+)	Brown (+)	Terpenoids
	8	0.40	-	Red (+)	-	Pink (++)	-	Terpenoids
	9	0.45	Green (+)	Blue (+)	-	-	-	-
	10	0.46	Green (+)	Red (+++)	-	Pink (+++)	-	Terpenoids
	11	0.52	-	Blue (+)	-	-	-	-
	12	0.60	Green (+)	-	-	-	-	Chlorophyll
	13	0.60	Yellow (++)	Red (+)	Blue (+)	-	-	-
	14	0.64	-	Red (+)	-	Pink (+++)	-	Terpenoids
	15	0.68	Yellow (++)	-	-	Pink (+++)	-	Terpenoids
	16	0.72	Yellow (+)	Blue (+)	-	-	-	-
	17	0.74	-	Red (+)	-	Pink (+++)	-	Terpenoids
	18	0.82	Yellow (++)	Red (++)	Blue (+)	Pink (+++)	-	Phenols
	19	0.89	Yellow (+++)	Red (+++)	Blue (+++)	Pink (++)	Green (+++)	Phenols
	20	0.93	-	blue (+++)	-	-	-	-

Colour intensity: (+) weak, (++) moderate, (+++) strong, (-) absent

Table 4.19 : Thin layer chromatography (TLC) profile for *I. aquatica* stem hexane (IA-SH) extract in 10 % methanol solvent.

Extract	Band	R_f	Observations					Remarks
			Visible light	UV light	Folin-Ciocalteu	Vanillin-sulphuric acid	Dragendorff	
I. aquatica stem hexane (IA-SH)	1	0.21	-	Blue (+)	-	Orange (+)	-	Terpenoids
	2	0.30	-	Red (+)	-	Orange (+)	Brown (+)	Phenols
	3	0.31	-	Blue (+)	-	Orange (+)	Brown (+)	Phenols
	4	0.37	-	Blue (+)	-	-	-	-
	5	0.47	-	Red (++)	-	Pink (++)	-	Terpenoids
	6	0.49	-	Blue (+)	-	-	-	-
	7	0.62	-	Blue (++)	-	Pink (++)	-	Terpenoids
	8	0.68	Yellow (+)	-	-	-	-	Xanthophylls
	9	0.88	-	Blue (++)	-	Pink (+)	-	Terpenoids
	10	0.92	Yellow (+)	Red (++)	-	Pink (++)	-	Terpenoids
	11	0.93	-	Blue (++)	-	-	-	-

Colour intensity: (+) weak, (++) moderate, (+++) strong, (-) absent

Table 4.20 : Thin layer chromatography (TLC) profile for *I. reptans* leaf hexane (IR-LH) extract in 10 % methanol solvent.

Extract	Band	R_f	Observations					Remarks
			Visible light	UV light	Folin-Ciocalteu	Vanillin-sulphuric acid	Dragendorff	
I. reptans leaf hexane (IR-LH)	1	0.09	-	Red (++)	-	-	-	-
	2	0.20	-	Red (++)	-	-	-	-
	3	0.38	-	Red (++)	-	Pink (+)	-	Terpenoids
	4	0.42	Green (+)	Blue (+)	-	-	-	-
	5	0.43	-	Red (+)	-	Pink (+)	-	Terpenoids
	6	0.49	-	-	-	Pink (++)	-	Terpenoids
	7	0.55	Yellow (+)	Red (+)	-	Pink (+)	-	Terpenoids
	8	0.62	-	Red (+)	-	Pink (+)	-	Terpenoids
	9	0.63	-	Blue (+)	-	-	-	-
	10	0.66	Yellow (+)	-	-	Pink (+)	-	Terpenoids
	11	0.74	-	Red (+)	-	Pink (++)	-	Terpenoids
	12	0.75	-	-	-	Orange (++)	-	Terpenoids
	13	0.82	Yellow (+)	Red (+++)	-	-	Brown (++)	Alkaloids
	14	0.86	Green (++)	Red (+++)	-	Pink (++)	-	Terpenoids
	15	0.89	Yellow (+++)	Red (+++)	Blue (+++)	Pink (+++)	-	Phenols
	16	0.91	-	blue (+++)	Blue (+++)	-	-	Phenols

Colour intensity: (+) weak, (++) moderate, (+++) strong, (-) absent

Table 4.21 : Thin layer chromatography (TLC) profile for *I. reptans* stem hexane (IR-SH) extract in 10 % methanol solvent.

Extract	Band	R_f	Observations					Remarks
			Visible light	UV light	Folin-Ciocalteu	Vanillin-sulphuric acid	Dragendorff	
I. reptans stem hexane (IR-SH)	1	0.65	Yellow (+)	-	-	-	-	Xanthophylls
	2	0.75	-	-	-	Pink (+++)	-	Terpenoids
	3	0.82	-	Red (+)	-	-	-	-
	4	0.87	-	Blue (+)	-	Pink (+)	-	Terpenoids
	5	0.89	-	Red (+)	-	Pink (+)	-	Terpenoids
	6	0.92	Yellow (+)	blue (+++)	-	Pink (+)	-	Terpenoids

Colour intensity: (+) weak, (++) moderate, (+++) strong, (-) absent

Table 4.22 : Thin layer chromatography (TLC) profile for *I. aquatica* leaf chloroform (IA-LC) extract in 10 % methanol solvent.

Extract	Band	R_f	Observations					Remarks
			Visible light	UV light	Folin-Ciocalteu	Vanillin-sulphuric acid	Dragendorff	
I. aquatica leaf chloroform (IA-LC)	1	0.14	-	-	-	Orange (++)	-	Terpenoids
	2	0.15	Green (++)	Red (+++)	Blue (++)	Brown (++)	Brown (+)	Phenols
	3	0.20	Green (+)	Red (+++)	Blue (++)	Brown (+++)	Green (+)	Phenols
	4	0.23	Green (+)	Red (+++)	Blue (++)	Brown (++)	Brown (+)	Phenols
	5	0.32	Green (++)	Red (+++)	Blue (++)	Brown (+++)	Brown (+)	Phenols
	6	0.37	Green (++)	Red (+++)	Blue (++)	Brown (+++)	-	Phenols
	7	0.41	-	Red (+)	-	Pink (+)	-	Terpenoids
	8	0.42	Green (++)	Red (+++)	-	-	Brown (+)	Alkaloids
	9	0.45	Green (++)	Red (+++)	Blue (++)	Brown (+++)	++	Phenols
	10	0.58	-	Blue (+++)	Blue (+)	Pink (+++)	-	Phenols
	11	0.61	-	Red (+)	-	-	-	-
	12	0.61	Yellow (++)		Blue (+++)	Blue (+++)	Green (+++)	Phenols
	13	0.61	-	Red (+)	Blue (+)	Brown (+++)	-	Phenols
	14	0.76	Green (++)	Red (+++)	Blue (+)	Green (+++)	-	Alcohols
	15	0.85	Green (+++)	Red (+++)	Blue (+++)	Green (+++)	Green (+++)	Alcohols

Colour intensity: (+) weak, (++) moderate, (+++) strong, (-) absent

Table 4.23 : Thin layer chromatography (TLC) profile for *I. aquatica* stem chloroform (IA-SC) extract in 10 % methanol solvent.

Extract	Band	R_f	Observations					Remarks
			Visible light	UV light	Folin-Ciocalteu	Vanillin-sulphuric acid	Dragendorff	
I. aquatica stem chloroform (IA-SC)	1	0.17	-	Blue (+)	-	-	-	-
	2	0.21	-	-	-	Orange (++)	-	Terpenoids
	3	0.29	Green (+)	Red (+++)	Blue (+)	Orange (+)	-	Terpenoids
	4	0.39	-	Blue (+)	-	-	-	-
	5	0.42	-	Red (++)	-	-	-	-
	6	0.44	-	-	-	Brown (++)	-	Terpenoids
	7	0.44	Green (+)	Red (+)	-	-	-	-
	8	0.54	-	Blue (+++)	-	-	-	-
	9	0.56	Green (+)	Red (++)	Blue (+)	Brown (++)	Brown (+)	Phenols
	10	0.62	-	Blue (+++)	-	Pink (+)	-	Terpenoids
	11	0.68	Yellow (+)	-	-	Blue (+)	-	Alcohols
	12	0.70	-	Red (+)	-	-	-	-
	13	0.81	-	-	-	Brown (++)	-	Terpenoids
	14	0.91	Green (+++)	Red (+++)	Blue (++)	Green (++)	Brown (++)	Alcohols

Colour intensity: (+) weak, (++) moderate, (+++) strong, (-) absent

Table 4.24 : Thin layer chromatography (TLC) profile for *I. reptans* leaf chloroform (IR-LC) extract in 10 % methanol solvent.

Extract	Band	R_f	Observations					Remarks
			Visible light	UV light	Folin-Ciocalteu	Vanillin-sulphuric acid	Dragendorff	
I. reptans leaf chloroform (IR-LC)	1	0.12	-	Red (+)	-	-	-	-
	2	0.19	-	Red (+)	-	Pink (++)	-	Terpenoids
	3	0.28	Green (+++)	Red (++)	Blue (+)	-	Brown (+)	Phenols
	4	0.28	-	Red (+)	-	Orange (+)	-	Terpenoids
	5	0.33	Yellow (+)	-	Blue (+)	-	-	Phenols
	6	0.34	Green (+)	Red (+)	Blue (+)	-	-	Phenols
	7	0.39	Green (+)	Red (+)	Blue (++)	-	-	Phenols
	8	0.46	Green (++)	Red (+++)	Blue (+)	Pink (++)	Brown (++)	Phenols
	9	0.48	-	Red (+)	-	Pink (+)	-	Terpenoids
	10	0.49	Yellow (+)	Red (+)	Blue (+)	-	-	Phenols
	11	0.52	-	Red (++)	-	-	-	-
	12	0.55	Green (+)	Red (+)	-	-	-	-
	13	0.55	-	Blue (++)	-	Pink (+)	-	Terpenoids
	14	0.63	-	Blue (++)	-	-	-	-
	15	0.64	Yellow (++)	-	Blue (++)	Blue (+++)	Green (+)	Phenols
	16	0.66	-	Red (++)	-	-	-	-
	17	0.76	Yellow (+)	Red (++)	-	Brown (+)	-	Terpenoids
	18	0.76	-	Red (++)	-	Pink (+)	-	Terpenoids
	19	0.83	Green (+)	-	Blue (+)	-	-	Phenols
	20	0.91	Green (+++)	Red (+++)	Blue (+++)	Green (+++)	Brown (+++)	Alcohols

Colour intensity: (+) weak, (++) moderate, (+++) strong, (-) absent

Table 4.25 : Thin layer chromatography (TLC) profile for *I. reptans* stem chloroform (IR-SC) extract in 10 % methanol solvent.

Extract	Band	R_f	Observations					Remarks
			Visible light	UV light	Folin-Ciocalteu	Vanillin-sulphuric acid	Dragendorff	
I. reptans stem chloroform (IR-SC)	1	0.26	-	Red (++)	-	-	-	-
	2	0.29	-	Blue (+)	-	-	-	-
	3	0.30	-	-	-	Orange (+)	-	Terpenoids
	4	0.30	-	Blue (+)	-	-	-	-
	5	0.44	-	Red (++)	-	-	-	-
	6	0.44	-	Red (+)	-	-	-	-
	7	0.52	-	Blue (++)	-	-	-	-
	8	0.54	Yellow (+)	-	-	Pink (+)	-	Terpenoids
	9	0.61	Green (+)	-	-	-	-	Chlorophylls
	10	0.62	-	Blue (++)	-	Pink (+)	-	Terpenoids
	11	0.67	Yellow (++)	Red (++)	-	-	-	-
	12	0.72	-	Red (++)	-	-	-	-
	13	0.81	-	-	-	Orange (+)	-	Terpenoids
	14	0.81	-	Red (++)	-	-	-	-
	15	0.92	Green (++)	Red (+++)	Blue (++)	-	Brown (++)	Phenols

Colour intensity: (+) weak, (++) moderate, (+++) strong, (-) absent

Table 4.26 : Thin layer chromatography (TLC) profile for *I. aquatica* leaf methanol (IA-LM) extract in 10 % methanol solvent.

Extract	Band	R_f	Observations					Remarks
			Visible light	UV light	Folin-Ciocalteu	Vanillin-sulphuric acid	Dragendorff	
I. aquatica leaf methanol (IA-LM)	1	0.07	-	Red (+)	-	Pink (+)	-	Terpenoids
	2	0.07	-	-	Blue (+++)	-	-	Phenols
	3	0.09	-	Blue (+++)	-	Pink (+++)	-	Terpenoids
	4	0.10	Green (+)	Blue (+)	-	-	-	-
	5	0.12	-	Blue (+)	-	-	-	-
	6	0.16	Yellow (+)	Blue (+)	-	-	-	-
	7	0.24	-	Red (+)	-	-	-	-
	8	0.28	Yellow (++)	Red (+)	-	-	-	-
	9	0.36	Green (+)	Red (++)	-	-	-	-
	10	0.37	-	Red (+)	-	-	-	-
	11	0.43	-	Blue (+)	-	-	-	-
	12	0.47	Yellow (+)	-	-	-	-	Xanthophylls
	13	0.50	Green (+)	Red (+++)	-	-	-	-
	14	0.55	-	Red (+)	-	-	-	-
	15	0.55	-	Blue (++)	-	-	-	-
	16	0.57	-	Blue (+)	-	-	-	-
	17	0.59	-	Blue (++)	-	-	-	-
	18	0.61	Yellow (+)	Red (+)	-	-	-	-
	19	0.65	-	Blue (++)	-	-	-	-
	20	0.69	-	Red (+)	-	Pink (+)	-	Terpenoids
	21	0.85	-	Red (+)	-	-	-	-
	22	0.91	Green (++)	Red (+++)	-	-	-	-
	23	0.94	Yellow (++)	Red (+)	-	-	-	-

Colour intensity: (+) weak, (++) moderate, (+++) strong, (-) absent

Table 4.27 : Thin layer chromatography (TLC) profile for *I. aquatica* stem methanol (IA-SM) extract in 10 % methanol solvent.

Extract	Band	R_f	Observations					Remarks
			Visible light	UV light	Folin-Ciocalteu	Vanillin-sulphuric acid	Dragendorff	
I. aquatica stem methanol (IA-SM)	1	0.10	-	Blue (+++)	-	Pink (+)	-	Terpenoids
	2	0.28	-	Blue (+)	-	-	-	-
	3	0.57	-	Blue (++)	-	-	-	-
	4	0.65	-	Blue (+++)	-	-	-	-
	5	0.73	-	Blue (+)	-	-	-	-
	6	0.93	Green (+)	Red (+++)	-	-	-	-
	7	0.97	-	Red (+)	-	-	-	-

Colour intensity: (+) weak, (++) moderate, (+++) strong, (-) absent

Table 4.28 : Thin layer chromatography (TLC) profile for *I. reptans* leaf methanol (IR-LM) extract in 10 % methanol solvent.

Extract	Band	R_f	Observations					Remarks
			Visible light	UV light	Folin-Ciocalteu	Vanillin-sulphuric acid	Dragendorff	
I. reptans leaf methanol (IR-LM)	1	0.05	-	Blue (++)	-	Pink (++)	-	Terpenoids
	2	0.07	-	Red (+)	-	-	-	-
	3	0.08	-	Blue (++)	-	Pink (++)	-	Terpenoids
	4	0.12	-	Red (+)	-	Pink (+)	-	Terpenoids
	5	0.15	-	Blue (+)	-	-	-	-
	6	0.20	-	Red (+)	-	-	-	-
	7	0.23	-	Red (+)	-	-	-	-
	8	0.28	Green (+)	Red (+++)	-	-	-	-
	9	0.39	-	Red (++)	-	-	-	-
	10	0.40	-	Blue (++)	-	-	-	-
	11	0.43	Green (+)	Red (+++)	-	-	-	-
	12	0.51	-	Blue (+)	-	-	-	-
	13	0.56	-	Red (+)	-	-	-	-
	14	0.56	-	Blue (+)	-	-	-	-
	15	0.64	-	Red (+)	-	-	-	-
	16	0.66	-	Red (+)	-	-	-	-
	17	0.76	-	Red (+)	-	-	-	-
	18	0.84	-	Red (+)	-	-	-	-
	19	0.94	Green (++)	Red (+++)	Blue (+)	-	Brown (+)	Phenols

Colour intensity: (+) weak, (++) moderate, (+++) strong, (-) absent

Table 4.29 : Thin layer chromatography (TLC) profile for *I. reptans* stem methanol (IR-SM) extract in 10 % methanol solvent.

Extract	Band	R_f	Observations					Remarks
			Visible light	UV light	Folin-Ciocalteu	Vanillin-sulphuric acid	Dragendorff	
I. reptans stem methanol (IR-SM)	1	0.07	-	Blue (+++)	-	-	-	-
	2	0.19	-	-	-	Pink (+)	-	Terpenoids
	3	0.39	-	Blue (+)	-	-	-	-
	4	0.49	-	Blue (+)	-	-	-	-
	5	0.55	-	Blue (+)	-	-	-	-
	6	0.92	-	Red (+)	-	-	-	-

Colour intensity: (+) weak, (++) moderate, (+++) strong, (-) absent

Table 4.30 : Thin layer chromatography (TLC) profile for *I. aquatica* leaf distilled water (IA-LD) extract in 10 % methanol solvent.

Extract	Band	R_f	Observations					Remarks
			Visible light	UV light	Folin-Ciocalteu	Vanillin-sulphuric acid	Dragendorff	
I. aquatica leaf distilled water (IA-LD)	1	0.45	-	Blue (+)	-	-	-	-
	2	0.52	-	Blue (+)	-	-	-	-

Table 4.31 : Thin layer chromatography (TLC) profile for *I. aquatica* stem distilled water (IA-SD) extract in 10 % methanol solvent.

Extract	Band	R_f	Observations					Remarks
			Visible light	UV light	Folin-Ciocalteu	Vanillin-sulphuric acid	Dragendorff	
I. aquatica stem distilled water (IA-SD)	1	0.04	-	Blue (++)	-	-	-	-
	2	0.48	-	Blue (++)	-	-	-	-
	3	0.58	-	Blue (+++)	-	-	-	-
	4	0.92	-	Red (+)	-	-	-	-

Colour intensity: (+) weak, (++) moderate, (+++) strong, (-) absent

Table 4.32 : Thin layer chromatography (TLC) profile for *I. reptans* leaf distilled water (IR-LD) extract in 10 % methanol solvent.

Extract	Band	R_f	Observations					Remarks
			Visible light	UV light	Folin-Ciocalteu	Vanillin-sulphuric acid	Dragendorff	
I. reptans leaf distilled water (IR-LD)	1	0.07	-	Blue (+++)	-	-	-	-
	2	0.97	-	Red (+)	-	-	-	-

Table 4.33 : Thin layer chromatography (TLC) profile for *I. reptans* stem distilled water (IR-SD) extract in 10 % methanol solvent.

Extract	Band	R_f	Observations					Remarks
			Visible light	UV light	Folin-Ciocalteu	Vanillin-sulphuric acid	Dragendorff	
I. reptans stem distilled water (IR-SD)	-	-	-	-	-	-	-	-

Colour intensity: (+) weak, (++) moderate, (+++) strong, (-) absen

4.3 Mass Spectrometry

4.3.1 Liquid Chromatography-Mass Spectrometry (LC-MS)

The liquid chromatography-mass spectrometry (LC-MS) analysis was performed to detect the presence of quercetin, tannic acid, gallic acid and phenylalanine in the selected *I. aquatica* and *I. reptans* extracts. The extracts were run against these standards and the concentration of each compound detected was calculated. Table 4.34 describes the concentration of the compounds detected in each of the extract sample analysed.

It can be seen that in all five extracts of *I. aquatica* and *I. reptans*, there were negligible traces of gallic acid. Varying amount of quercetin and phenylalanine detected in the *I. aquatica* and *I. reptans* extracts sent indicated that between plant species and parts of plant, there was a difference in the level of these compounds. Quercetin (13.36 mg/ml) and phenylalanine (25.95 mg/ml) were the highest in IA-LM. Higher level of quercetin and phenylalanine seen showed that methanol was more effective in extracting these compounds compared to distilled water. Methanol is known to be effective in dissolving sugar, amino acids, glycoside compounds and phenolic compounds with low and medium molecular weight and of medium polarity (Widyawati *et al.*, 2014). Besides, methanol is amphiphilic thus it is better at extracting polar and non-polar compounds such as flavanoid and phenyalanine.

The highest concentration of tannic acid (1.25 mg/ml) can be observed in IA-LD. The IA-LD had a higher concentration of quercetin (10.31 vs 1.46 mg/ml) and phenylalanine (20.01 vs 2.84 mg/ml) compared to IA-SD. This indicates that leaf extract in distilled water of *I. aquatica* had a higher level of quercetin and phenylalanine compared to the stem extract in distilled water of *I. aquatica.* IA-LM showed higher concentration of quercetin (13.36 vs 5.80 mg/ml) and phenylalanine (25.95 vs 11.25 mg/ml) compared to IR-LM while tannic acid remained similar in concentration. This shows that quercetin

and phenyalanine were higher in the methanolic leaf extract of *I. aquatica* compared to *I. reptans.*

Table 4.34 : The compounds detected in the LC-MS for the selected *I. aquatica* and *I. reptans* extracts. N/A indicates a negligible concentration.

Extract	Concentration (mg/ml)			
	Quercetin	**Tannic acid**	**Gallic acid**	**Phenylalanine**
IA-LM	13.36	0.68	N/A	25.95
IR-LM	5.80	0.68	N/A	11.25
IA-LD	10.31	1.25	N/A	20.01
IA-SD	1.46	0.93	N/A	2.84
IR-LD	7.44	0.17	N/A	14.44

IA-LM : *I. aquatica* leaf extract in methanol
IR-LM : *I. reptans* leaf extract in methanol
IA-LD : *I. aquatica* leaf extract in distilled water
IA-SD : *I. aquatica* stem extract in distilled water
IR-LD : *I. reptans* leaf extract in distilled water

4.3.2 Gas Chromatography-Mass Spectrometry (GC-MS)

The gas chromatography-mass spectrometry (GC-MS) performed suggested the presence of phenolic compounds, alkaloids, terpenes, sterol, esters, polyols, organic acids and fatty acids in all IA-LM, IR-LM, IA-LD, IA-SD and IR-LD. Among these compounds, more alkaloids and ester were detected. A greater level of phytochemical compounds were observed in *I. aquatica* compared to *I. reptans*. The IA-LD was shown to have a lower level of phytochemical compounds compared to IA-SD. This indicates that the stem extract in distilled water of *I. aquatica* contains more phytochemical compounds compared to its leaf extract in distilled water. Table 4.35 - 4.39 show the suggested compounds present in the extracts tested.

Table 4.35 : The suggested GC-MS result for *I. aquatica* leaf in methanolic (IA-LM) extract.

Compound name	Retention time	Area	Area (%)	Group
11-Hexadecenoic acid, 15-methyl-, methyl ester	4.27	7959	1.99	Ester
Benzyl propiolate	6.14	7904	1.97	Ester
Cholest-8(14)-ene-3,15-diol, diacetate, (3.beta-hydroxy-5 alpha-	12.92	8019	2.00	Sterol
Phenol, 2,4-bis(1,1-dimethylethyl)-	15.85	36071	9.01	Phenolic compound
6-[(Z)-1-Butenyl]-1,4-cycloheptadiene	16.70	9699	2.42	Terpene
Thiocarbamic acid, N,N-dimethyl, S-1,3-diphenyl-2-butenyl ester	17.52	7857	1.96	Ester
1- Benzazirene-1-carboxylic acid, 2,2,5a-trimethyl-1a-[3-oxo-1-butenyl] perhydro-	19.56	8089	2.02	Organic acid
Silicic acid, diethyl bis(trimethylsilyl) ester	20.48	8167	2.04	Ester
Phenol, 6-methyl-2-[(4-morpholinyl)methyl]-	21.25	7930	1.98	Phenolic compound
3,4,5-Tris(trimethylsiloxy)-1-cyclohexene-1-carboxylic acid	22.63	9591	2.40	Organic acid

Table 4.36 : The suggested GC-MS result for *I. reptans* leaf in methanolic (IR-LM) extract.

Compound name	Retention time	Area	Area (%)	Group
1,3-Propanediol, 2-methyl-2-propyl-	12.73	9538	3.16	Polyols
1- Benzazirene-1-carboxylic acid, 2,2,5a-trimethyl-1a-[3-oxo-1-butenyl] perhydro-	14.77	7969	2.64	Ester
Phenol, 2,5-bis(1,1-dimethylethyl)-	15.85	59172	19.63	Phenolic compound
Silicic acid, diethyl bis(trimethylsilyl) ester	17.59	8628	2.86	Ester
Methyl 3-(1-pyrrolo)thiophene-2-carboxylate	17.91	9414	3.12	Ester

Table 4.37 : The suggested GC-MS result for *I. aquatica* leaf in distilled water (IA-LD) extract.

Compound name	Retention time	Area	Area (%)	Group
Corydaldine	3.18	22416	8.88	Alkaloid
Ethyl 4-(N-(2-nitro)benzylidene)amino-benzoate	5.80	8257	3.27	Ester
4-Hexenoic acid, 6-hydroxy-4-methyl-, methyl ester, (E)-	7.90	10313	4.09	Ester
Phenol, 2,4-bis(1,1-dimethylethyl)-	15.85	30304	12.00	Phenolic compound
1-Benzazirene-1-carboxylic acid, 2,2,5a-trimethyl-1a-[3-oxo-1-butenyl] perhydro-, methyl ester	17.00	8085	3.2	Organic acid
Silicic acid, diethyl bis(trimethylsilyl) ester	18.73	11194	4.43	Ester
3-Methylindole-2-carboxylic acid, 4,5,6,7-tetrahydro-, ethyl ester	18.78	12319	4.88	Ester

Table 4.38 : The suggested GC-MS results for *I. aquatica* stem in distilled water (IA-SD) extract.

Compound name	Retention time	Area	Area (%)	Group
7-Norcarancarbonic acid,methyl ester	9.109	8899	2.33	Ester
Cholest-5-ene-3,20-diol, 3-acetate, (3.beta.,20R)-	11.727	7522	1.97	Polyol
Carbonic acid, methyl phenyl ester	12.304	8864	2.33	Ester
1,2,4-Benzenetricarboxylic acid, 4-dodecyl dimethyl ester	12.498	9010	2.36	Ester
Bromopropylate	13.23	7471	1.96	Ester
4-Dimethylamino-3,5-dinitrobenzoic acid	13.384	9107	2.39	Organic acid
Phenol, 2,4-dichloro-6-nitro-	14.676	9012	2.36	Phenolic compound
2-Nitro-4-(trifluoromethyl)phenol	16.653	7842	2.06	Phenol
Anthranilic acid, N-methyl-, butyl ester	17.459	7508	1.97	Ester
2-Nitro-4-(trifluoromethyl)phenol	17.722	19577	5.14	Phenolic compound
Demecolcine	18.362	7379	1.94	Alkaloid

Table 4.39 : The suggested GC-MS result for *I. reptans* leaf in distilled water (IR-LD) extract.

Compound name	Retention time	Area	Area (%)	Group
2-Chloroethyl oleate	7.92	13682	4.85	Ester
Phenol, 3,5-bis(1,1-dimethylethyl)-	15.84	47000	16.64	Phenolic compound
Phenol, 6-methyl-2-[(4-morpholinyl)methyl]-	16.01	8893	3.15	Phenolic compound
Silicic acid, diethyl bis(trimethylsilyl) ester	19.83	9095	3.22	Ester

4.4 Total Phenol Content (TPC) and Total Flavonoid Content (TFC)

The TPC was calculated based on the ascorbic acid standard curve generated, $y = 0.004x + 0.391$, $R^2 = 0.878$ (Appendix B) and was expressed as mg GAE/g. TFC was obtained from the quercetin standard curve, $y = 0.001x + 0.028$, $R^2 = 0.959$ (Appendix C) and expressed as mg QE/g.

Figure 4.1 and Figure 4.2 shows the TPC and TFC for all *I. aquatica* and *I. reptans* extract. A higher TPC and TFC can be observed in the non-polar extracts compared to the polar extracts for both plants. The highest TPC was observed in IR-LC (357.25 ± 3.88 mg GAE/g) followed by IR-SH (206.12 ± 9.85 mg GAE/g). TFC was the highest in IR-SH (3741.17 ± 214.14 mg QE/g) followed by IR-LC (3104.29 ± 135.48 mg QE/g). These indicate that the non-polar extracts of *I. aquatica* and *I. reptans* had a higher level of phenolic contents compared to the polar-extracts.

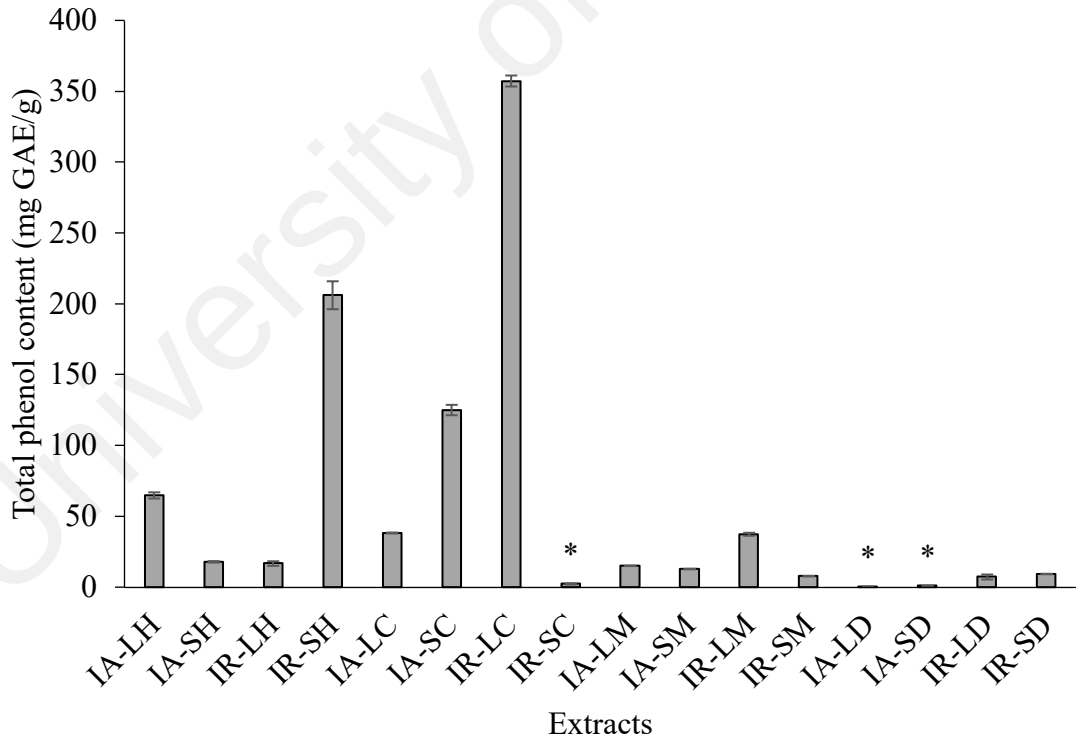

Figure 4.1 : TPC for *I. aquatica* and *I. reptans* extracts. All data were expressed as mean ± S.E. (n=3). Asterisk (*) indicates that the TPC was too small to be observed on the graph. All data were expressed as mean ± S.E. (n=3).

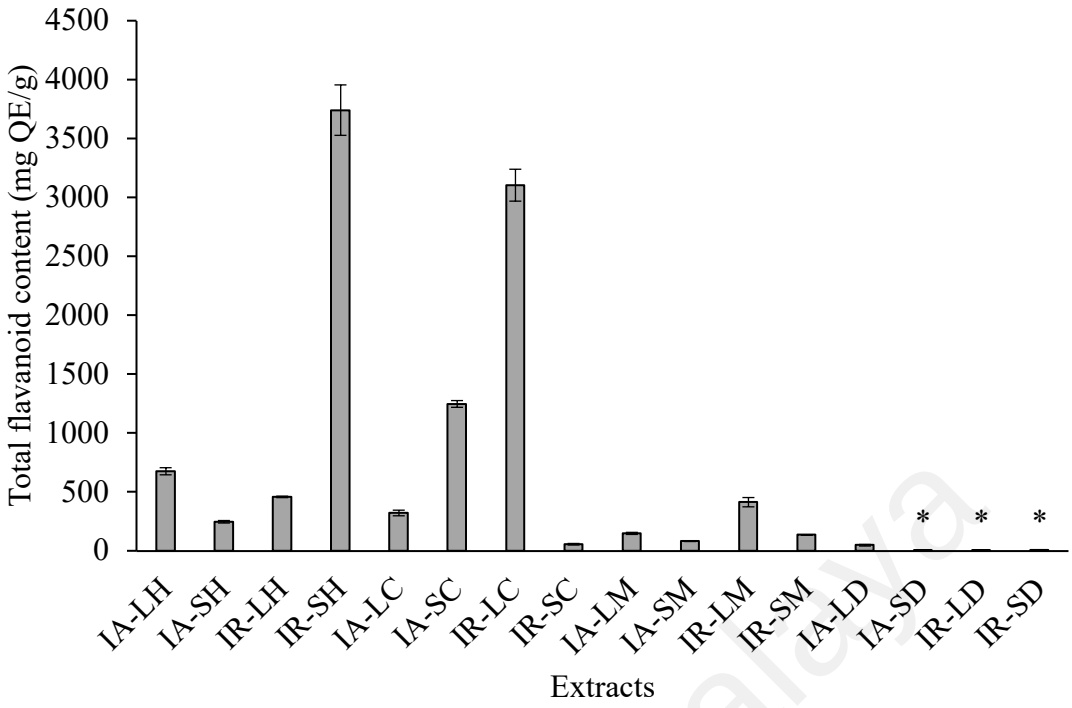

Figure 4.2 : TFC for *I. aquatica* and *I. reptans* extracts. All data were expressed as mean ± S.E. (n=3). Asterisk (*) indicates that the TFC was too small to be observed on the graph. All data were expressed as mean ± S.E. (n=3).

4.5 Antioxidant Assays

4.5.1 2,2-Diphenyl-1-picrylhydrazyl (DPPH) Radical Scavenging Assay

The ability to scavenge 2,2-Diphenyl-1-picrylhydrazyl (DPPH) radicals by *I. aquatica* and *I. reptans* extracts were measured by the changes in the absorbance of the samples at 517 nm. A colour change could also be observed in the sample solution turning from deep purple colour into bright yellow upon incubation with the extracts and DPPH solution after a few minutes.

Figure 4.3 shows the DPPH scavenging activity for all *I. aquatica* and *I. reptans* extract at 800 μg/ml. Ascorbic acid was used to generate a standard curve with an IC_{50} of > 19 μg/ml (Appendix F). Based on Figure 4.3, it can be observed that the methanolic extracts showed the greatest DPPH scavenging activity followed by distilled water extracts and chloroform extracts of both plants except in *I. reptans* stem. The highest DPPH scavenging activity was observed in IA-LM (71.4 ± 1.3 %) with IC_{50} at

> 200 µg/ml and IA-SM (62.5 ± 1.4 %) with an IC_{50} at > 300 µg/ml. These two extracts showed significantly similar scavenging activity as ascorbic acid at 800µg/ml. These show that the *I. aquatica* methanolic extracts had a high scavenging activity. It also indicates that the *I. aquatica* methanolic extracts had a higher level of phytochemical compounds to scavenge the DPPH radical compared to other extracts. Both IA-LM and IA-SM showed similar scavenging activity ($P < 0.05$) against DPPH which indicates that plant parts have no significant differences in their DPPH radical scavenging activity.

Distilled water extracts and chloroform extracts showed DPPH scavenging activity < 50 %. All hexane extract and IR-S had negligible scavenging activity against DPPH.

Lower scavenging activity seen in *I. reptans* extracts compared to *I. aquatica* extracts showed that *I. aquatica* has a better scavenging ability against DPPH. It also infers that both plants differ significantly in their phytochemical compounds content.

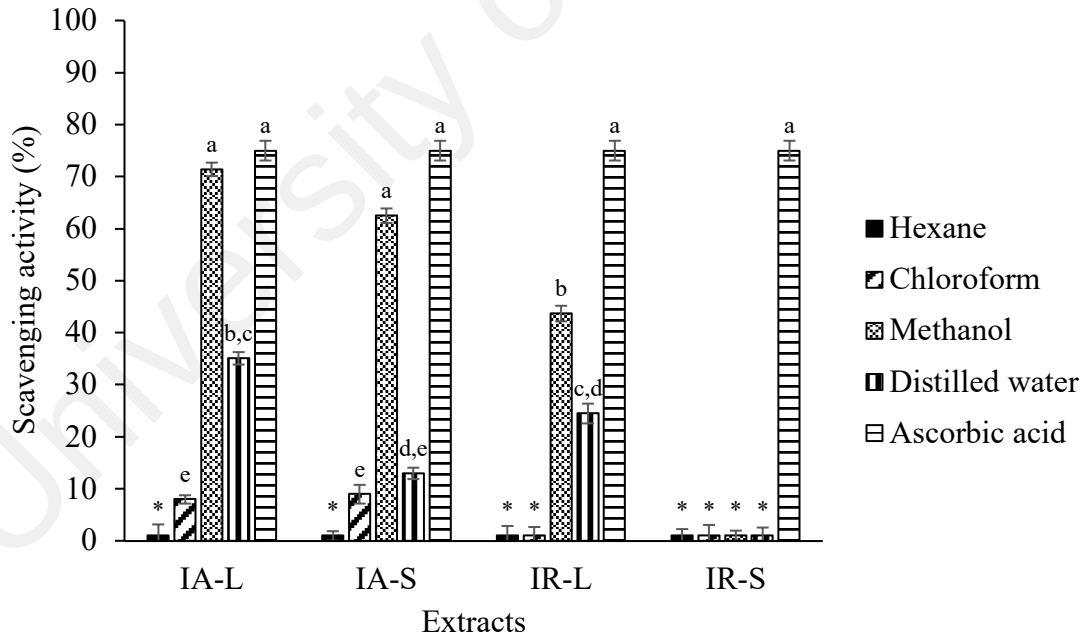

Figure 4.3 : The DPPH scavenging assay for the extracts of *I. aquatica* and *I reptans* at 800 µg/ml at 517 nm. All data were expressed as means ± S.E. (n=3). Means with differ lowercase letters (a, b, c, d, & e) are significantly different from each other ($P < 0.05$, using one-way ANOVA test followed by Duncan's post hoc multiple comparison test). Means with asterisk (*) indicates the activity is negligible.

4.5.2 Ferric Reducing Antioxidant Power (FRAP) Assay

Antioxidant reducing power of the *I. aquatica* and *I. reptans* extracts were measured as a change in the absorbance at 593 nm and were expressed as FRAP values. A colour change was also observed in the sample solutions, turning from colourless to blue or purple upon incubation with FRAP reagent and the extracts. Table 4.40 shows the FRAP values for the extracts expressed as mmol Fe^{2+}/ g dry extract ± S.E. Ferrous sulphate was used to construct the standard reference curve at $y = 0.2088x - 0.267, R^2 = 0.985$ where y is absorbance and x is concentration (Appendix G).

From the table, the non-polar extracts had a higher FRAP values compared to polar extracts for both plant extracts. The highest FRAP was observed in IR-SH (2.60 ± 0.01 mmol Fe^{2+}/g) followed by IR-LC (0.83 ± 0.02 mmol Fe^{2+}/g) and IA-SC (0.44 ± 0.07 mmol Fe^{2+}/g). Higher FRAP value indicates that the extract contains more antioxidants to reduce more ferric ion in the FRAP reagent. As the non-polar extracts for both plants had higher FRAP values, more antioxidants were present in these extracts compared to the polar extracts.

Table 4.40 : The FRAP value for *I. aquatica* and *I. reptans* extracts. All data were expressed as mean ± S.E. (n=3).

Extracts	FRAP value (mmol Fe2+/g)			
	Hexane	Chloroform	Methanol	Distilled water
IA-L	0.42 ± 0.11	0.19 ± 0.06	0.08 ± 0.10	0.20 ± 0.08
IA-S	0.14 ± 0.07	0.44 ± 0.07	0.09 ± 0.07	0.07 ± 0.11
IR-L	0.21 ± 0.02	0.83 ± 0.02	0.39 ± 0.09	0.28 ± 0.06
IR-S	2.60 ± 0.01	0.25 ± 0.02	0.06 ± 0.01	0.20 ± 0.01

IA-L : *I. aquatica* leaf
IA-S : *I. aquatica* stem
IR-L : *I. reptans* leaf
IR-S : *I. reptans* stem

4.5.3 Ferrous Ion (Fe^{2+}) Chelating Activity Assay

The ability of the extracts to chelate Fe^{2+} was measured through the changes in the absorbance reading at 562 nm. EDTA.Na_2 was used as a standard with and IC_{50} at > 100 µg/ml (Appendix H). A reduction in the red-coloured solution upon incubation with the extracts indicated chelating activity in the solution. Figure 4.4 displays the ability of each extract to chelate Fe^{2+} at 100 µg/ml.

The Fe^{2+} chelating ability for all *I. aquatica* and *I. reptans* extract was found to be comparatively low (< 40 %). Only three extracts showed significant activity; IA-SD (39.7 ± 4.0 %), IR-LD (24.1 ± 1.0 %) and IR-SC (10.3 ± 1.0 %). Other extracts showed negligible activity against Fe^{2+} chelation. Low chelating activity in the *I. aquatica* and *I. reptans* extracts indicates that the antioxidants present in the extracts had low iron binding ability. Therefore, less Fe^{2+} were chelated by the antioxidants present in the extracts.

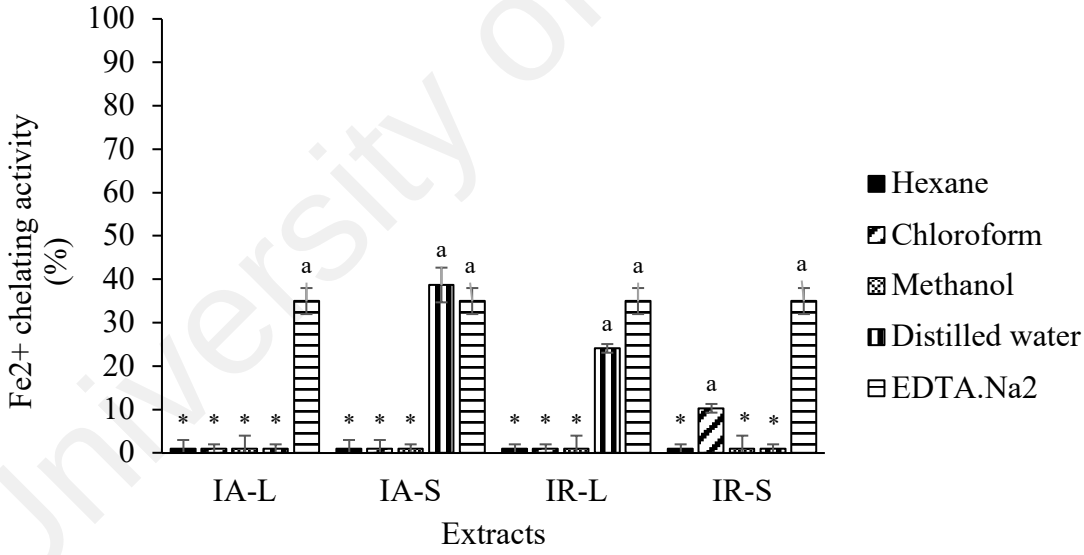

Figure 4.4 : The inhibition of $FeCl_2$ by different extracts of *I. aquatica* and *I reptans*. at 100 µg/ml at 562 nm. All data were expressed as means ± S.E. (n=3). Means with differ lowercase letters (a) are significantly different from each other ($P < 0.05$, using one-way ANOVA test followed by Duncan's post hoc multiple comparison test). Means with asterisk (*) indicates the activity is negligible.

4.5.4 Hydrogen peroxide, H_2O_2 scavenging assay

The extracts ability to scavenge hydrogen peroxide, H_2O_2 was measured as a reduction in the absorbance reading at 230 nm. Ascorbic acid was used as a standard with an IC_{50}

of > 100 µg/ml (Appendix I). Figure 4.5 describes the H_2O_2 scavenging activity of all extract at 100 µg/ml.

It was observed that IR-LH (55.5 ± 2.0 %) gave the highest H_2O_2 scavenging activity. The other extracts showed lower H_2O_2 scavenging activities (< 50 %) while IA-SC, IA-SM and IR-SM produced no comparable activity against H_2O_2 scavenging. The leaf extracts of *I. aquatica* and *I. reptans* tend to have a higher scavenging ability in the non-polar extracts. Conversely, the stem extracts of *I. aquatica* and *I. reptans* had a higher scavenging ability in the polar extracts. Since, the leaf and stem extracts of *I. aquatica* and *I. reptans* showed different scavenging activity in different solvent polarity, this indicates that different plant parts showed varied effectiveness in transforming H_2O_2 into water. This also denotes that different phytochemical compounds were involved in scavenging H_2O_2 in the leaf and stem extracts of *I. aquatica* and *I. reptans.*

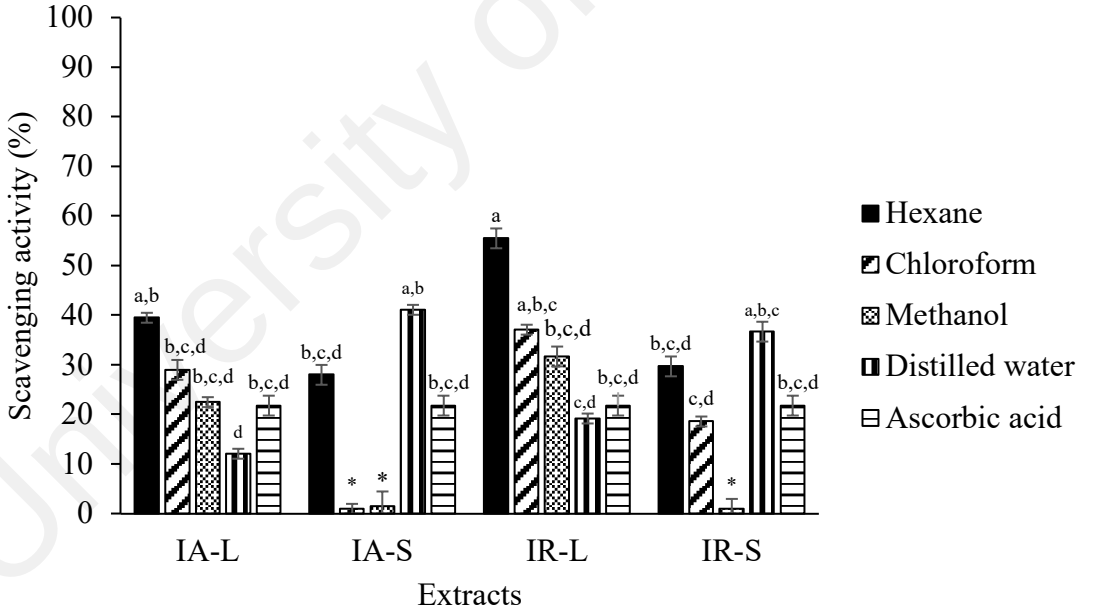

Figure 4.5 : The hydrogen peroxide, H_2O_2 scavenging activity by different extracts of *I. aquatica* and *I. reptans* at 100 µg/ml at 230 nm. All data were expressed as means ± S.E. (n=3). Means with differ lowercase letters (a, b, c, & d) are significantly different from each other ($P < 0.05$, using one-way ANOVA test followed by Duncan's post (cont.) hoc multiple comparison test). Means with asterisks (*) indicates that the scavenging activity is negligible.

4.6 Acetylcholinesterase Inhibitory Assay

The capability to inhibit AChE was measured as a reduction in the absorbance at 410 nm. Berberine was used as a standard with an IC_{50} of > 5 µg/ml (Appendix J). Figure 4.6 – 4.9 showed the AChE inhibition by all extracts.

It was observed that for both plant species, the chloroform extracts (70 – 100 %) showed the highest AChE inhibition followed by the methanolic extracts (< 30 %), the distilled water extracts (< 20 %) and the hexane extracts (< 20 %) for both *I. aquatica* and *I. reptans*. This indicates that the chloroform extracts of *I. aquatica* and *I. reptans* are the most effective at inhibiting AChE and the phytochemical compounds present in the chloroform extracts of both plants showed anticholinesterase properties. *I. aquatica* and *I. reptans* showed relatively similar inhibition against AChE. However, IR-SM observed a negligible activity against AChE inhibition.

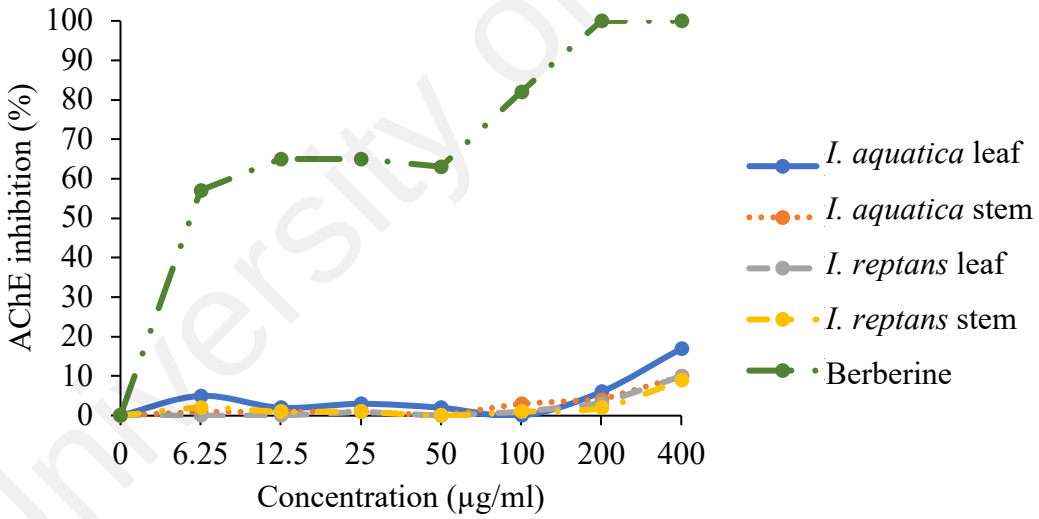

Figure 4.6 : The inhibition of *I. aquatica* and *I. reptans* hexane extracts on the activity of acetylcholinesterase measured at 410 nm. All data were expressed as means ± S.E. (n=3).

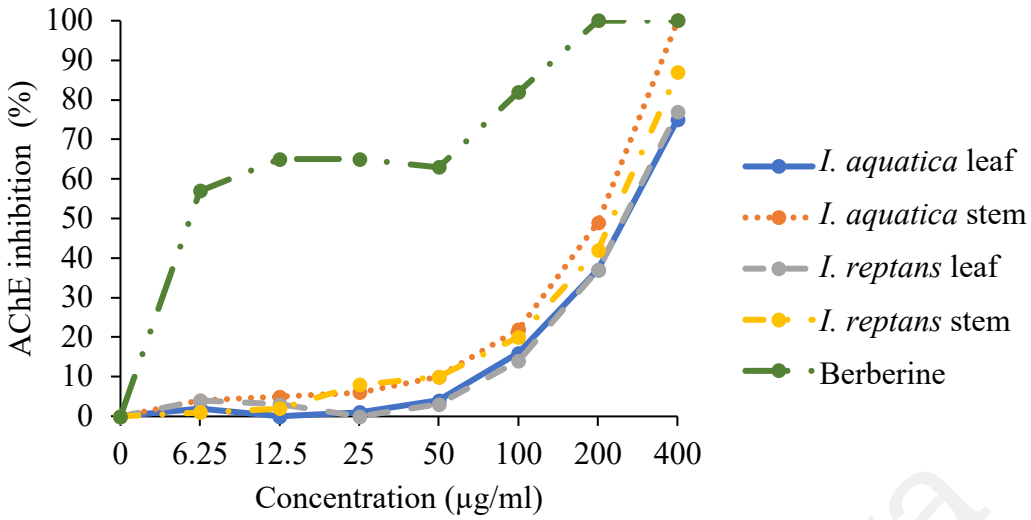

Figure 4.7 : The inhibition of *I. aquatica* and *I. reptans* chloroform extracts on the activity of acetylcholinesterase measured at 410 nm. All data were expressed as means ± S.E. (n=3).

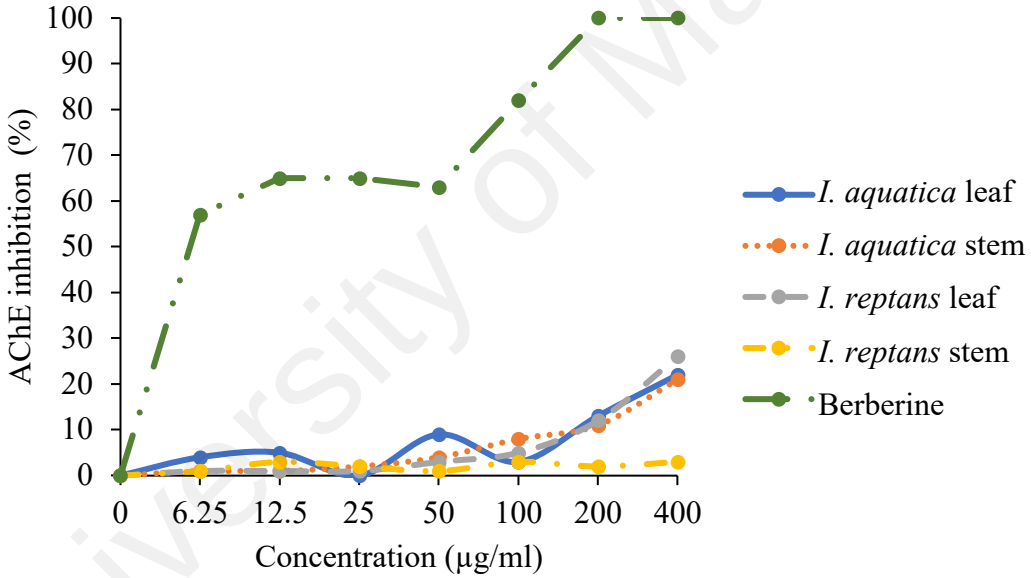

Figure 4.8 : The inhibition of *I. aquatica* and *I. reptans* methanolic extracts on the activity of acetylcholinesterase measured at 410 nm. All data were expressed as means ± S.E. (n=3).

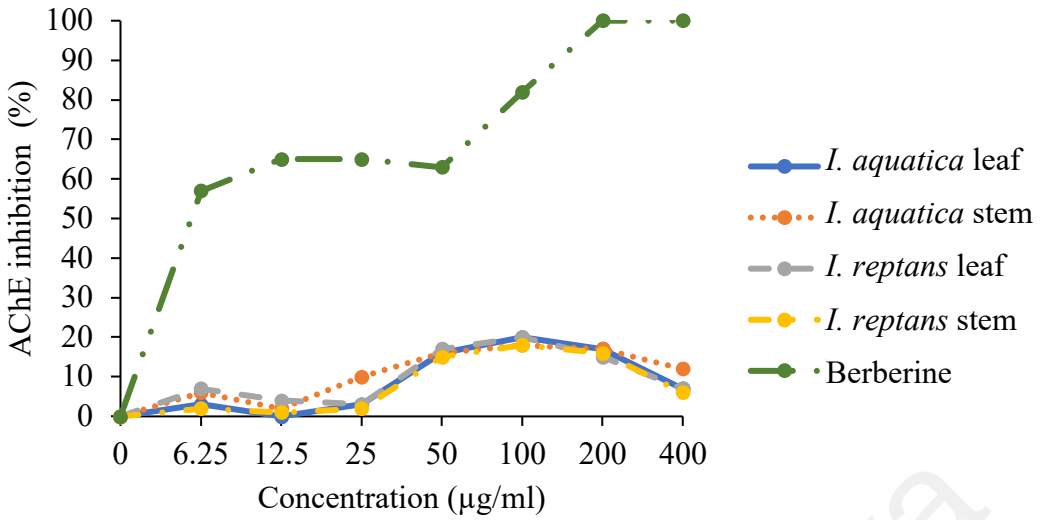

Figure 4.9 : The inhibition of *I. aquatica* and *I. reptans* distilled water extracts on the activity of acetylcholinesterase measured at 410 nm. All data were expressed as means ± S.E. (n=3).

4.7 Pearson Correlation Coefficient (R^2) Analysis

Positive value ($0 < R^2 \leq 1$) indicates there is a correlation between tested assays while negative value ($0 \leq R^2 \leq -1$) means there is no correlation between the assays. Table 4.41 shows the Pearson correlation coefficient (R^2) analysis for all the assays carried out.

Based on the table, it can be seen that the TPC assay has a positive correlation with the TFC assay ($R^2 = 0.961$, $P < 0.05$), showing that the level of the phenolic contents correlates with the level of the flavonoid contents present in the extracts of *I. aquatica* and *I. reptans*. Besides that, The FRAP assay ($R^2 = 0.751$, $P < 0.05$), the Fe^{2+} chelating assay ($R^2 = 0.622$, $P < 0.05$) and the cholinesterase inhibitory assay ($R^2 = 0.636$, $P < 0.05$) also have positive correlations with TPC assay indicating that the level of the phenolic compounds found present in the extracts of *I. aquatica* and *I. reptans* correlates with the activities seen in the assays. On the contrary, the DPPH assay ($R^2 = -0.523$, $P < 0.05$) and H_2O_2 assay ($R^2 = 0.026$) saw no correlation with TPC assay, indicating that the activities observed in the assays were not due to the action of the phenolic compounds present in the extracts of *I. aquatica* and *I. reptans.* Other phytochemical compounds might be responsible for the activity observed in the assays.

Table 4.41 : The Pearson correlation coefficient test for all seven assays carried out. All data were expressed as means ± S.E. (n=3).

Assay	Pearson correlation (R^2)					
	TPC	**TFC**	**DPPH**	**FRAP**	**Fe^{2+} chelating**	**H_2O_2 scavenging**
TPC		0.961^{**}				
DPPH	-0.523^{**}	-0.470^{**}				
FRAP	0.751^{**}	0.725^{**}	-0.737^{**}			
Fe^{2+} chelating	0.622^{**}	0.622^{**}	-0.431^{**}	0.401^{**}		
H_2O_2 scavenging	0.026	−0.021	−0.145	0.051	−0.045	
Cholinesterase inhibitory	0.636^{**}	0.623^{**}	0.021	0.177	0.257^{*}	0.085

** correlation is significant at the 0.05 level (1-tailed)

* correlation is significant at the 0.01 level (1-tailed)

CHAPTER 5 : DISCUSSION

5.1 Extraction of Plant Materials

Extracting phytochemical contents requires several considerations to ensure a proper extraction. Various factors have been identified to influence the extraction process such as pre-treatment and extraction process, solvents, temperature and time (Mojzer *et al.,* 2016). For instance, different pre-treatment processes such as milling, grinding, drying or freeze-drying can affect the phytochemical contents during the extraction process. Drying has been found to cause plant samples to be exposed to Maillard reaction (Sulaiman *et al.,* 2011). Maillard reaction or non-enzymatic browning produce compounds that enhance the antioxidant activity in the plant, consequently leading to overestimation of the polyphenolics and antioxidant activity of the plant (Sulaiman *et al.,* 2011; Phisut & Jiraporn, 2013). In the experiment, air-drying was chosen as it helped to preserve heat-labile compounds from degenerating (N, 2015). Grinding was carried out to ensure effective extraction and separation of compounds. Small particles improved surface area to volume ratio which provided more interactions between the compounds to be extracted and the solvent used, therefore enhancing the extraction process.

The type of solvent used also influence the extraction yield as each compound has its own chemical profile such as its solubility in solvent (Altemimi *et al.,* 2017). Polar solvents are effective in extracting polyphenols due to the strong hydrogen bond between the antioxidant polar sites and the solvent (Sultana *et al.,* 2009; Thouri *et al.,* 2017).

In the experiment, the highest yield was seen in the methanolic extracts for both *I. aquatica* and *I. reptans*. Polar extracts were seen to be better at extracting phytochemical compounds in *I. aquatica* and *I. reptans*. Similar findings were observed in the extraction of *Salacia chinensis* root and *Datura metel* (Dhawan & Gupta 2017; Ngo *et al.,* 2017). However, a higher yield in methanolic extracts could also be due to some low and high molecular weight phenolic compounds and highly soluble carbohydrates and proteins

being simultaneously extracted during the process (Prasad *et al.,* 2005; Do *et al.,*2014). As can be seen in the TPC assay carried out, higher yield of the methanolic extracts of both plants had a lower level of TPC and lower antioxidant activites.

5.2 Thin Layer Chromatography (TLC) Analysis

Thin layer chromatography (TLC) was conducted to enable preliminary screening on the phytochemical compounds present in the *I. aquatica* and *I. reptans* extracts. It functions to separate sample fractions and allow the observation of chemical reactions progressions (Meyers & Meyers, 2008). Through treatments with reagents and the calculation of the R_f value, the nature of the phytochemical compounds can be characterised and identified. Polar compounds produce lower R_f values in TLC assay as they travel up slowly across the TLC plate due to their strong adsorptivity. TLC is convenient, cheap, simple, produce rapid results and has high sensitivity and reproducibility (Santiago & Strobel, 2013).

The effectiveness of the sample fractions separation can be manipulated by the mixtures of the solvents used during the process. Polar solvents exhibited greater eluting strength compared to the less polar solvent. Selecting an appropriate solvent will result in good selectivity in separating the sample mixtures. The TLC conducted for the *I. aquatica* and *I. reptans* extracts observed more separation of compounds in 10 % methanol as solvents. This indicates that 10 % methanol, a polar solvent, was better at separating the *I. aquatica* and *I. reptans* extracts compared to chloroform, which is non-polar solvent.

Besides that, a greater amount of phytochemical compounds observed in non-polar extracts showed that the phytochemical compounds present are non-polar. For instance, terpene is known to be non-polar and very soluble in organic solvent such as hexane and chloroform (Jiang *et al.,* 2016). It is made up of hydrocarbon chains and can undergo modifications to generate a more complex terpenoid (Jiang *et al.,* 2016). Several studies

have shown the presence of terpenes in *I. aquatica* extracts (Shim, 2012; El-Sawi *et al.,* 2017).

5.3 Liquid Chromatography-Mass Spectrometry (LC-MS) and Gas Chromatography-Mass Spectrometry (GC-MS) Analyses

Liquid Chromatography-Mass Spectrometry (LC-MS) is commonly used to detect non-volatile and thermally labile compounds. LC-MS comprises of an autosampler, a high-performance liquid chromatography (HPLC) system, an ionization source and a mass spectrometer (MS) (Korfmacher, 2005). Ionization source functions as an interface between the liquid phase in the HPLC system and the gas phase in the mass spectrometer (Korfmacher, 2005). There are multiple types of ionization source that are available such as electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI). However, depending on the choice of the flow rates and mobile phase modifiers used, their performance may be restricted (Hanold *et al.,* 2004). The mobile phase in LC-MS is liquid. Water and methanol or acetonitrile are the common eluents used as a mobile phase in LC-MS (Korfmacher, 2005).

Quercetin, tannic acid and gallic acid are phenolic compounds usually found in plants. Phenylalanine is a type of amino acids that are important in many biological processes (Yoo *et al.,* 2013). Flavonoids, various phenolic compounds and isoflavonoids are some of the phenylalanine-derived compounds involved in plant defense mechanism, ultraviolet (UV) protection and signalling (Yoo *et al.,* 2013). The LC-MS conducted showed the presence of quercetin, phenylalanine and tannic acid in the extracts of both *I. aquatica* and *I. reptans*. Chu *et al.* (2000) and Lawal *et al.* (2017) documented the presence of various quercetin derivatives in *I. aquatica*. Men *et al.* (2010) and Doka *et al.* (2014) detected the presence of phenylalanine in *I. aquatica*.

Gas Chromatography-Mass Spectrometry (GC-MS) is usually used to detect volatile and semi-volatile compounds. Similar to LC-MS, the system involved a sampler, gas chromatography (GC) and detector, mass spectrophotometer (MS) (Hussain & Maqbool, 2014). Coupling of the two system enables simultaneous identification of the structural information of multiple compounds with great precision (Sneddon *et al.,* 2007). In GC-MS, the mobile phase used involved a carrier gas such as helium, nitrogen or argon. Different carrier gas used will have a different effectivity in separating the compounds present. The carrier gas will push the sample through a capillary column which contains a stationary phase, usually in a solid form, to separate the compounds in the sample (Hussain & Maqbool, 2014). Different compounds move at a different rate (retention time) through the column thus separating the sample into compound fragments and this is detected by the MS (Hussain & Maqbool, 2014). MS detects the fragments through ionization in which they will be organized according to mass to form a fragmentation pattern (Hussain & Maqbool, 2014). Fragmentation pattern and retention time are characteristic to each compound (Hussain & Maqbool, 2014). This enables the precise identification of a compound in a sample (Sneddon *et al.,* 2007).

The GC-MS carried out suggested the presence phenolic compounds, alkaloids, terpenes, sterol, esters, polyols, organic acids and fatty acids in all five extracts of *I. aquatica* and *I. reptans*. Sterols are a component of the cell membranes and involved in multiple biological functions. For instance, sterols help in plant growth and responses to biotic and abiotic pressures (Ferrer *et al.,* 2017). In plant, sterols can exist in free forms, as steryl esters, steryl glycosides and acyl steryl glycosides.

Alkaloids are heterocyclic nitrogen compounds known to have numerous pharmacological properties such as antimicrobial and antioxidant activities (Tiong *et al.,* 2013; Marutescu *et al.* 2017). Alkaloids can be divided into multiple classes which include pyrrolidine, isoquinolone, quinoline, indole, imine, imidazole, peperidine and pyridine (Iriti & Faoro, 2009).

Polyols are reduced ketose and aldehyde forms of sugars found in plants (Noiraud *et al.*, 2001). They are involved in the protection against biotic and abiotic stresses such as salt and photooxidative stress besides having antioxidant properties (Ehrenshaft, 2002).

5.4 Total Phenol Content (TPC) and Total Flavanoid Content (TFC) Assays Analyses

Folin-Ciocalteu's assay was adopted in performing the total phenol content (TPC) assay. The process involves electron reduction reactions between phenolic compounds and Folin-Ciocalteu's reagent under alkaline conditions. This resulted in colour changes of the reagent from yellow to blue solution (Sánchez-Rangel *et al*., 2013).

Total flavanoid content (TFC) quantification was performed based on the aluminium colorimetric assay which utilizes the formation of complexes between aluminium chloride with the keto- or hydroxyl groups of flavonoids under acidic conditions (Bhaigyabati *et al*., 2014).

The TPC and TFC assays for *I. aquatica* and *I. reptans* revealed that non-polar extracts had significantly higher phenolic and flavonoid contents compared to the other extracts. Similarly, the TLC assay performed showed more phenolic compounds in non-polar extracts compared to polar extracts of both plants. The LC-MS test done on five polar extracts also saw no to minimal traces of gallic acid and tannic acid and considerable level of quercetin in all five *I. aquatica* and *I. reptans* polar extracts sent, signifying low phenolic compounds level in these extracts.

A strong correlation was seen between the TPC and TFC ($R^2 = 0.961$, $P < 0.05$) assays for both plant extracts.

Nonetheless, the TFC was seen to be at a higher level compared to the TPC for both *I. aquatica* and *I. reptans* extracts. Flavonoid is one of the types of phenolic compounds.

The TPC level should be higher compared to the TFC level. An alternative is to replace quercetin with catechin as standard in the TFC assay (Singh *et al.,* 2015).

5.5 Antioxidant Analysis of Plant Extracts

There are multiple antioxidant assays available that can be used in antioxidant analysis. Each assay operates differently and depending on their usage, these assays can provide useful information on the antioxidant properties of the samples to be examined. Although a single assay can be employed, a combination of several assays may help in delivering a comprehensive understanding on the antioxidant profile of a sample (Genskowsky *et al.,* 2016). All four antioxidant assays were chosen to provide information on the antioxidant activities of *I. aquatica* and *I. reptans* in targeting free radicals forming agents.

5.5.1 2,2-Diphenyl-1-Picrylhydrazyl (DPPH) Assay Analysis

2,2-Diphenyl-1-Picrylhydrazyl (DPPH) assay is a type of test used to determine the antioxidant ability of individual phenolics, food and biological samples (Gülçin, 2012). It utilizes the reduction of the DPPH radical – a stable organic nitrogen radical with deep purple colour, in alcoholic solution by an antioxidant into diphenyl-picrylhydrazine which is yellow in colour (Gülçin, 2012). This indicates the hydrogen donating ability of the extracts and is measured by the change in absorbance of a sample by using a spectrometer (Rangasamy & Namasivayam, 2014).

A negative correlation between TPC and DPPH assays ($R^2 = -0.532$, $P < 0.05$) obtained showed that phenolic compounds did not contribute to the scavenging activity of the extracts. The higher TPC level in chloroform extracts of *I. aquatica* and *I. reptans* were observed to have a low DPPH scavenging activity. Other phytochemical compounds might be responsible for the high scavenging activity displayed in the methanolic extracts

such as amino acids. The LC-MS conducted observed a considerable amount of phenylalanine in the methanolic and distilled water extracts of both plants. Undertaking a phenylalanine content assay could be done to determine whether phenylalanine does contribute to the radical scavenging activity of these extracts. Meda *et al.* (2005) conducted a study on Burkina Fasan honey and found out that proline contributed strongly to the high radical scavenging activity observed. Similar result was seen in rapeseed protein hydrolysates in which high amino acid contents correlates with high antioxidant activity seen (Wang *et al.,* 2016).

Besides that, the polar extracts of *I. aquatica* and *I. reptans* were observed to have a better scavenging activity compared to the non-polar extracts. This indicates that the phytochemical compounds present in polar extracts of both plants have a higher hydrogen donating ability compared to those in non-polar extracts. Similar result was obtained in a previous study done on *I. aquatica* by Prasad *et al.* (2005).

No significant differences ($P < 0.05$) were observed in the DPPH scavenging activities of *I. aquatica* leaf and stem methanolic extracts which indicates they have similar scavenging ability. Nonetheless, Huang *et al.* (2005) saw differences in the radical scavenging activity between *I. aquatica* plant parts. The highest radical scavenging activity was observed in the ethanolic stem extract of *I. aquatica* compared to its leaf extracts (68.3 ± 0.7 % vs 63.9 ± 0.1 %). However, the extraction process, geographical locations and growth conditions might influence the differences in the phytochemical contents found in plants. Nevertheless, limited research has been done on the effects of these factors on *I. aquatica* and *I. reptans*.

5.5.2 Ferric Reducing Antioxidant Power (FRAP) Assay Analysis

Ferric Reducing Antioxidant Power (FRAP) assay defines an antioxidant reducing power through its ability to donate electrons and reduce ferric ion, Fe^{3+} to ferrous ion,

Fe^{2+} and is expressed as a FRAP value (Chen *et al.,* 2010; Benzie & Devaki, 2017). It provides a "quantitative correlation between the antioxidant capabilities and the redox potentials for reductant antioxidants" (Cheng & Li, 2004).

Reduction of Fe^{3+} to Fe^{2+} produces a blue-coloured complex, ferrous-tripyridyltriazine which can be measured by the change in absorbance at 593 nm (Benzie & Strain, 1996; Md. Irshad *et al.,* 2012). The FRAP assay is quick, simple and rapid (Benzie & Devaki, 2017).

A positive correlation with TPC assay ($R^2 = 0.725$, $P < 0.05$) indicates that the antioxidant reducing power in the extracts were contributed by the phenolic compounds. Higher FRAP values obtained in the non-polar extracts for both *I. aquatica* and *I. reptans* correlate with the high TPC observed in the extracts. A higher level of phenolic compounds in the non-polar *I. aquatica* and *I. reptans* extracts enabled more reduction of Fe^{3+} to Fe^{2+} through the donation of electrons, thereby forming more ferrous-tripyridyltriazine complex in the solution. This increased the absorbances for both plant extracts which were translated into higher FRAP values.

A negative correlation with DPPH assay ($R^2 = -0.737$, $P < 0.05$) means that the antioxidants involved in the activity of both assays were different significantly.

5.5.3 Fe^{2+} Chelating Assay Analysis

Iron is inevitably important in carrying out and maintaining a proper cell metabolism. Almost all cell directly or indirectly implicated in the cellular iron metabolism involving redox-reaction machinery (Bresgen & Eckl, 2015). Without appropriate regulation, this cellular machinery can be compromised, generate ROS and induce cellular damages (Galaris & Pantopoulos, 2008, Dixon & Stockwell, 2013). For instance, iron-catalyzed ROS is linked to cell necrosis, accumulation of intracellular ROS and lipid peroxidation (Gammella *et al.,* 2016). Antioxidants help in maintaining a proper level of ROS by acting

as iron chelators. They function through binding with the freely available iron in the body thereby forming stable complexes that can be passed out of the body through faeces or urine (Adjimani & Prince Asare, 2015). Consequently, this reduces the formation of ROS and prevent oxidative stress.

In Fe^{2+} chelating activity assay, the ability of antioxidant to chelate Fe^{2+} was measured. Ferrozine will react with ferrous chloride, $FeCl_2$, forming red colour complexes (Md Yusof *et al.,* 2013). Chelation of Fe^{2+} by antioxidants can be observed through decolorization of the red colour solution and can be measured using a spectrometer.

Positive correlations observed between Fe^{2+} chelating assay and the TPC ($R^2 = 0.622$, $P < 0.05$) and FRAP assay ($R^2 = 0.401$, $P < 0.05$) show that the chelating activities seen were due to the action of the phenolic compounds present in the extracts of *I. aquatica* and *I. reptans*.

Low chelating activities of Fe^{2+} by the all extract of *I. aquatica* and *I. reptans* denote that the phenolic compounds present in the extracts had low iron binding ability. This can be attributed to several factors. For instance, the antioxidant acitivity of a phenolic compound can be influenced by the number, position and binding site of the hydroxyl group and type of substituents on the aromatic ring (Andjelković *et al.,* 2006). Adjimani and Prince Asare (2015) found that phenolic compounds such as the 2,3-dihydroxybenzoic acid (DHBA) and caffeic acid had low iron binding ability owing to the structures bearing catechol groups. Previous studies showed that fractionated methanol extract of *I. aquatica* contained dihydroxybenzoic acid pentoside and di-pentoside and caffeic acid moeities (Lawal *et al.,* 2016; Gad *et al.,* 2017). However, Huang *et al.* (2005) reported that methanolic *I. aquatica* extracts had showed Fe^{2+}chelating activity.

5.5.4 Hydrogen Peroxide Assay Analysis

Hydrogen peroxide, H_2O_2 is a type of ROS that is "stable, diffusible and a non-radical oxidant" (Gough & Cotter, 2011). In low amount, H_2O_2 serve to regulate body metabolism and ensure proper functioning (Gough & Cotter, 2011). However, if not properly regulated, the molecule can oxidize numerous cellular components, inducing cell injuries and imposing damages to the DNA and lipid structures (Gough & Cotter, 2011). H_2O_2 scavenging assay measures the ability of antioxidants to transform H_2O_2 into water, H_2O through the donation of hydrogen atoms or electrons thereby reducing the concentration of H_2O_2 in the solution (Adjimani & Prince Asare, 2015). This is then scored by measuring the reduction in the absorbance of the sample at 230 nm.

No correlations seen between H_2O_2 scavenging assay and TPC and DPPH assays indicate that the antioxidants that contributed to the H_2O_2 scavenging activity were different.

The leaf and stem extracts of *I. aquatica* and *I. reptans* were shown to have different scavenging activities against H_2O_2. The leaf extracts were more effective in scavenging H_2O_2 in non-polar extracts while stem extracts had higher H_2O_2 scavenging activities in polar extracts for both plants. However, Dhanasekaran *et al.* (2015) observed differently in which the hydroalcoholic leaf extracts of *I. aquatica* had a higher H_2O_2 scavenging activity, attributed to the high TPC seen in the extract. This could be attributed to the geographical locations and growth conditions that might influence the differences in the phytochemical contents found in plants.

5.6 Anticholinesterase Assay Analysis

Acetylcholinesterase (AChE) enzyme belongs to the one of the two types of cholinesterase enzymes. In AD, overexpression of this enzyme has been found to be one of the main pathologies underlying the development of the disease. Hence targeting the

system by inhibiting the action of the enzyme may help in restoring the normal functioning of the mechanism. AChE inhibitory assay or Ellman assay is a cheap and convenient method in diagnosing, therapeutic monitoring and in vitro kinetic investigation for the interaction between compounds and AChE enzyme (Worek *et al.,* 2012). The assay employs the coupling of reaction between the hydrolyzation of acetylthiocholine by AChE into thiocholine and the reaction between thiocholine and 5,5′-Dithiobis (2-nitrobenzoic acid) (DTNB) to form a yellow complex (Ellman *et al.,*1961). The reduction in the yellow complex formed indicates the ability of a compound to inhibit the action of AChE enzyme and can be measured by the reduction in the absorbance.

Berberine is an alkaloid which belongs to protoberberine group (Wang *et al.,* 2018). It can be found in many types of plant including *Hydrastis canadensis* and *Coptis chinensis* (Wang *et al.,* 2018). Berberine is capable of inhibiting the action of AChE by inducing a conformational change in the structure of the enzyme therefore halting the activity of the enzyme (Xiang *et al.,* 2009). Research has been done in optimizing berberine into potential AD drug due to its low cytotoxicity, high efficacy and low adverse side effects (Xiang *et al.,* 2009)..

Based on the assay, a correlation with the TPC assay ($R^2 = 0.636$, $P < 0.05$) showed that the phenolic compounds present in the extracts of both plants displayed AChE inhibitory properties. A higher TPC level in the chloroform extracts for both *I. aquatica* and *I. reptans* was shown to exhibit higher AChE inhibitions. On the contrary, a low TPC level in the polar extracts for both plant species had a lower AChE inhibition. These show that AChE inhibition was influenced by the level of the phenolic compounds and that the phenolic compounds present had anticholinesterase activities. Dhanasekaran *et al.* (2015) revealed that a higher TPC level observed in hyroalcoholic extract of *I. aquatica* correlates with a higher inhibition against AChE activity.

A few phenolic compounds have been known to show anti-AChE activity such as hydroxyphenylpyruvic acid, caffeic acid, gallic acid, chlorogenic acid, quercetin and rutin (Szwajgier, 2015). These compounds particularly flavonoids, inhibit the action of AChE by forming hydrogen bonds between one of the aromatic rings bearing the OH groups with one of the amino acid residues in the active site of the enzyme (Roseiro *et al*., 2012; Ademosun *et al.,* 2016). This blocks the entry of other molecules into the enzyme active site thereby hindering the activity of the enzyme. *In silico* study demonstrated that chlorogenic acid and quercetin isolated from the hydroalcoholic extract of *I. aquatica* had considerable anti-AChE activity and was comparable to the activity of donepezil (Sivaraman *et al.,* 2014). The low concentration of quercetin (< 13.4 mg/ml) obtained through the LC-MS conducted in the polar extracts of *I. aquatica* and *I. reptans* could justify for the low AChE inhibition seen in these extracts. Moreover, phenolic compounds have variable efficiencies in inhibiting the action of AChE. Oboh *et al.* (2013) showed that caffeic acid had a greater inhibition against AChE than chlorogenic acid. Quercetin was found to be more effective in inhibiting the activity of AChE compared to rutin (Ademosun *et al.,* 2016).

CHAPTER 6 : CONCLUSION

I. aquatica and its relative, *I. reptans* were found to contain numerous nutrients and antioxidants such as terpenoids, phenolic compounds, flavonoid, alkaloid, polyols, organic acid, sterols and amino acids. However, *I. aquatica* was seen to have a higher antioxidant activity compared to *I. reptans*. Among all extracts, chloroform extracts for both *I. aquatica* and *I. reptans* showed the highest antioxidant activity except for in DPPH and hydrogen peroxide scavenging assays. This high antioxidant activity correlates with the high level of TPC observed in the chloroform extracts for both plant species. Nonetheless, other antioxidants and compounds such as alkaloids and amino acids might play a role in contributing to the scavenging activities seen in the DPPH and hydrogen peroxide assays. Further investigation can be done to determine whether the compounds display scavenging activities against DPPH radical and hydrogen peroxide. A correlation between TPC and acetylcholinesterase inhibition suggests phenolic compounds present in *I. aquatica* and *I. reptans* do possess anticholinesterase activities and are influenced by the level of the phenolic compounds present. Further optimization can be carried out to determine the active compounds that is responsible for the anticholinesterase activity seen. Therefore, good acetylcholinesterase inhibition in *I. aquatica* and *I. reptans* extracts suggest that the plants can be a potential source to be optimized into acetylcholinesterase inhibitor drug to treat Alzheimer's disease. Besides that, since there is a limited scientific information on the bioactivity of *I. reptans*, this research can help on elucidating the properties of the plant.

CHAPTER 7 : REFERENCES

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