Prospecting the Antidiabetic Potential of *Alisma orientale*

BY

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DISSERTATION

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T2DM	Type two diabetes mellitus
T1DM	Type one diabetes mellitus
TCM	Traditional Chinese medicine
GLUT4	Glucose type 4 receptor
HLA	Human leukocyte antigen
HBA1C	Glycated haemoglobin levels
IMA	Insulin mimetic agent
ISA	Insulin sensitizing agent
SGLT	Sodium-dependent glucose transport
CUHK	Chinese University of Hong Kong
HKCMMS	Hong kong chinese materia medica standards
TLC	Thin layer chromatography
HPLC	High performance liquid chromatography
HPTLC	High performance thin layer chromatography
PNPG	4-Nitrophenyl β-D-glucopyranoside
3T3-L1	Swiss albino mouse fibroblasts

DMEM	Dulbecco’s modified eagle’s medium
FBS	Fetal bovine serum
PBS	Phosphate buffered saline
CS	Calf serum
IBMX	Methyl isobutylxanthine
HEPES	Hydroxyethylpiperazine ethane sulfonic acid
2-DG	2-deoxy-D-glucose
CPM	Counts per minute
SGLT1	Sodium/D-glucose cotransporter 1
GLUT2	Glucose transporter type 2
BBMV	Double layer brush border membrane
TC	Total cholesterol
HDL-C	High density lipoprotein cholesterol
LDL-C	Low density lipoprotein cholesterol
TG	Triglyceride
MDA	Malondialdehyde

SOD	Superoxide dismutase
GSH-PX	Glutathione peroxidase
TNF-A	Tumor necrosis factor-A
IL-6	Interleukin-6
CRP	C-reactive protein
HE	Hematoxylin and eosin
IRS-1	Insulin receptor substrate type 1
AKT	Protein kinase B

ACKNOWLEDGMENTS

I would like to thank the members of my committee (Dr. Djaja Soejarto, Dr. Matthew Brady, Dr. Birgit Dietz, and Dr. James Graham) as well as my advisors Dr. Chun-Tao Che and the late Dr. Norman Farnsworth for their support of my research over the years, the production of this manuscript, and the development of me personally as a student in this field. I would also like to thank NIH for awarding me the National Research Service Award (NRSA) Pre-doctoral Fellowship (NIH 1F31AT00711701A1). None of this would have been possible without their help, and for that I am grateful.

A number of individuals, some already named, were instrumental in the collection of my data and need to be recognized specifically. Thanks to Dr. Soejarto for his assistance in obtaining a voucher herbarium specimen and authentication of our plant material. Thanks to Dr. Birgit Dietz for her guidance in my early days of cell culture. Thanks to Dr. Matthew Brady of the Department of Medicine-Section Endocrinology, Kovler Diabetes Center, University of Chicago for assistance in the glucose uptake and glycogen synthesis assays and showing me the impossible is possible in 3t3-L1 differentiation. Thanks also to Dr. Zhiqi Yin of the China Pharmaceutical University, Nanjing, China for conducting the animal study and determining the insulin mimetic activity of pure compounds. Thanks to Dr. Clara Lau of the Chinese University of Hong Kong, Hong Kong for conducting the glucose gut absorption assay. Thanks to Dr. Xiao-Qi Zhang for his assistance with isolation of the butanol-soluble partition. Thanks to Dr. Ming Zhao for instruction in a number of techniques and situations I encountered along the way. And lastly, thanks to Kelly Wescott and Holly Gunn for the illustrations.

JJG

CONTRIBUTION OF AUTHORS

Chapter 1 is a literature review with information on the species *A. orientale* as well as the disease diabetes and its current state of treatment. Chapter 2 is comprised of the authentication, extraction, and initial biological testing of *A. orientale*. As well as my own results from these processes it also contains results from the Chinese medicine experts at CUHK dealing with the authentication of the blue batch. Chapter 3 deals with the isolation of constituents from *A. orientale,* their structural characterization, and subsequent biological testing. Along with my own isolation work, nmr acquisitions and interpretations, and results of biological testing reported in this chapter it also contains the fruit of Dr. Xiao-Qi Zhang's labor who contributed in the isolation of compounds from the butanol fraction. Additionally, among the tested compounds which were pooled for this project were some isolates from Dr. Ming Zhao. Chapter 4 represents the work conducted with respect to *in vitro* 3t3-L1 glucose uptake studies. These were conducted by myself and Dr. Matthew Brady of the Department of Medicine-Section Endocrinology, Kovler Diabetes Center, University of Chicago. He and his team largely prepared the differentiated cells for these experiments, a significant and tedious aspect of this research. Chapter 5 consists of glucose gut absorption studies performed on my group of pooled isolated compounds and was performed by Dr. Clara Lau of the Chinese University of Hong Kong, Hong Kong. Chapter 6 deals with an animal study performed on an ethyl acetate partition of *A. orientale* I created and was performed by Dr. Zhiqi Yin of the China Pharmaceutical University, Nanjing, China. Chapter 7 is the conclusion of the totality of experiments present within the project including findings, importance, problems, and suggestions for future study.

STATEMENT OF THE PROBLEM

Diabetes mellitus is a group of devastating and potentially deadly diseases plaguing our society. It is currently the seventh leading cause of death in the United States, and all indications suggest that this number will not improve without the development of new and better drugs. Plants, especially those with a history of use to treat diabetes, are an excellent source for the discovery of lead compounds, which may be developed into a new generation of pharmaceuticals. Furthermore, the standardization by active ingredients of herbal preparations in use to treat diabetes is expected to lead to safe and effective treatment options.

The plant species *Alisma orientale* has a long history of traditional use and has been shown in previous studies to have antidiabetic properties both *in vivo* and *in vitro,* acting through a number of mechanisms potentially useful for the treatment of the disease. However, the antidiabetic principles of this plant were largely unknown.

This realization led to the questions: Can this species' antidiabetic activity be reproduced? Can the traditional use of this plant against diabetes be justified? What compounds cause the antidiabetic activity of this species as a whole? What mechanisms of action do they work through? How might this species be standardized to help ensure its antidiabetic activity?

It is our hope that in the course of investigation our findings will help to answer these questions and provide valuable research information towards the discovery of natural antidiabetic leads.

SUMMARY

This thesis concerns antidiabetic studies on the plant species *Alisma orientale* which has a long history of being used traditionally for this purpose. The aim of this research is to analyze the antidiabetic activity previously reported, to determine active principles that help to explain this species' biological activity as a whole, to provide justification for the traditional use of this plant, and to explore leads for the development of antidiabetic remedies, as well as the standardization of herbal complexes which utilize in part this species in the treatment or prevention of diabetes and its complications. Such information is potentially useful for a number of populations including traditional healers, researches, and practitioners.

The project employed various chemical separation techniques including extraction, partitioning, open column chromatography, thin-layer chromatography, and high-performance chromatography, as well as numerous detection and characterization methods including spray reagents, UV-Vis, mass spectrometry, and nuclear magnetic resonance spectroscopy. Additionally, various biological tests were conducted including an *in vivo* streptozotocin animal study, and *in vitro* glucose-gut absorption, glucose-uptake induction, and α-glucosidase inhibition assays.

In the end the project observed possible antidiabetic activity of *Alisma orientale* and its components *in vivo* and *in vitro* in the glucose uptake, glucose gut absorption, and α-glucosidase inhibition assays. These results may help to explain the bioactivity previously reported for this species and provide information for further study of this species with respect to its potentially antidiabetic properties.

CHAPTER I. LITERATURE REVIEW

1.1. *ALISMA ORIENTALE*

1.1.1. BOTANY

1.1.1.1. TAXONOMY AND DISTRIBUTION

Alisma orientale (Sam.) Juz. is a semi-aquatic plant belonging to the family Alismataceae assigned by Sergei V. Juzepczuk. It's basionym is *Alisma plantago-aquatica* L. var. *orientale* Sam. The plant is classified in the class Equisetopsida, sub-class Magnoliidae, superorder Lilianae, and order Alismatales.[1]

A. orientale is mainly distributed in China but can be found from northern Europe through southeast Asia. Countries in which *A. orientale* have been documented include China, Japan, Myanmar (formerly Burma), Thailand, Vietnam, Laos, Mongolia, Russia, Korea, Nepal, and India.[2, 3]

1.1.1.2. MORPHOLOGY AND MICROSCOPIC FEATURES

A. orientale is a semi-aquatic perennial herb (FIGURE 1) found growing in mud and shallow water[4] in the margins of freshwater bogs and marshes.

Its Chinese name "Zexie" translates to "marsh drain"[5] and the genus name "Alisma" traces back to the Celtic word for water.[6] The specific epithet "plantago" derives from the Latin word for the sole of a foot, while "aquatica" in Latin means living in water.[7]

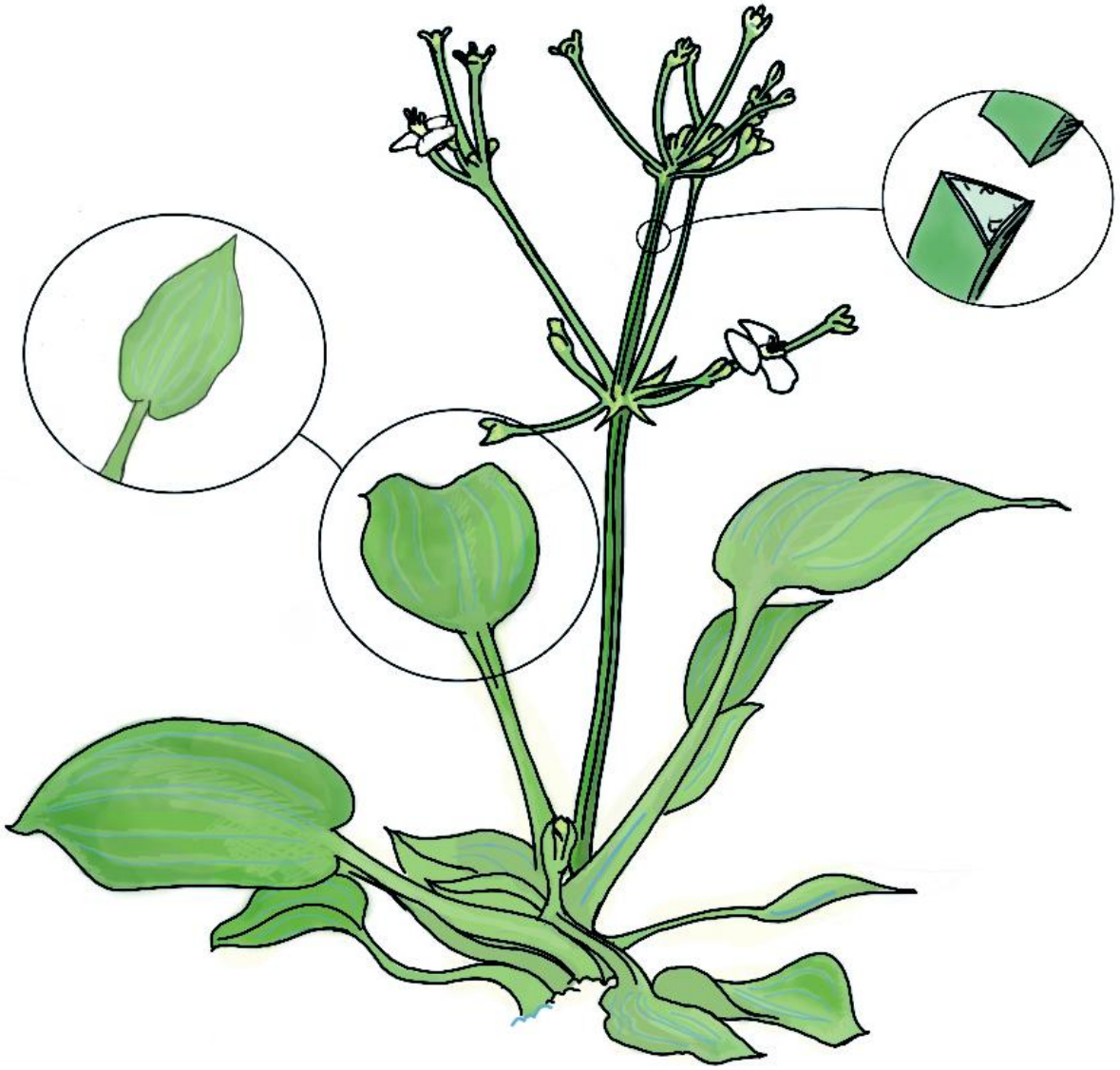


FIGURE 1: WHOLE PLANT OF *A. ORIENTALE*[8] ILLUSTRATION BY KELLY WESCOTT AND HOLLY GUNN, ADAPTED FROM IMAGE BY BFF CC BY-SA 3.0 (HTTPS://CREATIVECOMMONS.ORG/LICENSES/BY-SA/3.0).[9]

The morphological features of *A. orientale* is described as follows: Plant rhizomatous, height < 1 m, stem triangular.[6] Leaves basal, aerial blades, lanceolate to elliptical, base acuminate, apex acuminate.[3] Inflorescence paniculate[6], branches and

secondary branchlets of the panicles whorled, flowers small (a feature which separates *Alisma plantago-aquatica* var. orientale from its European counterpart[10]), sepals ovate[3], petals rounded, white or pale purple[6] to pinkish, margins undulate and irregular. Carpels many, apocarpus, in a ring, styles erect, stigmatose. Fruit an achene, grooved, pericarp thick and opaque.[3] Rhizome 2-8 cm in length and 20-70 mm in diameter, spherical, egg-shaped, or ellipsoid, and covered in yellow/brown cortex and fibrous root hairs.[3, 6, 11] The dried rhizomes and their slices as used in Chinese medicine are presented in FIGURE 2.

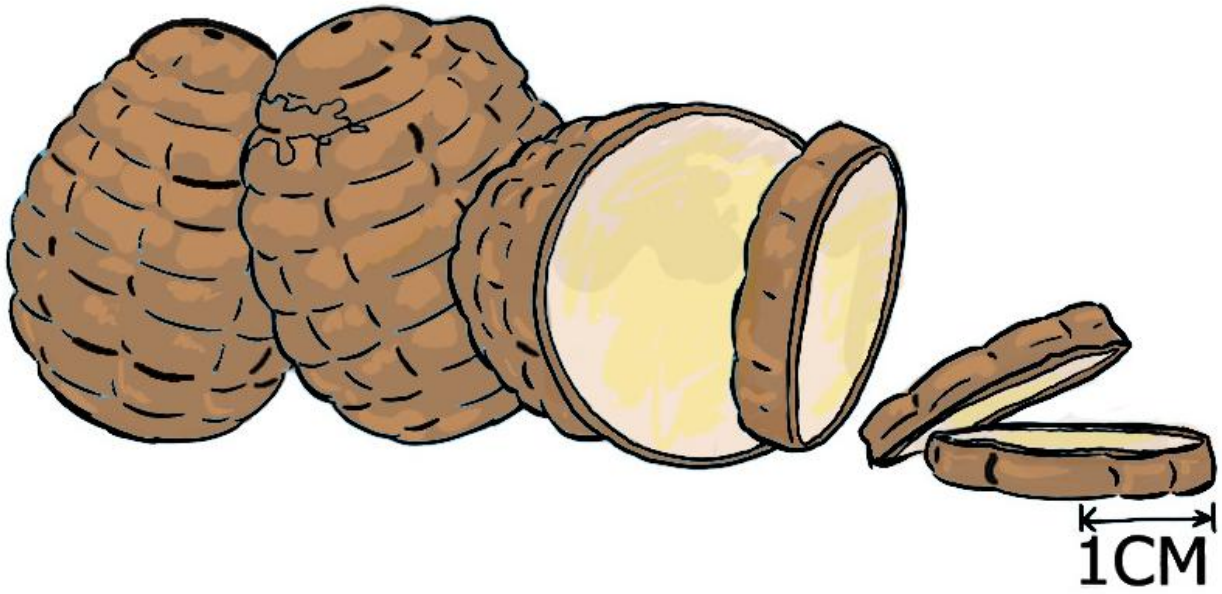


FIGURE 2: RHIZOME (A) AND SLICED HERB DRUG (B) OF *A. ORIENTALE.*[12-13] ILLUSTRATION BY KELLY WESCOTT AND HOLLY GUNN, ADAPTED FROM IMAGE BY TING TIAN JOURNAL OF ETHNOPHARMACOLOGY 158 (2014) 373-387.[14]

Transverse section of *A. orientale* rhizome is characterized by the presence of oil-secretory glands, amphivasal vascular bundles, a lignified row of endodermis, and sometimes residual cortex material (FIGURE 3).

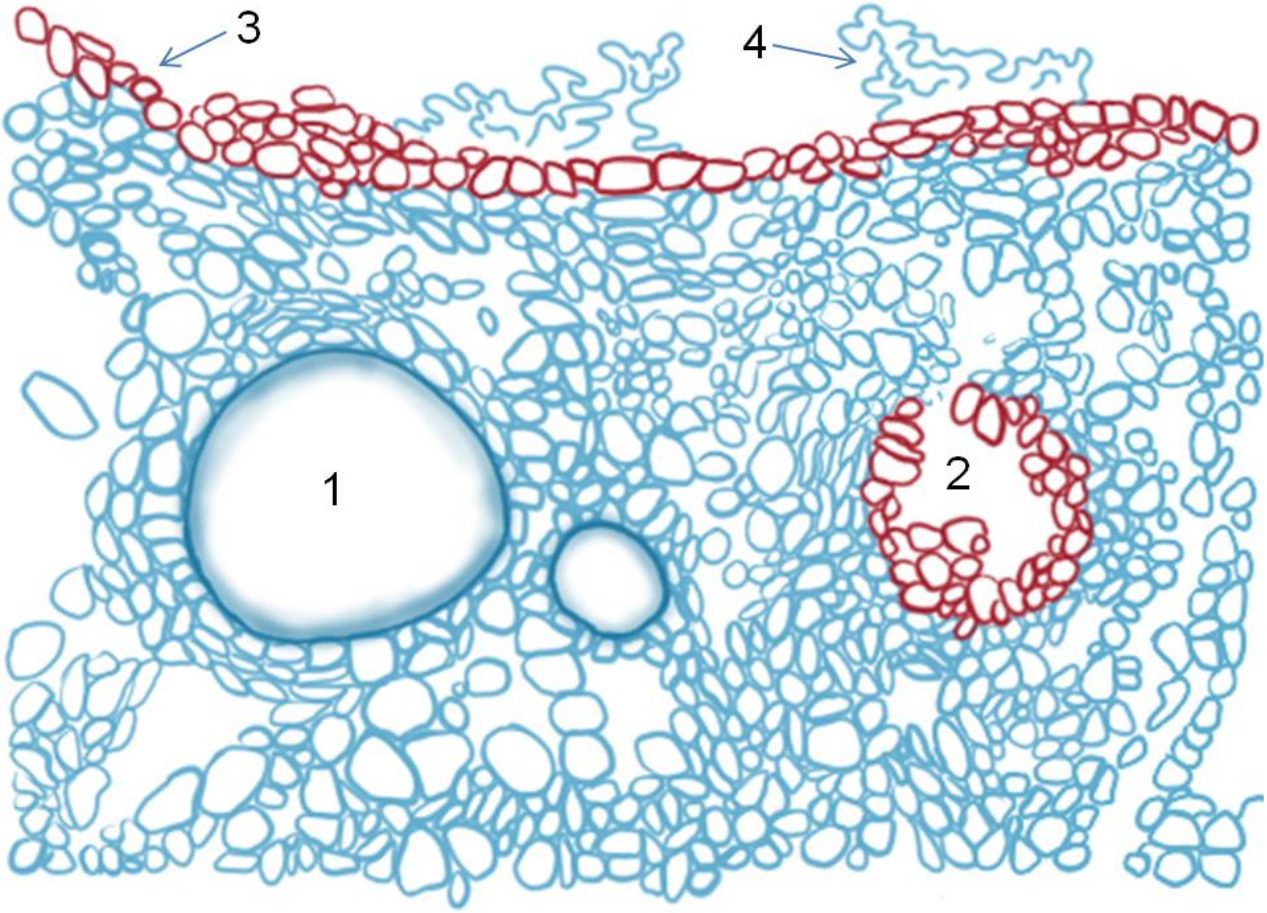


FIGURE 3: CHARACTERISTIC FEATURES FROM THE TRANSVERSE SECTION OF THE RHIZOME OF *A. ORIENTALE.* 1) OIL-SECRETORY GLANDS, 2) AMPHIVASAL VASCULAR BUNDLES, 3) LIGNIFIED ROW OF ENDODERMIS, 4) RESIDUAL CORTEX MATERIAL. ILLUSTRATION BY KELLY WESCOTT AND HOLLY GUNN, ADAPTED FROM IMAGE FROM THE HONG KONG CHINESE MATERIA MEDICA STANDARDS, VOL 1: RHIZOMA ALISMATIS.[15]

Powdered drug is yellow/brown in color. Parenchyma cells contain numerous starch grains which show a characteristic cross shape when examined under polarized light. Pitted parenchyma, endodermis, and mostly broken oil-secretory glands, spiral vessels, and thick fibers are also present[11] (FIGURE 4).

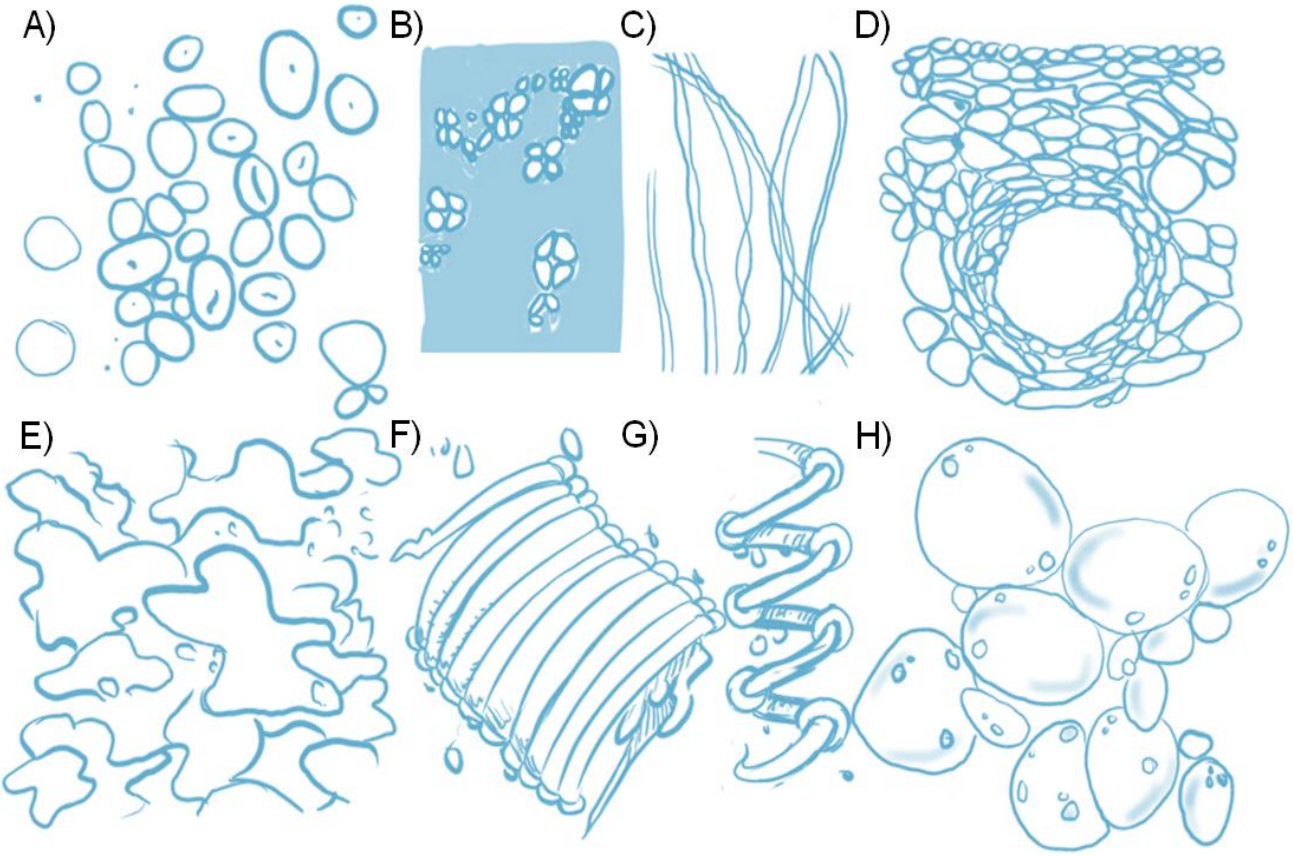


FIGURE 4: CHARACTERISTIC FEATURES FROM THE GROUND RHIZOME OF *A. ORIENTALE.* A) STARCH GRAINS, B) STARCH GRAINS UNDER POLARIZED LIGHT, C) FIBERS, D) OIL VESSICLES, E) PARENCHYMA, F) ANNULAR VESSEL ELEMENTS G) SPIRAL VESSELS, H) STARCH GRAINS IN PARENCHYMA. ILLUSTRATION BY KELLY WESCOTT AND HOLLY GUNN, ADAPTED FROM IMAGE FROM THE HONG KONG CHINESE MATERIA MEDICA STANDARDS, VOL 1: RHIZOMA ALISMATIS.[15]

The dried rhizome tastes bland, slightly sweet and bitter. It has a slight odor that is earthy and musky.[11, 4]

1.1.2. ETHNOBOTANICAL USES

Alisma orientale has a long history in Traditional Chinese Medicine (TCM) where it goes by the name of *Alismatis Rhizoma* or its Chinese name "Zexie".[14] It is designated as a cold dry medicine used to remove excess heat and dampness (such as Wu Ling San and Zhu Ling Tang) within TCM.[13] It is most notably used as part of the well-known TCM prescription "Liuwei Dihuang Wan", which means "six ingredients with rehmania", i.e. *Rehmania preparata, Cornus officinalis, Schisandra chinensis, Dioscorea opposite, Alisma orientalis,* and *Poria cocos.* This mixture is often used in the treatment for yin deficiency,[16] which is linked by TCM theory with endocrine and metabolic disorders, including diabetes mellitus.[17] Although *A. orientale* is normally used in multi-plant mixtures, it is considered an antidiabetic drug individually as well.[14] Additionally, the rhizome of *A. orientale* is used in the traditional herbal recipe Piweiwan,[18] as well as the Japanese formula Gosha-jinki-gan for diabetic complications.[19] *A. orientale* is also officially approved for use in the treatment of diabetes individually by the Chinese Health Regulatory Agency and is routinely prescribed for this purpose.[13, 20]

In addition to specific history of uses for the treatment of diabetes this species is also recorded to have broader ethnobotanical use in South Korea as a diuretic and for the treatment of hyperlipidemia and fatty liver disease,[21] Taiwan for the treatment of liver disease,[22] and the U.S.S.R. for the treatment of rabies and mammary congestion.[23]

1.1.3. CHEMISTRY OF *A. ORIENTALE*

Various databases, including Napralert[24], SciFinder[25], and Google Scholar[26] were searched to access the current state of phytochemical knowledge about *A. orientale.* According to these databases, there have been close to 100 compounds isolated from *A. orientale,* and over 110 isolated from the genus *Alisma*. Among these, 84 terpenes account for the major chemical class of this species.[27] The chemical constituents also include non-alkaloid nitrogen heterocycles, phenolic compounds, and various primary metabolites such as lipids, carbohydrates, and proteins.[24]

1.1.3.1. PRIMARY METABOLITES

As with any plant products, there are a variety of primary metabolites present within the rhizome of *A. orientale*. These compounds are often not reported because of their ubiquitous nature and general lack of bioactivity, however they are listed here for sake of completion and as reference to researchers studying this species as they have the possibility of displaying biological activity (TABLE I).

1.1.3.2. DITERPENES

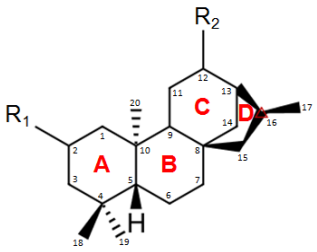
A. orientale rhizome is known to contain two types of diterpenes: Kaurane-type[28] and Orieditерpene-type diterpenes[29] (FIGURE 5). The kaurane-type diterpenes found in *A. orientale* consist of three 6-carbon rings (designated A, B, and C rings), with a fourth cyclic unit attached on carbons 8 and 13 (D ring), occasionally together with the presence of ketone groups attached to carbon 2 of the A ring, and/or carbon 12 of the C ring, and four methyl groups (C17, C18, C19, and C20). Orieditерpene-type diterpenes

TABLE I. PRIMARY METABOLITES FROM *A. ORIENTALE*

COMPOUND	COMPOUND CLASS	MOLECULAR FORMULA	PLANT PART	REF
TRICOSANE,N:	LIPID	$C_{23}H_{48}$	DRIED RHIZOME	30
DOCOSAN-1-OL	LIPID	$C_{18}H_{38}O$	DRIED RHIZOME	31
ALISMAN S-I	CARBOHYDRATE	$C_{366}H_{732}O_{21}$	DRIED TUBER	32
DULCITOL	CARBOHYDRATE	$C_6H_{14}O_6$	DRIED RHIZOME	31
INOSITOL,MYO: HEXAPHOSPHATE	CARBOHYDRATE	$C_6H_{18}O_{24}P_6$	DRIED RHIZOME	33
LACTOSE HEXAPHOSPHATE	CARBOHYDRATE	$C_{18}H_{39}O_{35}P_6$	FRESH RHIZOME	34
METHYL-BETA-D-FRUCTOFURANOSIDE	CARBOHYDRATE	$C_7H_{14}O_6$	DRIED RHIZOME	35
ALISMAN P-II	CARBOHYDRATE	n/a	TUBER	34
THYMIDINE	NITROGEN HETEROCYCLE	$C_{10}H_{14}N_2O_5$	DRIED RHIZOME	36
URIDINE	NITROGEN HETEROCYCLE	$C_9H_{12}N_2O_6$	DRIED RHIZOME	35

n/a = information not available

KAURANE-TYPE DITERPENE



ORIEDITERPENE-TYPE DITERPENE

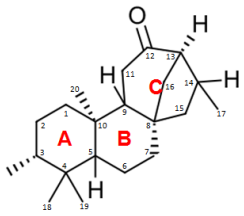
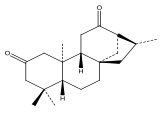
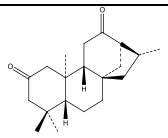
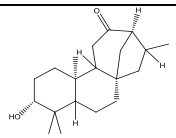
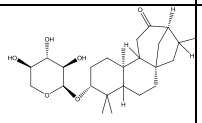


FIGURE 5: THE TWO TYPES OF DITERPENES PRESENT WITHIN *A. ORIENTALE*

triterpenes found in *A. orientale* are derivatives of alisol B 23-acetate, which is found in high abundance in young plants.[37]

They differ from karuane-type in the C ring, which is a 7-carbon ring with a ketone group at C12, with a carbon bridge between carbons 8 and 13, and an oxygenated group located on carbon 3. The R group can be a hydroxyl group or a sugar linkage. TABLE II lists the diterpenes that have been reported from *A. orientale*.

TABLE II. DITERPENES FROM *A. ORIENTALE*

COMPOUND	COMPOUND CLASS	STRUCTURE	MOLECULAR FORUMULA	PLANT PART	REF
KAURANE-2-12-DIONE,16(R):	DITERPENE		$C_{20}H_{30}O_2$	FRESH/ DRIED RHIZOME	38
KAURANE-2-12-DIONE,16(R): ENT:	DITERPENE		$C_{20}H_{30}O_2$	DRIED RHIZOME	39
ORIEDITERPENOL	DITERPENE		$C_{20}H_{32}O_2$	DRIED RHIZOME	39
ORIEDITERPENOSIDE	DITERPENE		$C_{25}H_{40}O_6$	DRIED RHIZOME	39

1.1.3.3. TRITERPENES

Triterpenes found in *A. orientale* are of the Protostane structural class. Protostane-type triterpenes are named as such because their base skeleton is

considered the classic, or “prototype”, steroid structure (FIGURE 6).

Fifty six protostane triterpenes have been isolated from *A. orientale*[29] (TABLE III),

PROTOSTANE TRITERPENE DAMMARANE

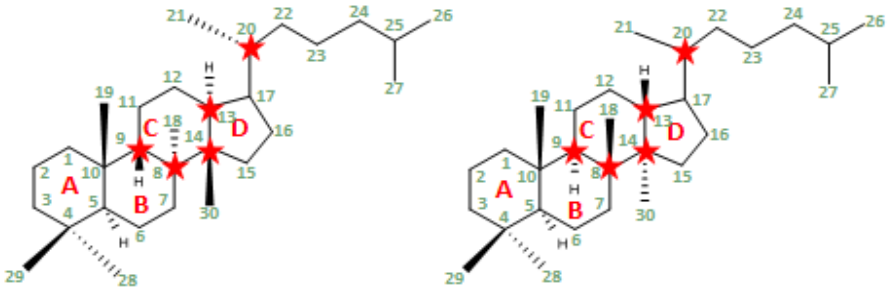
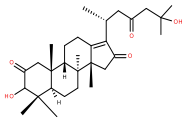
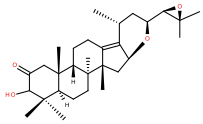
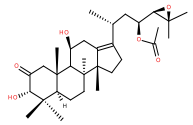
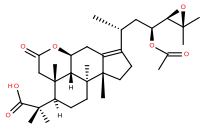
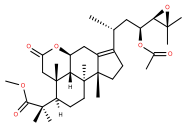
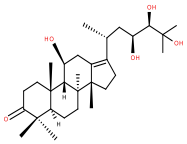
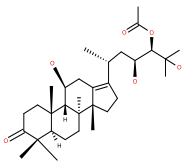
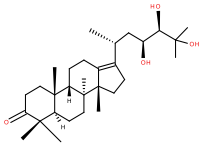


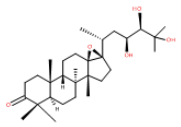
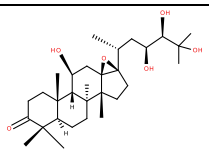
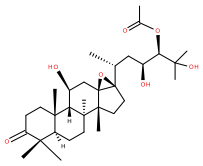
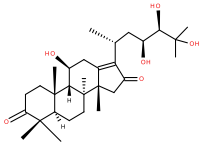
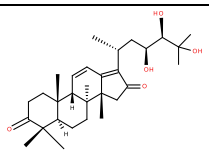
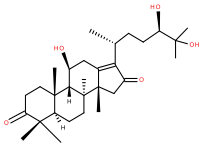
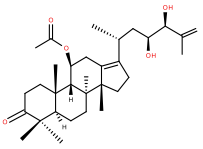
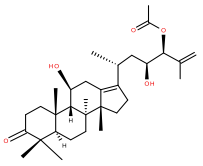
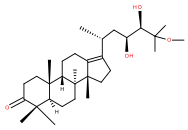
FIGURE 6: PROTOSTANE TRITERPENES COMPARED TO DAMMARANE, A CLASSIC STEROID

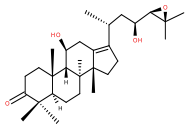
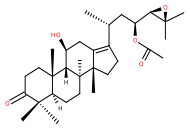
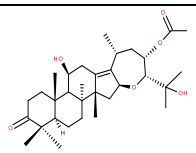
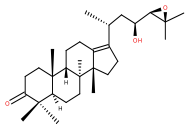
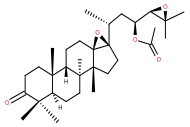
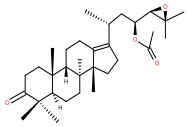
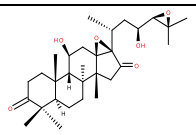
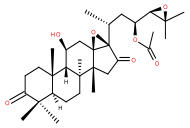
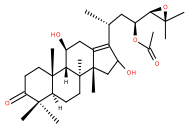
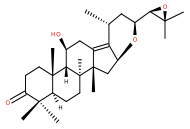
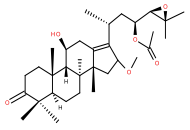
and referred to as variations of alisols A-P,[14] but they are generally discernible as one of six types: alisol A, B, or E series, and *Seco, Nor, or* Rearranged Protostanes[29] (FIGURE 7). They are tetracyclic stereoisomers of dammarane that have characteristic geometries at carbons 8, 9, 13, 14, and 21.[14, 37, 40]

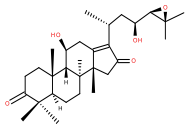
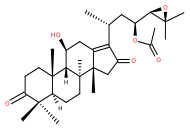
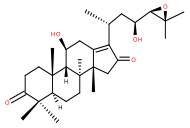
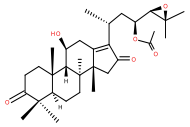
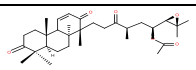
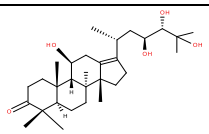
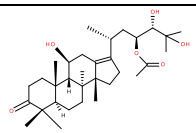
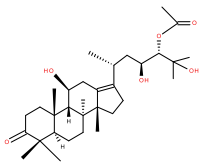
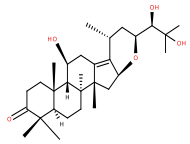
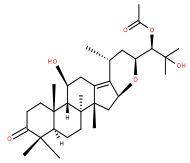
The alisol A series consist of the first protostane-type triterpenes found within *A. orientale* (alisol A), as well as compounds later isolated with related structures.

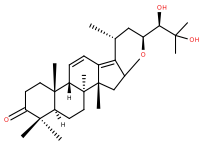
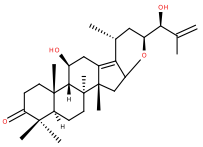
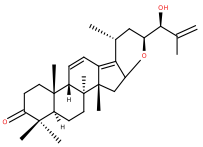
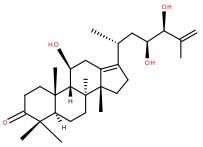
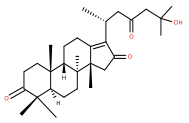
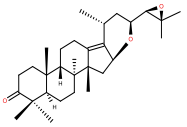
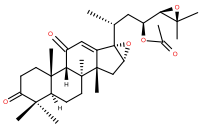
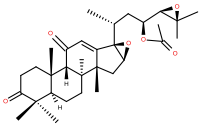
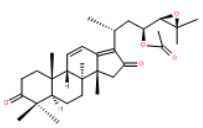
TABLE III. TRITERPENES FROM *A. ORIENTALE*

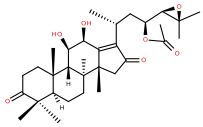
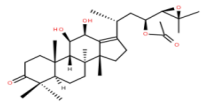
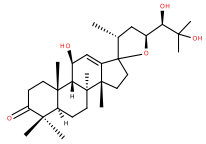
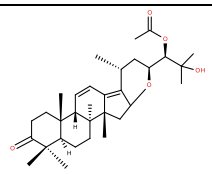
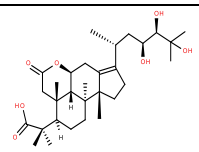
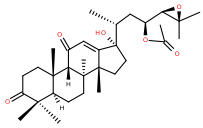
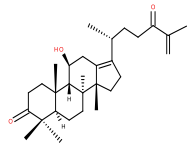
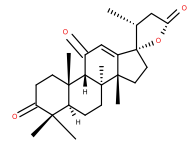
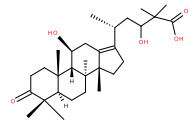
COMPOUND	CLASS	STRUCTURE	MOLECULAR FORMULA	PART	REF
ALISMAKETONE H	A-SERIES		$C_{30}H_{46}O_5$	DRIED RHIZOME	41
ALISMAKETONE I	B-SERIES		$C_{31}H_{50}O_4$	DRIED RHIZOME	41
ALISMAKETONE A, 23-ACETATE	B-SERIES		$C_{33}H_{54}O_6$	n/a	41
ALISMALACTONE 23-ACETATE	E-SERIES		$C_{33}H_{52}O_7$	DRIED RHIZOME	41
ALISMALACTONE ,3-METHYL: 23-ACETATE	E-SERIES		$C_{34}H_{54}O_7$	DRIED RHIZOME	42
ALISOL A	A-SERIES		$C_{30}H_{50}O_5$	FRESH AND DRIEND RHIZOME, DRIED ENTIRE PLANT	43
ALISOL A 24-ACETATE	A-SERIES		$C_{32}H_{52}O_6$	FRESH AND DRIED RHIZOME, DRIED ENTIRE PLANT	44
ALISOL A,11-DEOXY:	A-SERIES		$C_{31}H_{54}O_4$	FRESH RHIZOME	38

COMPOUND	CLASS	STRUCTURE	MOLECULAR FORMULA	PART	REF
ALISOL A,11-DEOXY: 13-BETA-17-BETA-EPOXY:	A-SERIES		$C_{31}H_{54}O_5$	FRESH RHIZOME	38
ALISOL A,13-17-EPOXY:	A-SERIES		$C_{31}H_{54}O_5$	DRIED RHIZOME	45
ALISOL A,13-17-EPOXY: 24-ACETATE	A-SERIES		$C_{33}H_{56}O_7$	DRIED TUBER	46
ALISOL A,16-OXO:	A-SERIES		$C_{31}H_{52}O_6$	COMMERCIAL SAMPLE OF RHIZOME	45
ALISOL A, 16-OXO, 11-ANHYDRO	A-SERIES		$C_{31}H_{50}O_5$	DRIED RHIZOME	47
ALISOL A, 16-OXO, 23-DEOXY	A-SERIES		$C_{31}H_{52}O_5$	n/a	47
ALISOL A,25-ANHYDRO: 11-ACETATE	A-SERIES		$C_{32}H_{50}O_5$	DRIED ENTIRE PLANT	48
ALISOL A,25-ANHYDRO: 24-ACETATE	A-SERIES		$C_{31}H_{54}O_5$	DRIED ENTIRE PLANT	48
ALISOL A,25-O-METHYL:	A-SERIES		$C_{32}H_{56}O_4$	FRESH RHIZOME	38

COMPOUND	CLASS	STRUCTURE	MOLECULAR FORMULA	PART	REF
ALISOL B	B-SERIES		$C_{30}H_{48}O_4$	DRIED RHIZOME AND INFLORE SCENCE	44
ALISOL B 23-ACETATE	B-SERIES		$C_{32}H_{50}O_5$	FRESH AND DRIED RHIZOME	44
ALISMAKETONE B 23-ACETATE	A-SERIES		$C_{32}H_{50}O_6$	n/a	42
ALISOL B,11-DEOXY:	B-SERIES		$C_{31}H_{52}O_3$	FRESH AND DRIED RHIZOME	49
ALISOL B,11-DEOXY: 13-BETA-17-BETA-EPOXY: 23 ACETATE	B-SERIES		$C_{33}H_{54}O_5$	FRESH RHIZOME	38
ALISOL B,11-DEOXY: 23-ACETATE	B-SERIES		$C_{33}H_{54}O_4$	FRESH AND DRIED RHIZOME	49
ALISOL B,13-BETA-17-BETA-EPOXIDE	B-SERIES		$C_{31}H_{50}O_6$	FRESH RHIZOME	38
ALISOL B,13-BETA-17-BETA-EPOXY: 23-ACETATE	B-SERIES		$C_{33}H_{52}O_7$	FRESH RHIZOME	50
ALISOL B,16(A)-HYDROXY: 23-ACETATE	B-SERIES		$C_{33}H_{54}O_6$	TUBER	38
ALISOL B,16-23-OXIDO:	B-SERIES		$C_{31}H_{50}O_4$	RHIZOME	38
ALISOL B, 16-METHOXY, 23-ACETATE	B-SERIES		$C_{34}H_{56}O_6$	n/a	51

COMPOUND	CLASS	STRUCTURE	MOLECULAR FORMULA	PART	REF
ALISOL C	B-SERIES		$C_{31}H_{50}O_5$	FRESH AND DRIED RHIZOME	38
ALISOL C 23 ACETATE	B-SERIES		$C_{33}H_{52}O_6$	FRESH AND DRIED RHIZOME	38
ALISOL C, 11-DEOXY	B-SERIES		$C_{30}H_{46}0_5$	n/a	50
ALISOL C,11-DEOXY: 23-ACETATE	B-SERIES		$C_{32}H_{48}O_6$	FRESH RHIZOME	38
ALISMAKETONE C, 23-ACETATE	SECO-SERIES		$C_{34}H_{54}0_6$	n/a	42
ALISOL E	E-SERIES		$C_{31}H_{54}O_5$	DRIED RHIZOME	42
ALISOL E 23-ACETATE	E-SERIES		$C_{33}H_{56}O_6$	DRIED RHIZOME	49
ALISOL E 24-ACETATE	E-SERIES		$C_{33}H_{56}O_6$	n/a	46
ALISOL F	A-SERIES		$C_{30}H_{48}O_5$	DRIED RHIZOME	52
ALISOL F 24-ACETATE	A-SERIES		$C_{32}H_{50}O_6$	TUBER	53

COMPOUND	CLASS	STRUCTURE	MOLECULAR FORMULA	PART	REF
ALISOL F 24-DEACETYL, O-11-ANHYDRO	A-SERIES		$C_{30}H_{46}0_4$	n/a	40
ALISOL F 25-ANHYDRO	A-SERIES		$C_{31}H_{50}O_4$	n/a	54
ALISOL F 11,25-ANHYDRO	A-SERIES		$C_{31}H_{48}0_3$	n/a	55
ALISOL G	A-SERIES		$C_{30}H_{48}O_4$	DRIED RHIZOME	43
ALISOL H	A-SERIES		$C_{30}H_{46}O_4$	DRIED RHIZOME	56
ALISOL I	B-SERIES		$C_{31}H_{50}O_3$	DRIED RHIZOME	56
ALISOL J 23-ACETATE	B-SERIES		$C_{33}H_{50}O_6$	DRIED RHIZOME	56
ALISOL K 23-ACETATE	B-SERIES		$C_{33}H_{50}O_6$	DRIED RHIZOME	56
ALISOL L 23-ACETATE	B-SERIES		$C_{33}H_{50}O_5$	DRIED RHIZOME	56

COMPOUND	CLASS	STRUCTURE	MOLECULAR FORMULA	PART	REF
ALISOL M 23-ACETATE	B-SERIES		$C_{33}H_{52}O_7$	DRIED RHIZOME	56
ALISOL N 23-ACETATE	B-SERIES		$C_{33}H_{54}O_6$	DRIED RHIZOME	56
ALISOL,NEO:	A-SERIES		$C_{30}H_{48}O_5$	DRIED ENTIRE PLANT	48
ALISOL O	A-SERIES		$C_{32}H_{48}O_5$	n/a	53
ALISOL P	SECO-SERIES		$C_{31}H_{52}O_7$	n/a	57
ALISOL Q	B-SERIES		$C_{33}H_{52}O_6$	n/a	58
ALISOL X	A-SERIES		$C_{30}H_{46}O_3$	n/a	59
ALISOLIDE	NOR-SERIES		$C_{27}H_{40}0_4$	n/a	57
3-OXO-13B-23-DIHYDROXY-24,24-DIMETHYL-26,27-DINOR PROTOST-13(17) -EN-25-OIC ACID	REARRANGED PROTOSTANE		$C_{31}H_{52}O_5$	n/a	57

n/a = information not available

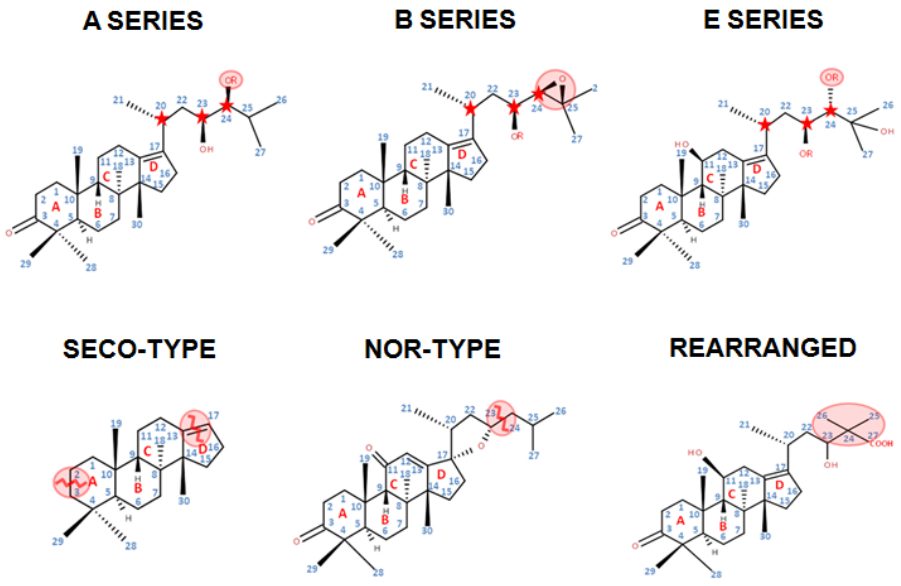


FIGURE 7: THE SIX TYPES OF TRITERPENES PRESENT WITHIN *A. ORIENTALE.*

Diagnostic characteristics include a (20R, 23S, 24R) absolute stereochemical configuration and a hydrocarbon side chain located on carbon 17 which lacks a 24,25 epoxy group. Variations within compounds that belong to the A-Series of protostane triterpenes include one or more of the following: Deoxylation, acetylation, or reduction of carbon 11, reduction of carbon 12, an epoxy group between carbons 13 and 17, a ketone group at carbon 16, an epoxy group between carbon 23 and carbon 16 or 17, the acetylation of carbon 23, a ketone or deoxylation at carbons 23 and/or 24, and the reduction, deoxylation, or cyclization of carbon 25. Together these variances constitute the diversity of this class of protostane triterpenes.

The alisol B series is characterized by the presence of a 24,25-epoxy group. Variations within compounds that belong to the B series of protostane triterpenes

include one or more of the following: The reduction of the ketone group on carbon 3 to a hydroxyl group, a ketone group, deoxylation, or reduction of carbon 11, reduction or hydroxylation of carbon 12, an epoxy group between carbons 13 and 17, a ketone or methoxy group at carbon 16, an epoxy group between carbon 23 and carbon 16, an epoxy group between carbon 16 and carbon 17, hydroxylation of carbon 17, the acetylation of carbon 23, a ketone or deoxylation at carbons 23 and/or 24, and the reduction, deoxylation, or cyclization of carbon 25.

The alisol E series, like the alisol A series, also lacks a 24,25-epoxy group, but differs in having a (20R, 23S, 24S) absolute stereochemical configuration.

There have been three protostane triterpenes isolated from *A. orientale* which are categorized as the E series. They differ only in the acetylation and hydroxylation patterns on carbon 23 and carbon 24, one with both hydroxylated, and the other two possessing one carbon hydroxylated and one carbon acetylated.

Seco-Protostanes are a group of triterpenes that are characterized by the cleavage of either carbons 2 and 3 of the A ring or carbons 13 and 17 of the D ring.

Four *seco*-protostane triterpenes have been isolated from *A. orientale.* Three are compounds that result from cleavage of the A ring between carbon 2 and carbon 3. They differ between epoxy and hydroxyl versions of carbon 24. The epoxy version has two versions: One containing a carboxylic end at carbon 3, and the other containing a acetyl group at this same position. The fourth *seco*-protostane also contains an epoxy between carbon 24 and carbon 25 but has a ring break between carbon 13 and carbon 17, leaving a nine carbon side chain containing a ketone and acetyl group.

Nor-protostanes are a class of triterpenes that have carbons eliminated during biosynthesis. An example of this skeleton type is 24,25,26,27-tetra-*nor*-protostane. Additionally, the protostane triterpene 3-oxo-11β,23-dihydroxy-24,24-dimethyl-26,27-dinorprotost-13(17)-en-25-oic acid was isolated from *A. orientale* which has undergone rearrangement during biosynthesis are referred to as rearranged protostane triterpenes.

1.1.3.4. SESQUITERPENES

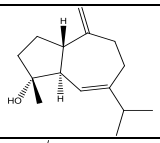
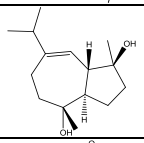
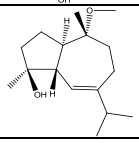
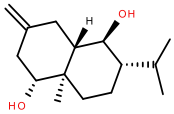
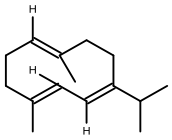
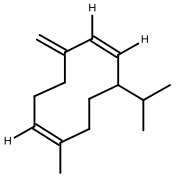
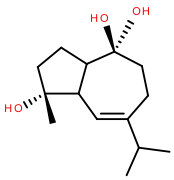
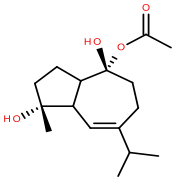
Eighteen sesquiterpenes have been isolated from *A. orientale* (TABLE IV). They consist of two types of structural subclasses: Guaiane-type and germacrene-type[14] (FIGURE 8).

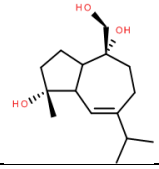
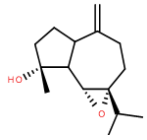
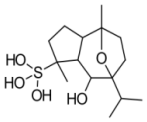
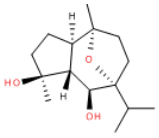
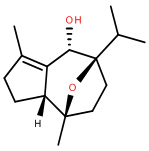
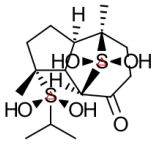
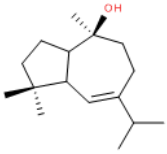
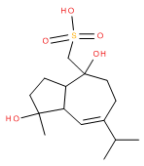
Despite their differences, both types are related products derived from E,E-farnesyl cation cyclized to a germacryl cation. From here the germacryl cation is either protonated and cyclized to form a germacrene skeleton and further modified or the germacryl cation goes through an anti-Markovnikov addition and cyclizes to form a guaiane skeleton where it is further modified.

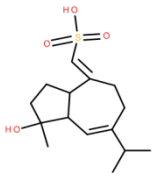
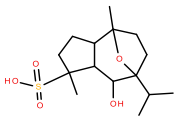
There are three germacrene sesquiterpenes that have been isolated from *A. orientale*. They differ in the presence and location of double bonds within the ring, as well as alkene, alkane, and hydroxyl additions.

There have been 13 guaiane-type sesquiterpenes that have been isolated from *A. orientale.* They differ by the presence and location of alkane, ketone, hydroxy, and epoxy groups on both A and B rings, as well as acetylation.

TABLE IV. SESQUITERPENES FROM *A. ORIENTALE*

COMPOUND	CLASS	STRUCTURE	MOLECULAR FORMULA	PART	REF
ALISMOL	GUAIANE-TYPE		$C_{15}H_{24}O$	FRESH AND DRIED RHIZOME	38
ALISMOXIDE	GUAIANE-TYPE		$C_{15}H_{26}O_2$	FRESH AND DRIED RHIZOME	52
ALISMOXIDE,10-O-METHYL:	GUAIANE-TYPE		$C_{16}H_{28}O_2$	FRESH RHIZOME	38
EUDESM-4(14)-ENE-1-BETA-6-ALPHA-DIOL	GERMACRENE-TYPE		$C_{15}H_{26}O_2$	RHIZOME	38
GERMACRENE C	GERMACRENE-TYPE		$C_{15}H_{24}$	FRESH RHIZOME	49
GERMACRENE D	GERMACRENE-TYPE		$C_{15}H_{24}$	FRESH RHIZOME	49
ORIENTALOL A	GUAIANE-TYPE		$C_{14}H_{24}O_3$	DRIED RHIZOME	60
ORIENTALOL A, 10-ACETATE	GUAIANE-TYPE		$C_{16}H_{26}O_4$	n/a	60

COMPOUND	CLASS	STRUCTURE	MOLECULAR FORMULA	PART	REF
ORIENTALOL B	GUAIANE-TYPE		$C_{15}H_{26}O_3$	DRIED RHIZOME	60
ORIENTALOL C	GUAIANE-TYPE		$C_{15}H_{24}O_2$	DRIED RHIZOME	52
ORIENTALOL D	GUAIANE-TYPE		$C_{15}H_{27}O_5S$	DRIED RHIZOME	42
ORIENTALOL E	GUAIANE-TYPE		$C_{15}H_{26}O_3$	DRIED TUBER	61
ORIENTALOL F	GUAIANE-TYPE		$C_{15}H_{24}O_2$	n/a	62
ORIENTANONE	GUAIANE-TYPE		$C_{15}H_{25}S_2O_5$	FRESH ENTIRE PLANT	63
SULFOORIENTALOL A	GUAIANE-TYPE		$C_{16}H_{28}O$	DRIED RHIZOME	36
SULFOORIENTALOL B	GUAIANE-TYPE		$C_{15}H_{26}O_5S$	DRIED RHIZOME	36

COMPOUND	CLASS	STRUCTURE	MOLECULAR FORMULA	PART	REF
SULFOORIENTALOL C	GUAIANE-TYPE		$C_{15}H_{24}O_4S$	DRIED RHIZOME	36
SULFOORIENTALOL D	GUAIANE-TYPE		$C_{15}C_{26}O_5S$	DRIED RHIZOME	36
n/a = information not available					

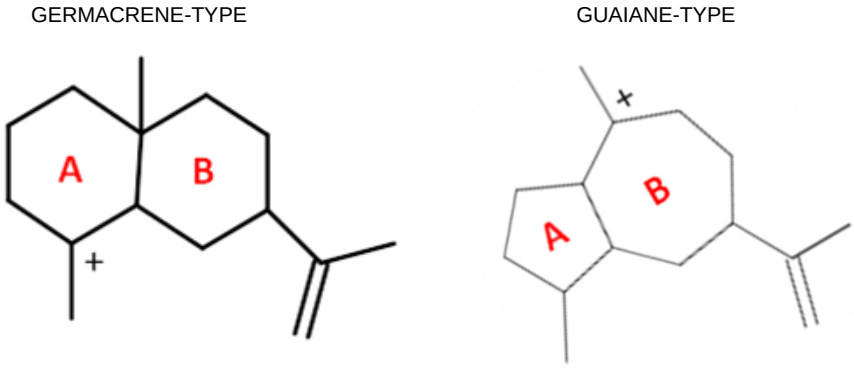


FIGURE 8: THE TWO TYPES OF SESQUITERPENES PRESENT WITHIN *A. ORIENTALE*

1.1.4. BIOLOGICAL ACTIVITIES

1.1.4.1. STUDIES ON MIXTURES CONTAINING *A. ORIENTALE*

The TCM formula Liuwei-Dihuang-Wan, of which *A. orientale* rhizome is an ingredient, has shown convincing evidence for its usefulness in regiments used to treat

Diabetes Mellitus and its complications through a number of mechanisms as determined by *in vivo* and *in vitro* studies.[64-65]

1.1.4.2. *IN VITRO* STUDIES ON *A. ORIENTALE*

A number of *In vitro* studies have been conducted on *A. orientale* which helped to inform this project. Below is a brief description of these studies.

This species has been tested in the BBMV glucose gut absorption inhibition assay. At a concentration of 1 mg/ml a decoction significantly reduced absorption.[66]

Studies revealed that in the 3T3-L1 glucose uptake model decoctions as well as aqueous ethanolic extracts of *A. orientale* exhibited insulin dependent glucose uptake induction in Glucose Transporter Type-4 (GLUT4) containing 3T3-L1 adipocytes at concentrations 0.01 mg/ml and 25 µg/ml, respectively.[66-67] A series of pure TABLE IV. compounds isolated from *A. orientale* including alisol C, alisol C 23-acetate, alisol A, alisol A 24-acetate, 16-ketoalisol A, alisol B 23-acetate, alisol B, and alisol L were screened for bioactivity at doses between 5 and 20 µM.[68] The last two of these isolated were considered inactive in the screen, and the rest displayed variable weak activity. Follow-up studies on the isolates from this screen were never published. Additionally, studies have shown that the aqueous ethanolic and butanol extracts of *A. orientale* cause α-glucosidase inhibition in kinetic studies at concentrations of 25 µg/ml and 0.002 mg/ml, respectively[67, 69], and the compound alisol F was determined to be weakly active in this assay at a concentration of 0.125 mM.[40] A summary of these studies, as well as their results, can be found in TABLE V**.**

TABLE V. *IN VITRO* STUDIES ON *A. ORIENTALE*

STUDY	FRACTION	DOSE	RESULT	REFERENCE
BBMV	H_2O	1 MG/ML	ACTIVE	[66]
3T3-L1	H_2O	0.01 MG/ML	ACTIVE	[66]
3T3-L1	70% ETOH	25 µG/ML	ACTIVE	[67]
α-GLUC	70% ETOH	25 µG/ML	ACTIVE	[67]
α-GLUC	BUTANOL	.002 MG/ML	ACTIVE	[69]

1.1.4.3. *IN VIVO* STUDIES ON *A. ORIENTALE*

Several *in vivo* studies have been conducted on *A. orientale* to determine its antidiabetic potential. The result of these studies are summarized in TABLE VI.

TABLE VI: *IN VIVO* STUDIES ON *A. ORIENTALE*

ANIMAL MODEL	**EXTRACT**	**ROUTE ADMIN.**	**DOSE**	**RESULTS**	**REFERENCE**
STREPTOZOTOCIN	H20	I.G.	1 G/KG	-	[70]
STREPTOZOTOCIN	H20/ETOH	I.G.	1.5 G/KG	+	[71]
STREPTOZOTOCIN	CH_2CL_2	I.G.	100 MG/KG	+	[72]
NORMAL	N/R	I.G.	20 G/KG	+	[73]
NORMAL	BuOH	I.G.	N/R	+	[69]
ALLOXAN	N/R	I.G.	20 G/KG	+	[74]
ADRENALINE	N/R	I.G.	20 G/KG	+	[74]
N/R = information not recorded					

Extracts of the rhizome were tested in streptozotocin, alloxan, and adrenaline-induced diabetic mice, as well as in normal mice. One report found no antidiabetic effect in the streptozotocin model.[70] However, these findings have been disputed by other studies using different extracts in the same model.[71-72] Extracts have also shown antihyperglycemic effects in the Alloxan[74] and Adrenaline models,[74] and have shown hypoglycemic effects in normoglycemic mice.[69, 73]

1.1.4.4. CLINICAL STUDIES ON *A. ORIENTALE*

A case study on 110 hyperlipidemic patients on *A. orientale* individually for the treatment of high cholesterol and triglyceride levels and a rudimentary human study on 40 patients in a formulation with other herbal drugs for the treatment of diabetic foot ulcers have been conducted. Both studies displayed evidence of effectiveness against these ailments. Although the former was only an observational study with little statistical validity or high-level evidence of effect, and the latter was not a double-blind, randomized, or placebo controlled clinical study, these studies serve as background information for the potential effect of *A. orientale.* [75-76]

1.1.4.5. TOXICITY STUDIES ON *A. ORIENTALE*

The toxicity of *A. orientale* rhizome is largely unknown. One study reported no toxic effects at 33.3 g/kg doses in mice.[77] However, long-term use as well as overdoses of this species have been shown to cause hepatotoxicity and nephrotoxicity in one study. A case report from Queen Mary Hospital in Hong Kong detailed how a patient died of hepatic and renal failure after taking 8 doses daily for three weeks of a traditional

Chinese medicinal formulae containing 11 different herbs, one of which was *A. orientale*.[12] Despite these conflicting reports *A. orientale* has been used by millions of people and is considered to be generally nontoxic with most use not resulting in adverse effects or contraindications.[14, 78-79]

1.1.4.6. STUDIES ON PURE COMPOUNDS FROM *A. ORIENTALE*

A number of compounds isolated from *A. orientale*, or alternatively isolated from other sources but known to be present within *A. orientale*, have been tested in antidiabetic assays. The results of these studies, most of all which found weak or no activity, are found in TABLE VII.

1.2. DIABETES MELLITUS

1.2.1. OVERVIEW AND IMPACT

Approximately 29 million Americans have diabetes according to a 2012 study.[80] This is up from 26 million in 2010. Of these 29 million, approximately 28 million had Type Two Diabetes Mellitus (T2DM). These numbers assume a full quarter, 8 million people, have the disease but are undiagnosed[81]. Additionally, it is estimated that in 2012 86 million Americans over 20 years of age are prediabetic, a number that has increased from 79 million in 2010.[80]

Youth are most commonly diagnosed with type 1 diabetes (T1DM), with over 3 times the rate as T2DM, although the incidence of T2DM within youth is a growing concern in recent years.[82]

TABLE VII: STUDIES ON PURE COMPOUNDS FROM *A. ORIENTALE*

STUDY	FRACTION	DOSE	RESULT	REFERENCE
3T3-L1	ALISOL C	20 µG/ML	WEAKLY ACTIVE	[68]
3T3-L1	ALISOL C 23-ACETATE	20 µG/ML	WEAKLY ACTIVE	[68]
3T3-L1	ALISOL A	20 µG/ML	WEAKLY ACTIVE	[68]
3T3-L1	ALISOL A 24-ACETATE	10 µG/ML	WEAKLY ACTIVE	[68]
3T3-L1	16-KETOALISOL A	20 µG/ML	WEAKLY ACTIVE	[68]
3T3-L1	ALISOL B 23-ACETATE	10 µG/ML	WEAKLY ACTIVE	[68]
3T3-L1	ALISOL B	20 µG/ML	INACTIVE	[68]
3T3-L1	ALISOL L	20 µG/ML	INACTIVE	[68]
α-GLUC	CYTIDINE	2.5 mM	WEAKLY ACTIVE	[83]
α-GLUC	ALISOL F	0.125 mM	ACTIVE	[40]
α-GLUC	16-OXO-11-ANHYDROALISOL A 24-ACETATE	100 µM	INACTIVE	[84]
α-GLUC	16-OXO-11-ANHYDROALISOL A	100 µM	INACTIVE	[84]
α-GLUC	13β,17β-EPOXY-24,25,26,27-TETRANOR-ALISOL A 23-OIC ACID	100 µM	INACTIVE	[84]
α-GLUC	1α-H,5αH-GUAIA-6-ENE-4β-10β-DIOL	100 µM	INACTIVE	[84]
α-GLUC	ALISGUAIAONE	100 µM	INACTIVE	[84]
α-GLUC	ALISOL A	100 µM	INACTIVE	[84]
α-GLUC	ALISOL A 24-ACETATE	100 µM	INACTIVE	[84]
α-GLUC	25-O-ETHYLALISOL A	100 µM	INACTIVE	[84]
α-GLUC	11-DEOXYALISOL A	100 µM	INACTIVE	[84]

STUDY	FRACTION	DOSE	RESULT	REFERENCE
α-GLUC	ALISOL E 24-ACETATE	100 μM	INACTIVE	[84]
α-GLUC	ALISOL G	100 μM	INACTIVE	[84]
α-GLUC	ALISOL B 23-ACETATE	100 μM	INACTIVE	[84]
α-GLUC	10-HYDROXY-7,10-EPOXYSALVIALANE	100 μM	INACTIVE	[84]
α-GLUC	25-ANHYDROALISOL F	100 μM	INACTIVE	[84]
α-GLUC	ALISOL F	100 μM	INACTIVE	[84]
α-GLUC	13β,17β-EPOXYALISOL A	100 μM	INACTIVE	[84]
α-GLUC	ALISOL F 24-ACETATE	100 μM	INACTIVE	[84]
α-GLUC	ALISOL C 23-ACETATE	100 μM	INACTIVE	[84]
α-GLUC	ALISMAKETONE B 23-ACETATE	100 μM	INACTIVE	[84]
α-GLUC	ALISMOXIDE	100 μM	INACTIVE	[84]
α-GLUC	4β,10β-DIHYDROXY-1αH,5βH-GUAIA-6-ENE	100 μM	INACTIVE	[84]
α-GLUC	ORIENTALOL A	100 μM	INACTIVE	[84]
α-GLUC	ORIENTALOL B	100 μM	INACTIVE	[84]
α-GLUC	ORIENTALOL E	100 μM	INACTIVE	[84]
α-GLUC	ENT-OPLOPANONE	100 μM	INACTIVE	[84]

Minorities are at particular risk for developing diabetes (and specifically T2DM). While the rate of diabetes for non-Hispanic whites lies at around 7.6%, rates for American Indians/Alaskan Natives, non-Hispanic blacks, and Hispanics are nearly

double being estimated at 15.9%, 13.2%, and 12.8%, respectively. A slight increase in incident rates can also be seen among Asian Americans, with particular vulnerability within the Filipino and Asian Indian population.[82] T2DM affects 246 million people worldwide.[85]

T2DM accounts for 1 in 20 deaths throughout the world, and ranks as the 7th leading cause of death in the U.S., killing over 72,000 people every year.[86] From 2003-2006 it was estimated that deaths due to cardiovascular disease were 1.8 times higher within those who have diabetes when compared to those who do not. In 2010 it was estimated that those who are diabetic are 1.5 times more likely to have a stroke, although morbidity rates among these groups were not assessed.[82]

For many individuals diabetes mellitus is not only a serious health risk, but it is also a distinct financial burden. Medical expenditures alone are estimated to be 2.3 times higher among those suffering from diabetes mellitus when compared to those who do not.[87]

Nationally, it was estimated that the direct medical cost of treating diabetes mellitus in the United State was $176 billion in the year 2012. Additionally, in the same year it was estimated that it cost an additional $69 billion in reduced productivity. This adds to a total annual cost of $245 billion in 2012.[86]

1.2.2. CAUSES

Cells throughout the body require energy to function. This need is fulfilled by the consumption of simple sugars such as glucose or through the breakdown of complex

carbohydrates consumed in food into glucose. Once consumed or produced through digestion, blood glucose concentration is highly and biochemically regulated in a negative feedback loop caused by the stimulus of β-cells in the pancreas that release insulin. Insulin causes specialized insulin-responsive glucose transport proteins (GLUT4) to uptake glucose in peripheral cellular tissue for use as energy, as well as the uptake of glucose by the liver to be stored as glycogen, both of which decrease blood glucose levels back to its homeostatic level. During times of famine, or after a person has skipped a meal, blood glucose can dip lower than the homeostatic level and cause a feedback loop whereby α-cells of the pancreas are stimulated to release glucagon, which signals the liver to break down glycogen into glucose and release it into the blood stream, which causes the blood glucose level to rise, returning to its homeostatic set point. In this way blood glucose concentrations can be raised or lowered to remain as close to the set point as possible at all times as well as to actively utilize glucose, and therefore energy, present within the blood, excess glucose within the blood, and situations where not enough glucose is present in the blood to provide the energy needs of the cells of the body.[88]

Diabetes mellitus is a group of metabolic diseases classified as endocrine disorders and characterized by abnormal blood glucose homeostasis. Although T1DM and T2DM have different causes, both produce the same outcome: The β-cells within the pancreas are unable to produce insulin. Thus both require intervention which includes insulin. In T2DM this situation is complicated by the presence of insulin resistance.

T1DM is caused by an immune reaction of T-cells to destroy the β-cells within the islet tissue of the pancreas. Over the course of several years this reaction causes complete cessation of the production of insulin and the onset of a diabetic state. The exact causes for this immune system response within T1DM are not fully understood but there is strong evidence of inheritance and thus at least a partial genetic component. One current theory is that people with genes for several identified human leukocyte antigen (HLA) complexes on chromosome 6 can trigger immune responses leading to the development of various autoimmune disorders, including T1DM.[89] Another theory is that some viruses also have the ability to trigger T-cell destruction of β-cells. The viruses which have been identified as having antigens similar to that of β-cells include the B4 strain of coxsackie B virus, German measles, mumps, and rotavirus.[90]

T2DM is the result of a pre-diabetic condition caused by an inability to store and use glucose.[91] Defects in this process, due to insulin resistance, cause excessive accumulation of glucose in the blood (hyperglycemia).[92] Progression of the pre-diabetic state involves the overproduction of insulin to compensate for the resistance, a condition called hyperinsulinemia.[93] This ultimately leads to pancreatic damage and the decline of insulin production, at which point patients can be diagnosed as having T2DM.[94]

If left untreated, diabetes mellitus causes the inability to lower blood glucose levels back to its homeostatic set point such as during a postprandial blood glucose spike. The resulting excess glucose can cause damage to nearly every organ system and tissue type. A lack of insulin also causes cells which have energy needs satisfied

by the uptake of blood glucose to be starved of this energy, which also causes extensive and widespread cell death. Together these effects can result in a myriad of health consequences including hypoglycemia and chronic hyperglycemia which can lead to complications such as hypertension, dyslipidemia, retinopathy,[95] neuropathy[96] and nephropathy,[97] kidney disease, amputations, heart attack, and stroke.

1.2.3. INSULIN MIMETICS AND INSULIN SENSITIZERS

1.2.3.1. TREATMENT OPTIONS

Currently there are numerous options for treating diabetes mellitus, both individually and also concurrently. Exercise and diet are at the forefront, especially in the prevention of diabetes for which it is a known cause, but sometimes these measures are not enough. Additionally there are a range of known insulin secretagogues, insulin mimetics and insulin sensitizers, glucose gut absorption inhibitors, and α-glucosidase inhibitors.

Diet and exercise is an integral part of both the prevention and treatment of diabetes mellitus, be it T1DM or T2DM. The incidence of T2DM has risen in tandem with obesity rates. Additionally, the implementation of diet and exercise programs has been shown to decrease the incidence of T2DM in high-risk populations, and interventions which include diet and exercise components have been shown to reduce glycated haemoglobin (HbA1c) levels in T2DM and reduce mortality in those with T1DM. Because of the strong link between diet and exercise and the development and progression of the disease, doctors often advise patients to lose weight and improve their metabolism.[98]

Exercise is generally recommended in all cases of T2DM and in the case of pre-diabetics if at all possible. Even in cases where mobility is limited or complications prevent one from strenuous activity, light activity such as walking is recommended. For those who are able moderate to high-activity is recommended.

Insulin secretagogues are drugs that cause the β-cells of the pancreas to release and produce more endogenous insulin. They act by closing K(ATP) channels in the β-cells' plasma membrane. This release of insulin causes an increase in blood insulin which has a hypoglycemic effect overall. This is produced by the glucose uptake in cells of the body with GLUT4 transporters giving cells the energy they need and storing excess blood sugar as fat and glycogen. This effect does not occur in patients with advanced diabetic states as the β-cells tend to naturally undergo gradual depletion over time causing this type of intervention to be less effective. Treatment with insulin secretagogues itself contributes to this decline. Insulin secretagogues are also known to cause weight gain in some individuals and concerns have been raised that some insulin secretagogues may impair circulation by acting as an inhibitor of ATP-sensitive potassium channels in blood vessels. There are currently three classes of known insulin secretagogues: Sulfonylureas, meglitinides, and D-phenylalanine derivatives.[99]

Insulin mimetic agents (IMAs) are substances which cause the same type of glucose uptake stimulation as insulin. They work by moving glucose from the blood into cells via the GLUT4 receptor. IMAs act on the insulin receptor, causing a signal transduction that causes the release of intracellular GLUT4 and its translocation to the plasma membrane where it acts as a glucose transporter.[100] There are currently no

FDA approved clinical IMAs on the market although there are a number known both synthetic and natural in origin. Currently, insulin is the first-line treatment for T2DM.[101]

Insulin sensitizing agents (ISAs), on the other hand, are substances which act to increase cellular responses to the presence of endogenous and exogenous insulin. ISAs are commonly used in patients which are trying to lose weight, as it decreases the amount of insulin needed in the blood and facilitates cellular glucose uptake. Recent research suggests they may also down-regulate genes involved in the storage of fat. Additionally, their use has also been shown to reduce the risk of cardiovascular problems such as blood clotting, blood pressure, thickening of the heart, and improve lipid profiles and lipoprotein, C-reactive protein, and serum fibrinogen levels. Some studies conducted on ISAs currently on the market have shown additional independent actions that can inhibit glycosylation of proteins throughout the body and inhibit the progression of diabetic kidney disease. There are three commonly prescribed ISAs on the market: Metformin (Glucophage), and the thiazolidinediones Rosiglitizone (Avandia), and Pioglitazone (Actos). Troglitazone, another ISA, was prescribed but recently taken off the market because of hepatotoxicity.[102]

Another popularly prescribed class of antidiabetic drugs are sodium-dependent glucose transport (SGLT) inhibitors. One commonly used example of drugs in this category is that of SGLT2 inhibitors which block glucose reuptake by the kidney thereby increasing glucose in the urine to combat hyperglycemia. Another example in this category, albeit less commonly used today, is that of the SGLT1 glucose gut absorption inhibitors. These drugs prevent the rise of blood glucose from the small intestine either

by inhibiting the breakdown of complex carbohydrates consumed in the diet or by inhibiting the absorption of glucose into the bloodstream through the small intestine.[103]

α-glucosidase inhibitors are a class of T2DM drugs that act on the breakdown of complex carbohydrates. These drugs, which often resemble carbohydrates themselves, act to competitively bind to the endogenous enzyme α-glucosidase thereby preventing the breakdown of said carbohydrates into glucose. The overall effect of such an action is to reduce the rate and extent of the postprandial glucose spike which occurs after the intake of, especially, highly processed carbohydrate rich foods. α-glucosidase inhibitors are frequently given in combination with other antidiabetic drugs, especially in the case of poor dietary habits. The known α-glucosidase inhibitors are often poorly tolerated, as bacteria in the gut breaks down remaining polysaccharides producing unpleasant gastrointestinal effects such as gas and diarrhea. Side effects of α-glucosidase inhibitors include diarrhea, flatulence, and abdominal distention. Examples of clinically used α-glucosidase inhibitors are acarbose and miglitol.

Drugs that slow the absorption of glucose into the bloodstream within the small-intestine are referred to as amylin-mimetics. Amylin is a hormone released by the pancreas in reaction to rising blood glucose concentrations. This hormone is part of a negative feedback loop that causes an increase in stomach emptying time and slows the absorption of glucose through the small intestine into the blood. Some side effects of amylin mimetics are nausea, dizziness, and vomiting. An example of a clinical amylin-mimetic drug is Pramlintide.[104]

1.2.3.2. THE NEED FOR NEW TREATMENTS

Current drug therapy targeting key mechanisms involved in hyperglycemia, as well as its detrimental effects, have been unable to prevent and offset the impact of these diseases. Indeed six people continue to die per minute due to their effects, accounting for 1 in 20 deaths worldwide.[86] Aside from issues with the general success of current drugs there are specific issues with current therapies. Because insulin is not orally active and must be injected, the development of orally active IMAs is highly desirable for augmenting or even replacing insulin therapy.[105] A variety of IMAs are being investigated experimentally but none have yet made it into the clinic.[106] Also of interest for development are ISAs. Biguanides (e.g. metformin and phenformin[94]) and Thiazolidinediones (e.g. pioglitazone and rosiglitazone[107]) are among the clinically used ISAs. Metformin has been a very successful drug for controlling diabetes[108] although the majority of its hypoglycemic action does not result from insulin sensitization.[109] Several other drugs in this category have earned unwanted attention for safety concerns.[110-111] For all these reasons new and better drugs could and should be developed for the treatment of diabetes.

1.3. PLANTS AS A SOURCE OF NEW DRUGS

1.3.1. PLANTS THAT HAVE PRODUCED CLINICAL DRUGS

Natural products are a viable source for novel leads in many disease models, diabetes included. Some 900 plants have been recorded as being used for the treatment of diabetes. They include plants with ethnobotanical histories, as well as those which have been assayed through blind screening to show antidiabetic activity in

in vitro or *in vivo* tests or clinical trials. Through this effort a number of active compounds have been discovered allowing for the standardization of therapies utilizing plants as well as allowing for analogues of unique structure to be made.[24]

1.3.2. METFORMIN: FROM PLANT TO CLINIC

One example of the potential success of plants in the treatment of diabetes currently sits at the front of the market: Metformin. Its premier as a drug started in 1958 in the United Kingdom National Formulary, and was an analog developed by Jean Sterne from galegine, derived from the plant *Galega officinalis*. This species has a history of being used traditionally for the treatment of diabetes in Europe, Japan, and Chile.[18] It was approved by the FDA in 1994 for the treatment of T2DM and is currently being marketed by Bristol-Myers Squibb under the name originally given by its discoverer: Glucophage™. Metformin serves as a prototype, an indication that making a naturally derived clinically useful drug for diabetes is a possibility.[112]

CHAPTER II. EXTRACTION, FRACTIONATION, AND BIOLOGICAL EVALUATION OF A. ORIENTALE RHIZOME

2.1. INTRODUCTION

In order to study the antidiabetic potential of the rhizome of *A. orientale* it was first necessary to obtain authentic material from this species and prepare it for study, ensuring it is the correct species and that it displays the biological activity sought in this study as a whole. To achieve this, plant material was acquired and authenticated using a series of procedures to test its macroscopic, microscopic, and chemical attributes against that of previously authenticated material, as well as comparing it to previously established standards for the authentication of this species. Once assured of its correct identity plant material was extracted, partitioned, fractionated, and tested for biological activity.

2.2. EXPERIMENTAL PROCEDURES

2.2.1. PROCUREMENT OF PLANT MATERIAL

Batches of rhizome of *A. orientale* (Designated Blue, Red, Green, and Yellow batch) were obtained from reputable suppliers in Hong Kong through the School of Chinese Medicine, the Chinese University of Hong Kong (CUHK) and Nam Bac Hang medicinal herb supplier Chicago, Il. Additionally, taxonomically authenticated material (rhizome) was made available through a specimen in deposit at the Field Museum of Natural History, Chicago, Il (Collector Walter Koelz no. 4202, FM accession no. 149316, Punjab, India, 2000 ft., April 22, 1933). (FIGURE 9)

Authenticated Material		Voucher rhizome specimen removed from Walter Koelz-4202: FM 149316 in deposit at the Field Museum, Chicago, U.S.A.
Blue batch		Commercial sample from Zisun Chinese Pharmaceutical Co., Ltd. Guangzhou, China.
Yellow batch		Commercial sample of Royal King product of China from Kowk Shing Hong, Inc. New York, U.S.A.
Green batch		Commercial sample of Herbal Doctor product of China from Murray International Trading Co., Inc. New York, U.S.A.
Red batch		Commercial sample of Tsang Fook Kee Sailing Boat Brand product of China from Sunlike Trading Inc. New York, U.S.A.

FIGURE 9: SOURCE PLANT MATERIALS OBTAINED

FOR AUTHENTICATION AND EXTRACTION IN THIS PROJECT.

2.2.2. AUTHENTICATION OF PLANT MATERIAL

2.2.2.1. MACROSCOPIC AND ORGANOLEPTIC EXAMINATION

The blue batch was initially identified and authenticated to be of the highest commercial grade by the Chinese medicine experts at CUHK and underwent a vigorous authentication process once received in our facilities to ensure sample integrity. At UIC pharmacognostic authentications were conducted for all samples acquired. Specifically,

the macroscopic and organoleptic properties and microscopic features (histology-anatomy) of cross-sections and powders were characterized and compared with the previously authenticated blue batch, the rhizome of Walter Koelz-4202: FM 149316 removed from a voucher herbarium specimen at the Field Museum of Natural History (Permit attached in Appendix), and the characteristics established in the Chinese Pharmacopoeia and the Hong Kong Chinese Materia Medica Standards (HKCMMS).[15] Additionally, both thin-layer and high-performance liquid chromatographic (TLC and HPLC) fingerprinting profiles of this species were established.

For macroscopic characterization of *A. orientale* batches samples were initially examined in the state in which they were obtained for broad characteristics of size, shape, color, texture, taste, and smell.

The diagnostic features of the whole dried sliced rhizome as stated in the HKCMM Standards are as follows: Rhizome diameter 20-70 mm, externally yellowish or yellow/brown in color, textured with possible remnants of transverse-annular furrows and/or bud scars, yellow/white starchy interior with extensive small pores, slight odor, and slightly bitter taste.[15]

Commercial samples from the blue, yellow, green, and red batches were roughly similar in all aspects and comparable to the previously mentioned attributes stated in the HKCMM standards. Briefly, they all measured roughly 50 mm in diameter, with the green and yellow batches being slightly larger on average than the rest, and exhibited a light to medium yellow/brown, coarse exterior, the yellow and red batches of which contained moderately more cortex material, with a light yellow/tan/starchy/porous

interior, a slight musky/hay-like odor, and a slight sweet/bitter taste, the strongest of which were found in the yellow and red batches.

The rhizome of the Field Museum voucher specimen also matched these same attributes but was noticeably smaller, measuring in at just under 20mm, likely due to the age at which it was collected, and had a darker exterior which included the root hairs as it, unlike the commercial samples, had not been processed for use. It also exhibited less taste and odor, although some remained and was consistent with the other batches and the HKCMM Standards. This lack of taste/odor is possibly attributable to the age of the specimen at time of collection, as well as the significant period of time between the date of collection (1933) and our analysis of this sample. A summary of the results of the macroscopic evaluation in the authentication of *A. orientale* samples can be found in TABLE VIII.

2.2.2.2. MICROSCOPIC CHARACTERIZATION OF RHIZOME CROSS-SECTION AND OF POWDER

After macroscopic examination cross-sections of dried sliced rhizomes were made by soaking samples in water overnight and sectioning free-hand using a razor blade. For analysis of powdered samples dried sliced rhizomes were ground using a traditional grinding wheel mortar and pestle to semi-fine powder. In either case samples were then cleared using a chloral hydrate solution to remove debris. No staining was used in these examinations.

After clearing, cover slips were placed on the slides over the specimens and mounted on the microscope stage. From there the lighting was adjusted accordingly,

TABLE VIII: MACROSCOPIC AUTHENTICATION OF *A. ORIENTALE* SAMPLES IN COMPARISON WITH THE DIAGNOSTIC FEATURES STATED IN THE HKCMMS

SAMPLE	SIZE	EXTERIOR FEATURES	INTERIOR FEATURES	ODOR	TASTE
VOUCHER (FM 149316)	✓	✓	✓	✓*	✓*
BLUE BATCH	✓	✓	✓	✓✓*	✓✓*
YELLOW BATCH	✓	✓	✓	✓✓✓*	✓✓✓*
GREEN BATCH	✓	✓	✓	✓✓*	✓✓*
RED BATCH	✓	✓	✓	✓✓✓*	✓✓✓*
✓= match, ✓* = slight, ✓✓* = moderate, ✓✓✓ * = strong					

and the magnification was set first to 4x magnification to roughly home-in on the part of the specimen being examined and to locate and center features and then increasing magnification to 100x in the case of cross-section analysis and 400x in the case of powdered drug analysis and fine-tuning lighting to allow for better examination.

In line with the diagnostic features of the cross-sections as stated in the HKCMM Standards we detected possible residual cortex, an endodermis composed of a row of cells with thick lignified reinforced cell wall, amphivasal vascular bundles, and subobicular oil-secretory cavities.[15]

Diagnostic features of powdered material being sought in comparison to the HKCMM Standards are as follows: Numerous roughly spherical starch grains both externally and within parenchymatous cells, lignified endodermous cells, oil-secretory cavities either intact or broken, spiral vessels, and thick fibers.[15]

The cross-sections of samples of dried sliced rhizomes were comparable across samples including the Walter Koelz-4202: FM 149316 sample removed from a voucher herbarium specimen as well as the blue batch which was previously authenticated by the Chinese medicine experts at CUHK and were consistent with characteristics outlined in the HKCMM Standards The cross-section from the voucher specimen had significantly more cortex material present on the exterior, as did the red batch although to a lesser degree, and the oil-secretory cavities were generally smaller and fewer in number in the voucher specimen than those present within the commercial samples. This is likely due to the age of the plant at time of collection. The yellow and red batch exhibited slightly more and larger oil glands than the other commercial batches. A summary of the results from the cross-section analysis of samples of *A. orientale* are listed in TABLE IX.

In addition to assessing diagnostic features present in the cross-sections of rhizome, analysis of the features within powdered samples of these same specimens was also conducted.

The features observed within each batch, including the blue batch, as well as the voucher specimen, were generally comparable to each other and the characteristics laid out in the HKCMM Standards for powdered specimen authentication.

TABLE IX: MICROSCOPIC AUTHENTICATION OF *A. ORIENTALE RHIZOME* SAMPLE CROSS-SECTIONS

SAMPLE	CORTEX	ENDODERMIS	VASCULAR BUNDLES	OIL GLANDS
VOUCHER (FM 149316)	✓	✓	✓	✓
BLUE BATCH	✓	✓	✓	✓
YELLOW BATCH	✓	✓	✓	✓
GREEN BATCH	✓	✓	✓	✓
RED BATCH	✓	✓	✓	✓

A notable difference was that the voucher specimen powdered material contained more fibers than the commercial samples. Within the commercial samples, the yellow and red batch had slightly more fiber. These differences were attributed to the presence of more cortex material in these samples. The powdered specimen analysis, like that of the cross-section analysis, also found fewer oil-secretory gland remnants in the voucher sample. A particular abundance was found in the red and yellow batches. Additionally, less starch grains were seen in the overview of the voucher when compared to the commercial batches, again attributable to age. Within the commercial samples the blue and green batches were observed to contain more

starch grains overall. The powdered material analysis in the authentication of *A. orientale* samples and their results are found in TABLE X.

TABLE X: MICROSCOPIC AUTHENTICATION OF POWDERED *A. ORIENTALE* SAMPLES

SAMPLE	STARCH GRAINS	ENDODERMIS	OIL GLANDS	SPIRAL VESSELS	FIBERS
VOUCHER (FM 149316)	✓	✓✓	✓	✓✓	✓✓✓
BLUE BATCH	✓✓✓	✓✓	✓✓	✓✓	✓
YELLOW BATCH	✓✓	✓✓	✓✓✓	✓✓	✓✓
GREEN BATCH	✓✓✓	✓✓	✓✓	✓✓	✓
RED BATCH	✓✓	✓✓	✓✓✓	✓✓	✓✓
✓ = low quantity, ✓✓ = medium quantity, ✓✓✓ = high quantitity					

2.2.2.3. THIN-LAYER CHROMATOGRAPHIC (TLC) AUTHENTICATION

Plant material obtained from the blue batch (1.0 g) was sonicated in 30 ml methanol for 30 minutes and dried using a rotary evaporator. The extract was then

brought back up in 1 ml methanol and run through a reverse phase C-18 column pre-washed with 60 to 80% aqueous methanol. The standard solution of alisol B 23-acetate was prepared by dissolving 1 ml of a commercial sample of alisol B 23-acetate standard in 2 ml methanol.

The test solution and alisol B 23-acetate standard solution were spotted on a high performance thin layer chromatographic (hptlc) plate. The plate was developed with a mixture of petroleum ether and ethyl acetate (8:9 v/v) and sprayed with a solution made from 50% sulphuric acid and 2% p-hydroxybenzaldehyde in methanol (1:10 v/v) and heated slowly until the appearance of color. The test solution was done in duplicate, and the layout included a blank as well as a spiked sample. The results of this analysis indicate the presence of alisol B 23-acetate in the blue batch of *A. orientale* (FIGURE 10).

TLC analysis was also performed to determine the presence of alisol B 23-acetate in the red, green, and yellow batches (not shown).

2.2.2.4. HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC (HPLC) FINGERPRINTING

A solution was made by sonicating 1 g of powdered material in 20 ml methanol followed by centrifugation at 3k x g for 5 min and filtration through a 0.45 µm filter.

An alisol B 23-acetate standard solution was made by diluting the standard stock 10:1 using methanol and filtered using a 0.45 µm filter.

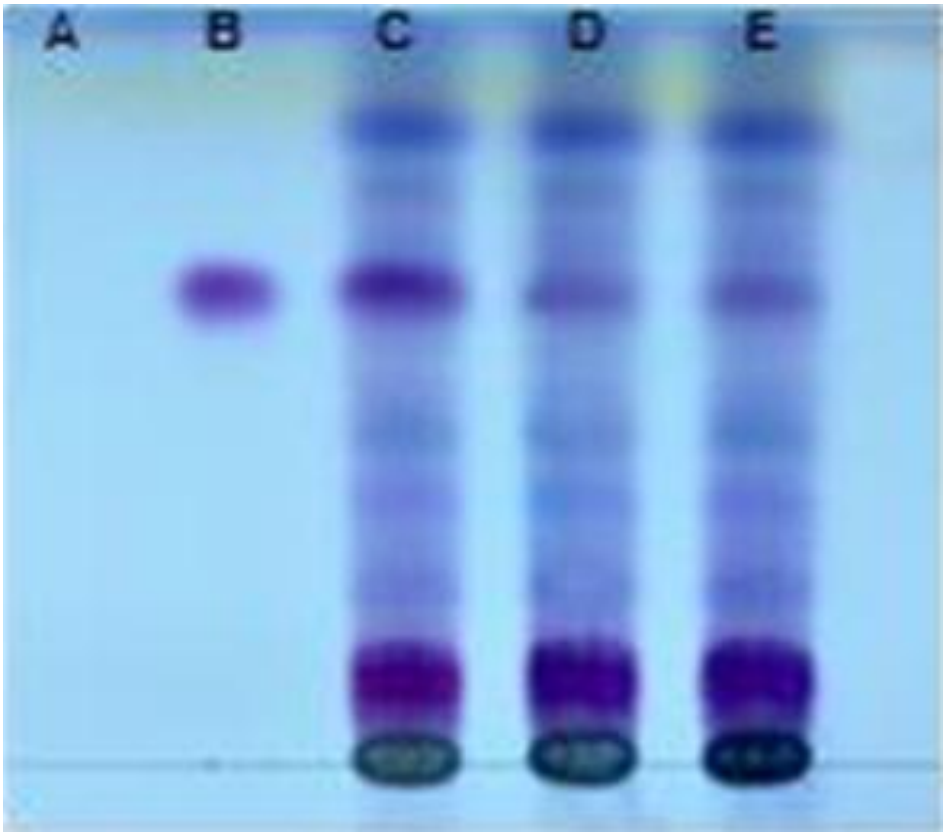


FIGURE 10: TLC AUTHENTICATION AND BATCH COMPARISON OF *A. ORIENTALE* USING STANDARD ALISOL B ACETATE. 1) A = BLANK, B = ALISOL B 23-ACETATE, C = SPIKED SAMPLE, D, E = SAMPLE.

The HPLC analysis followed a slightly modified method from the Hong Kong Chinese Materia Medica Standards. The elution program is shown in TABLE XI.

The retention times and fingerprint at 210 nm obtained for all batches of samples were consistent with one another and showed the presence of alisol B 23-acetate (FIGURE 11).

TABLE XI: HPLC ELUTION PROGRAM FOR THE AUTHENTICATION OF THE RHIZOME OF *A. ORIENTALE*

TIME (MIN)	0.1% ACETIC ACID (% V/V)	ACETONITRILE (% V/V)	ELUTION
0-10	80	20	ISOCRATIC
10-45	80-0	20-100	LINEAR GRADIENT
45-60	0	100	ISOCRATIC

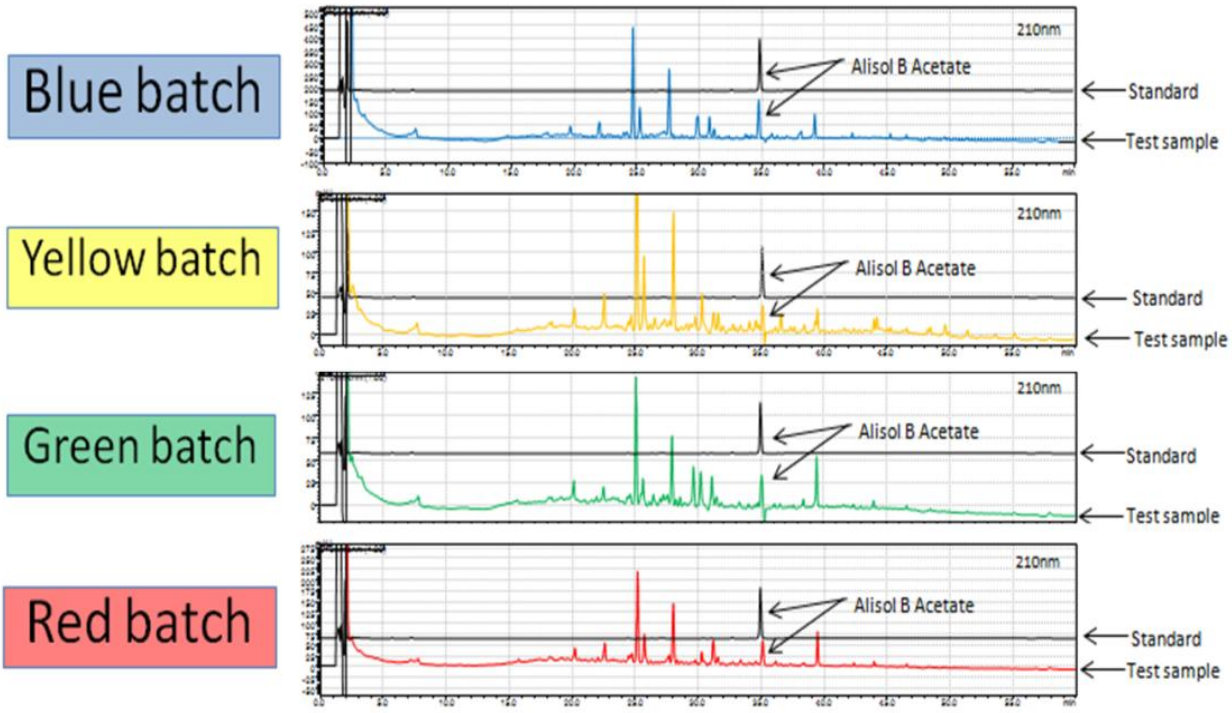


FIGURE 11: HPLC VALIDATION OF BATCHES OF *A. ORIENTALE* RHIZOME USING THE 1) HKCMMS STANDARD PROCEDURE USING ALISOL B 23-ACETATE AND 2) BATCH COMPARISON USING SLIGHT MODIFICATION OF HKCMMS PROCEDURES.

2.2.3. EXTRACTION AND FRACTIONATION

Plant material from the different batches were pooled, ground into powder, and extracted by percolation with 95% ethanol. Excessive solvent was removed and the dried extract was subsequently brought up in water and partitioned with ethyl acetate.

The ethyl acetate part was removed in stages, combined and dried, and taken on for further separation. The water part was repeatedly partitioned with butanol, combined, and dried for further separation (DIAGRAM 1).

The ethyl acetate fractions were subjected to a flash silica column to produce the E series with six distinct regions, designated E1-E6 for testing (DIAGRAM 2).

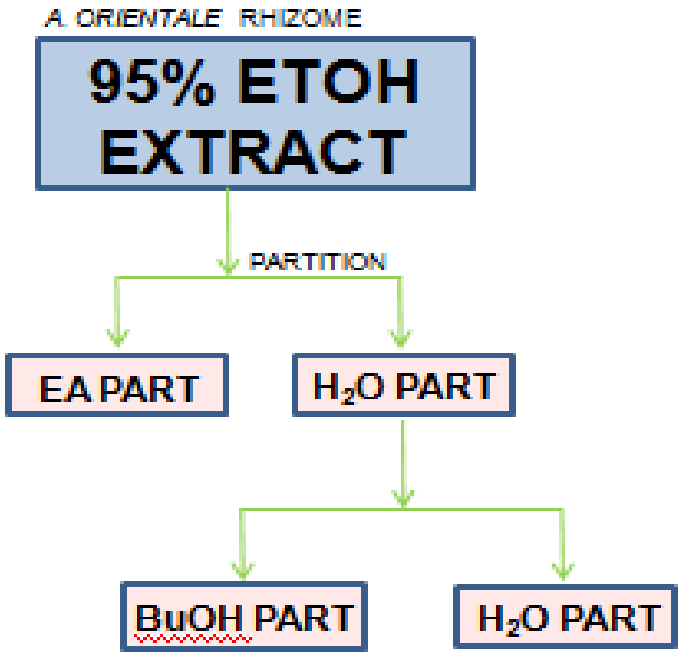


DIAGRAM 1: THE INITIAL EXTRACTION AND PARTITIONING OF *A. ORIENTALE*.

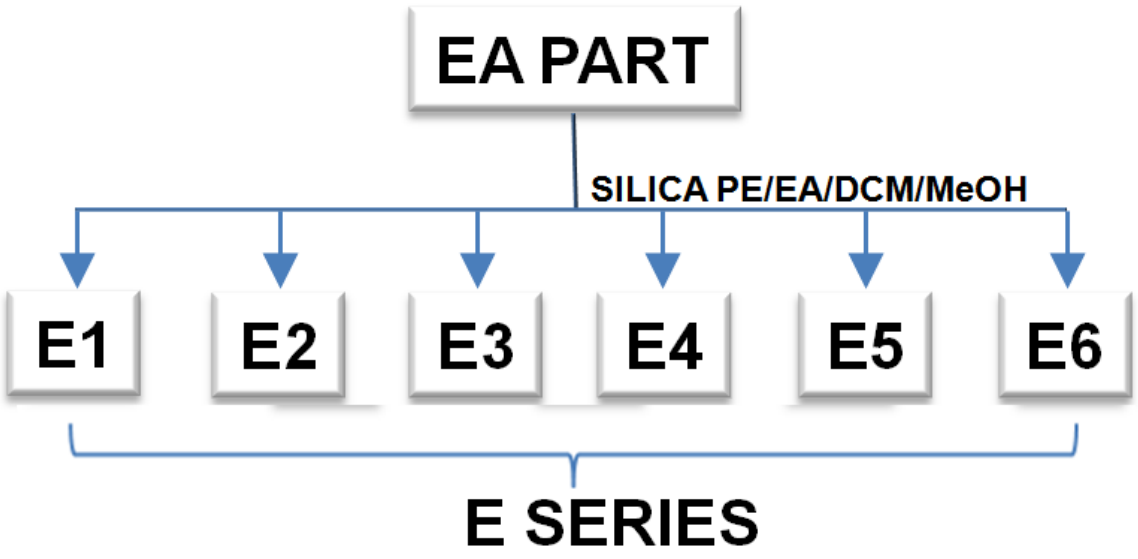


DIAGRAM 2: GENERAL LAYOUT AND TLC ANALYSIS OF THE FIRST PHASE OF FRACTIONATION OF THE EA PART OF *A. ORIENTALE.*

The butanol partition underwent an initial crude separation using a reverse-phase column and an H_20/ETOH mobile phase. The gradient moved from H_2O to 95% ETOH in 4 steps from 100% H_20 to 95% ETOH. Each step was combined to form the B series made up of fractions B1-B5 for testing (DIAGRAM 3).

2.2.4. DETERMINATION OF α-GLUCOSIDASE INHIBITING ACTIVITY

The α-glucosidase inhibition assay is a kinetic assay used to determine the ability of extracts, partitions, fractions, and pure compounds to inhibit the breakdown of trisaccharides and disaccharides into glucose by the endogenous enzyme α-glucosidase (DIAGRAM 4).

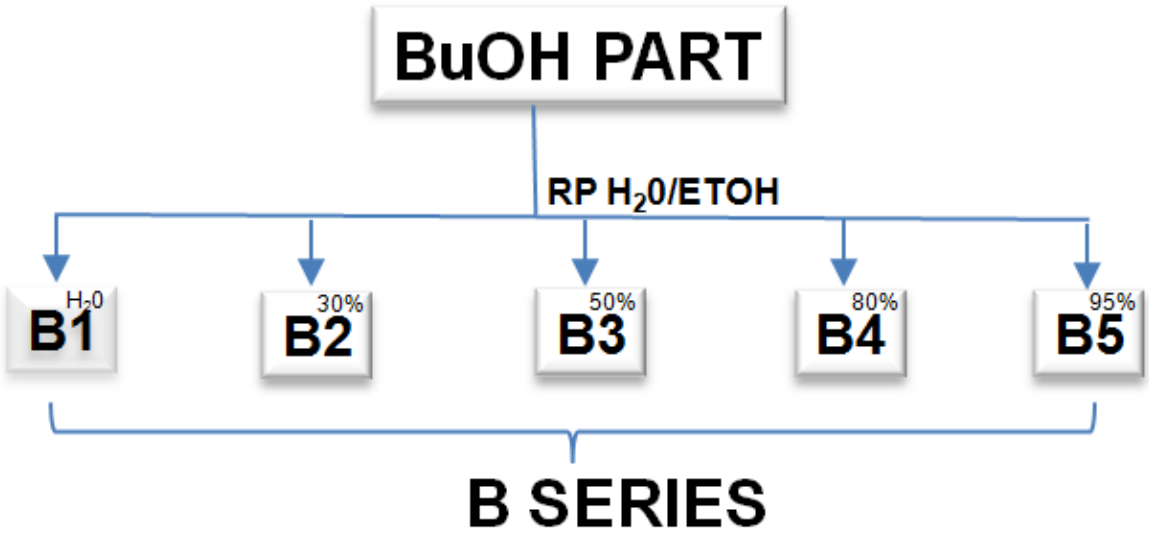


DIAGRAM 3: SCHEME FOR THE PRODUCTION OF THE B SERIES AS A PART OF THE BIOACTIVITY-GUIDED FRACTIONATION OF THE BUTANOL PARTITION.

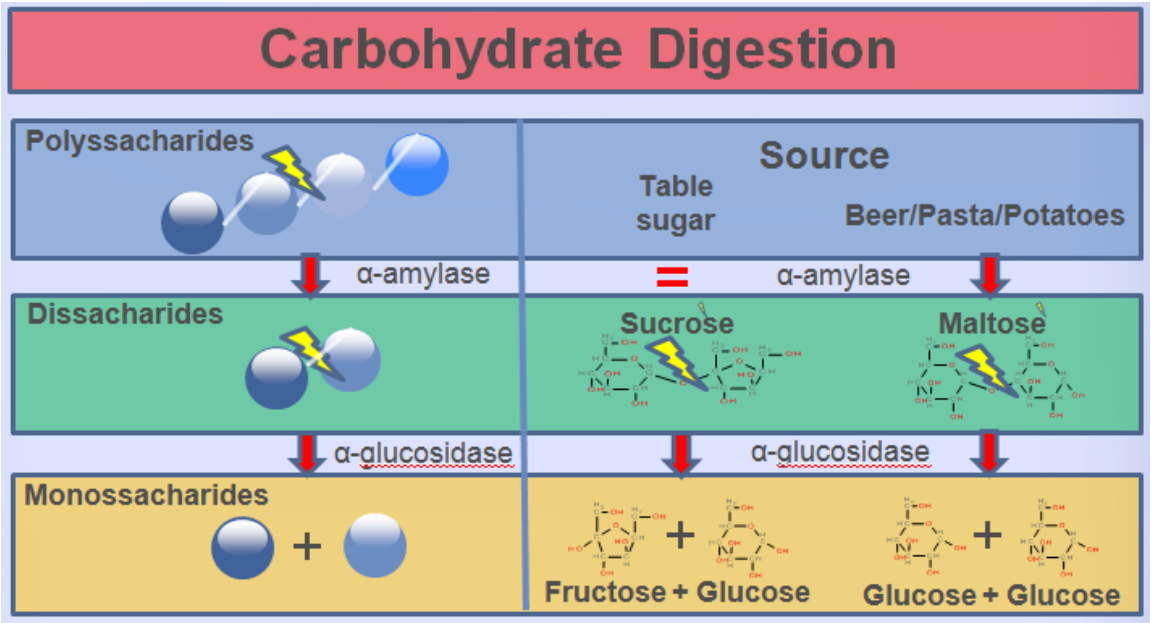


DIAGRAM 4: REPRESENTATION OF THE PROCESS BY WHICH COMPLEX CARBOHYDRATES ARE BROKEN DOWN INTO MONOSACCHARIDES SUCH AS GLUCOSE.

This biological process is relevant to the treatment of diabetes because it is the cause of postprandial glucose spikes and therefore a target for preventing them. α-glucosidase enzymes of both yeast and animal source were used in versions of this assay. Animal-derived α-glucosidase models measure the same activity as yeast-source models but have the added benefit of more closely approximating the enzyme types found in the human intestine. For our purposes this assay was used to check that our leads were viable and not the result of activity that is likely not relevant to humans.

These assays utilize the modified sugar 4-Nitrophenyl β-D-glucopyranoside (PNPG), which contains a sugar moiety as well as a conjugated moiety that, when cleaved by enzyme, turns yellow in color and strongly absorbs light at 400 nm. These assays are performed in a 96-well format that uses twin sample and blank wells. The blank wells are designed to measure the inherent absorbance of the components of the assay, especially the treatments being tested (e.g. extracts, partitions, and fractions which are often colored). Thus by introducing test samples and monitoring this process, inhibitors of these enzymes can be identified.

In both versions fractions and compounds were first thoroughly dried using a rotovap in combination with P_20_5 under vacuum over night, or lyophilization for at least three days. Samples were typically weighed and dissolved in 100% DMSO to 100 mg/ml. Samples were then diluted in water using serial dilution to 10x the final concentration desired for the test.

In the yeast-source enzyme version of the assay A MAK123 α-glucosidase activity assay kit (Sigma Aldrich, St. Louis, MO) was used according to the

recommended procedures with slight modification. Sample wells contain the test sample (20 µl), and a mixture of 3 mM glutathione (20 µl) and 0.7 U/ml α-glucosidase (20 µl) in 67 mM potassium phosphate buffer (pH = 6.8)(140 µl) at 37 °C. Wells in the blank group have water (20 µl) substituted in for the α-glucosidase enzyme.

A literature search for the first 15 publications[113-127] that report the IC_{50} of acarbose in assays which use α-glucosidase enzyme sourced from Sigma Aldrich yielded acarbose IC_{50}'s ranging from 0.051[125] to 3.52[117] mM with a mean of 0.59 mM and a median of 0.34 mM. This is in line with the 0.15 mM IC_{50} of acarbose reported in this project.

The animal-source enzyme version roughly follows that of Jo[128] and Mohamed[129]. Acarbose IC_{50}'s have similar values between Jo, Mohamed, and the procedure used (0.05mM, 0.053mM, and ~0.05mM respectively).

In this version sample wells consist of test sample (20 µl), 3 mM glutathione (20 µl), an α-glucosidase solution (Enzyme) made from 50 mg/ml rat intestinal powder in 50 mM potassium phosphate buffer (40 µl), in 0.1 M potassium phosphate buffer (120 µl). Blank wells substitute the enzyme with water (20 µl).

In both forms of the assay to initiate the reaction PNPG (20 µl) is added to both sample and blank wells, and plates were briefly agitated by tapping/swirling.

The raw data from the α-glucosidase inhibition test is read using a Biotek Synergy H4 Hybrid Multi-Mode Microplate spectrophotometer. There are two types of data recorded, time and absorbance at 400 nm. Times aligned in columns represent the different read sequences. For the α-glucosidase inhibition study reads occurred

every one minute starting at 0:02 seconds. The values within each row represent absorption values within each read sequence. All wells in the 96-well plate are read each sequence (each minute) in the same order. Reported within each row are the reads taken for a particular well in the 96-well plate. There are 41 rows in each column containing data for each of the 41 reads, once per minute over 40 minutes. The last run (time 0:39:02) is used in the production of the bar graph and for the One-Way ANOVA Dunnette's Multiple Comparison Test in Graphpad Prism by comparing sample values against the no treatment group at 40 min. Error bars were added from the statistical analysis to represent standard deviation.

2.3. RESULTS AND DISCUSSION

Aqueous ethanol (70%) extracts of the red, green, blue, and yellow batches were all tested in the yeast source α-glucosidase inhibition assay (FIGURE 12) at concentrations of 1, 5, and 10 mg/ml.

The assay resulted in a 25% enzyme activity with 5 mM acarbose compared to the no treatment group indicating a working assay.

The red batch saw a slight increase in enzymatic activity at a dose of 1 mg/ml that was statistically significant. The higher doses of 5 and 10 mg/ml did not affect the enzyme appreciably.

All three concentrations of the green batch caused a strong, statistically significant decrease in α-glucosidase activity. These reductions were dose dependent and resulted in total enzyme activities of 58, 52, and 44% at concentrations of 1,5, and 10 mg/ml, respectively.

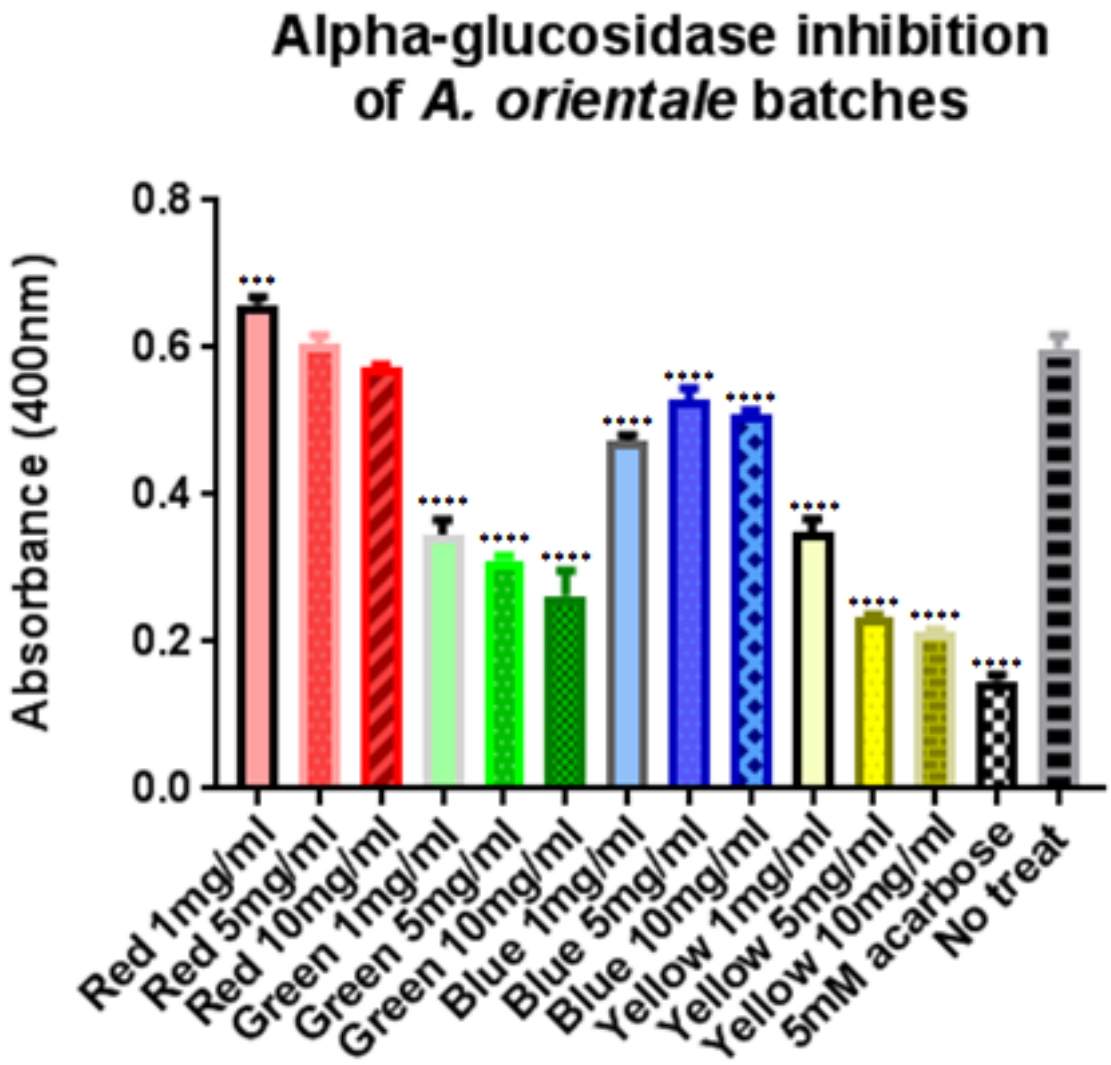


FIGURE 12: α-GLUCOSIDASE INHIBITION OF THE DIFFERENT BATCHES OF *A. ORIENTALE* USING YEAST SOURCE ENZYME. NS (NO MARK) = P > 0.05, *** = P ≤ 0.001, **** = P ≤ 0.0001 COMPARED TO NO TREATMENT.

The blue batch displayed moderate inhibition across the board, the most potent of which was seen at the low dose of 1 mg/ml which caused an enzyme activity of 79%.

The yellow batch brought about significant reductions in α-glucosidase enzyme activity at all three concentrations that were dose dependent and statistically significant. At a concentration of 1 mg/ml enzyme activity was 58%, at 5 mg/ml it was 39%, and at 10 mg/ml it was 36%.

In addition to testing in the yeast-source α-glucosidase inhibition test, the red, green, blue, and yellow batches were tested in an animal-source α-glucosidase inhibition test (FIGURE 13).

The animal-source α-glucosidase assay reported a 21% enzyme activity using 5 mM acarbose when compared to the no treatment group. This indicates a working assay.

All batches, at all doses displayed the ability to reduce α-glucosidase enzyme activity, albeit a few were found to not be statistically significant upon detailed analysis.

The red batch showed a concentration-dependent reduction in enzyme activity with 56, 31, and 28% activity at concentrations of 0.1, 0.5, and 1 mg/ml, respectively.

The green batch displayed concentration-dependent α-glucosidase inhibition. At a concentration of 0.1 mg/ml enzyme activity was measured at 69%. At a concentration of 0.5 mg/ml enzyme activity was measured at 37%, and at a concentration of 1 mg/ml it was reduced to 10% activity.

The blue batch exhibited significant α-glucosidase inhibition at all doses. These ranged from 60% activity at 0.1 mg/ml to 46% at 0.5mg/ml to 36% at 1 mg/ml.

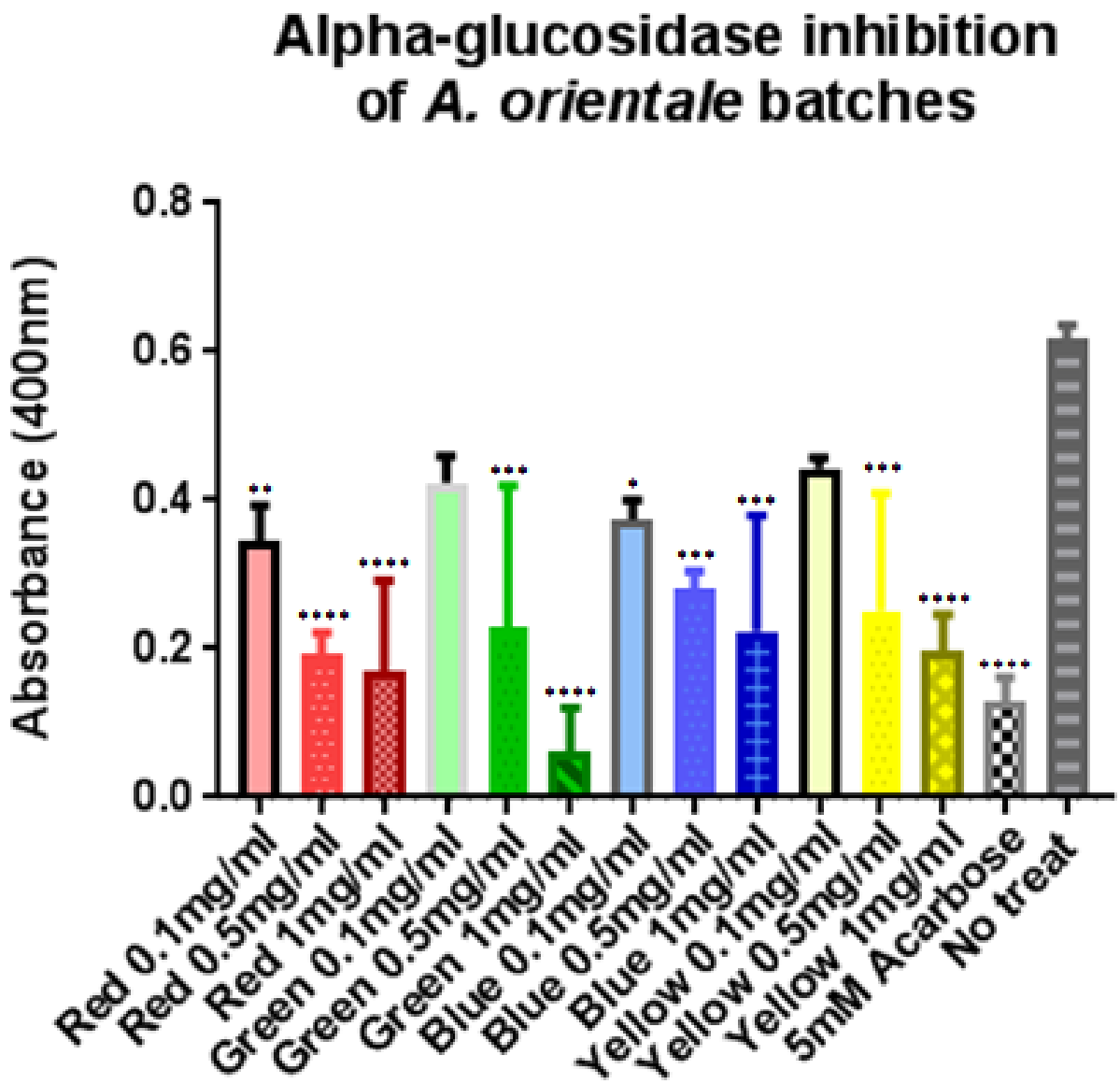


FIGURE 13: α-GLUCOSIDASE INHIBITION OF THE DIFFERENT BATCHES OF *A. ORIENTALE* USING ANIMAL SOURCE ENZYME. NS (NO MARK) = P > 0.05, * = P ≤ 0.05, ** = P ≤ 0.01, *** = P ≤ 0.001, **** = P ≤ 0.0001 COMPARED TO NO TREATMENT.

The yellow batch was observed producing α-glucosidase inhibition in a dose dependent manner. At a concentration of 0.1 mg/ml enzyme activity was measured at

72%. Through statistical analysis this moderate inhibition was not revealed to be significant. However, at concentrations of 0.5 and 1 mg/ml enzyme activity was measured at 41 and 32% and these results were found to be statistically significant.

Enzyme inhibitory activity was then determined using the EA and Bu partitions. The EA part showed a moderate inhibition of 62.5% at a concentration of 10 µg/ml, reaching 92.1% inhibition at 25 µg/ml, and causing as high as 97.5% enzyme inhibition at a concentration of 75 µg/ml. Similarly, the Bu part was observed to cause 10.5% inhibition at 10 µg/ml, a 63.4% enzyme inhibition at a concentration of 25 µg/ml, and reaching 84.7 and 92.5% at the highest concentrations of 50 and 75 µg/ml. The H_2O part was less active, producing a 12.2, 7.3, 30.9, and 79.7% enzyme inhibition at 10, 25, 50, and 75 µg/ml concentrations, respectively (FIGURE 14). Based on these results, subsequent studies were focused on the EA and Bu partitions.

Testing of the butanol fraction resulting in significant enzyme inhibition potential within fractions B1 and B3 of the butanol fraction (FIGURE 15). More specifically, the results of this test indicate there is statistically significant α-glucosidase inhibition in all five fractions. However, fractions B1 and B3 emerged as fractions of interest because of their activity.

Fraction B1 had the greatest inhibitory potential reducing enzyme activity to a startling 12.5% at 50 µg/ml, and reducing it further to 11.7% at 75 µg/ml. Fraction B3 also displayed modest but nonetheless notable inhibition more than halfing enzyme activity at 43.8 and 42.2% at these same concentrations.

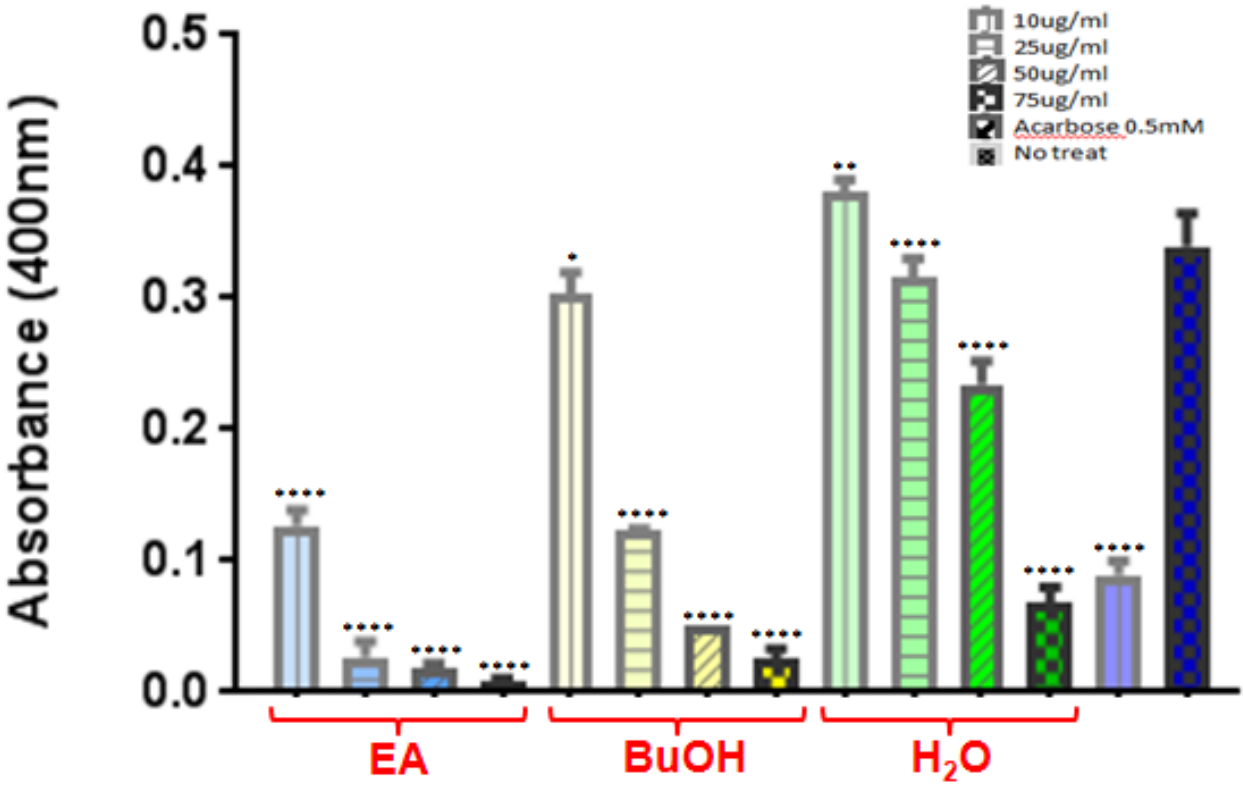


FIGURE 14: α-GLUCOSIDASE INHIBITION RESULTS FROM THE EA, BU, AND H20 PARTITIONS.

* = P ≤ 0.05, ** = P ≤ 0.01, **** = P ≤ 0.0001 COMPARED TO NO TREATMENT.

Significant enzyme inhibitory activity was also observed in fractions E2, E3, and E5 of the EA partition. Fraction E2 exhibited α-glucosidase inhibition activity at all doses in a dose-dependent and statistically significant way. Enzyme activity was measured at 35, 10, 3, and 2% at concentrations of 10, 25, 50, and 75 µg/ml, respectively.

Fraction E3 also displayed inhibition that was both dose-dependent and statistically significant. For this fraction enzyme activity at 10, 25, 50, and 75 µg/ml concentrations were 27, 11, 5, and 3% of the control.

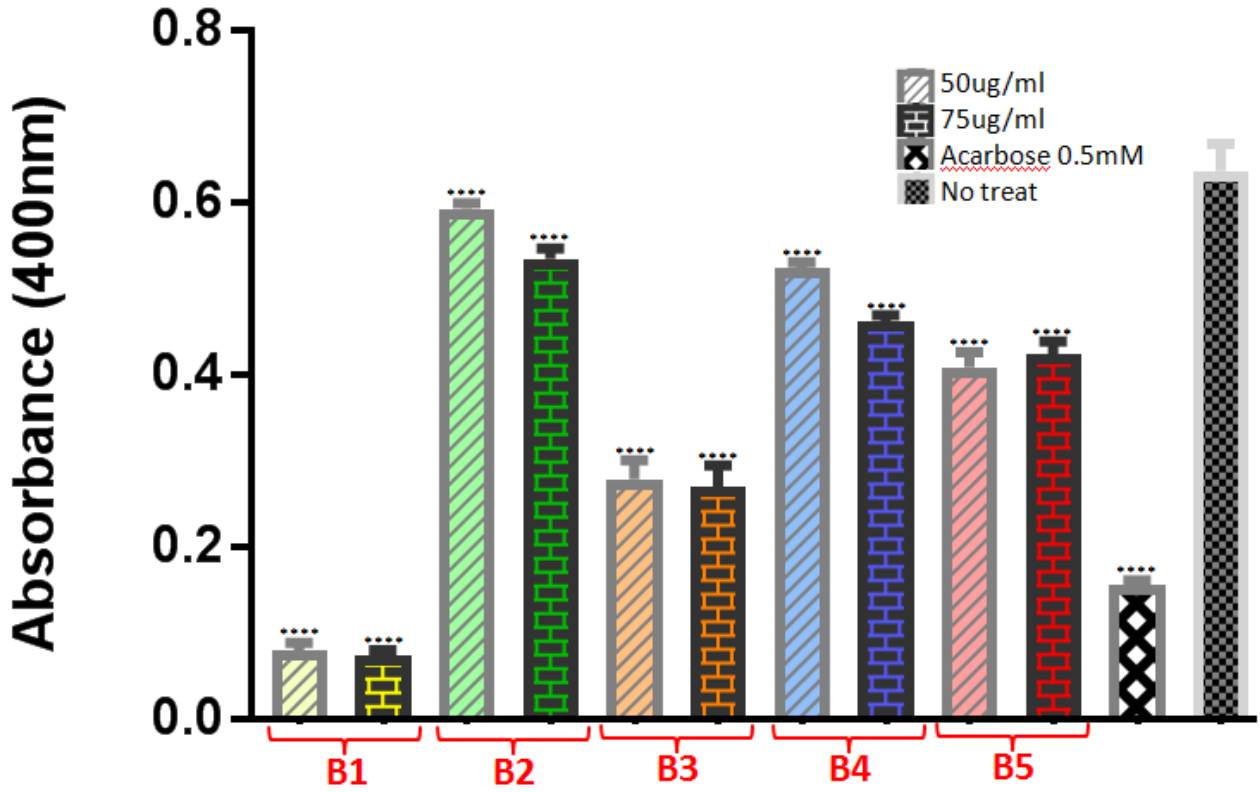


FIGURE 15: α-GLUCOSIDASE INHIBITION RESULTS FOR FRACTIONS B1-B5 OF THE BUTANOL PARTITION. **** = P ≤ 0.0001 COMPARED TO NO TREATMENT.

Fraction E5 displayed statistically significant enzyme reduction at all concentrations. These were measured at 83, 71, 54, and 49% enzyme activity for the concentrations tested, respectively (FIGURE 16).

2.4. CONCLUSION

The preceding experiments demonstrate the possession of materials from a fully authenticated plant species with demonstrated biological activity for use in isolating

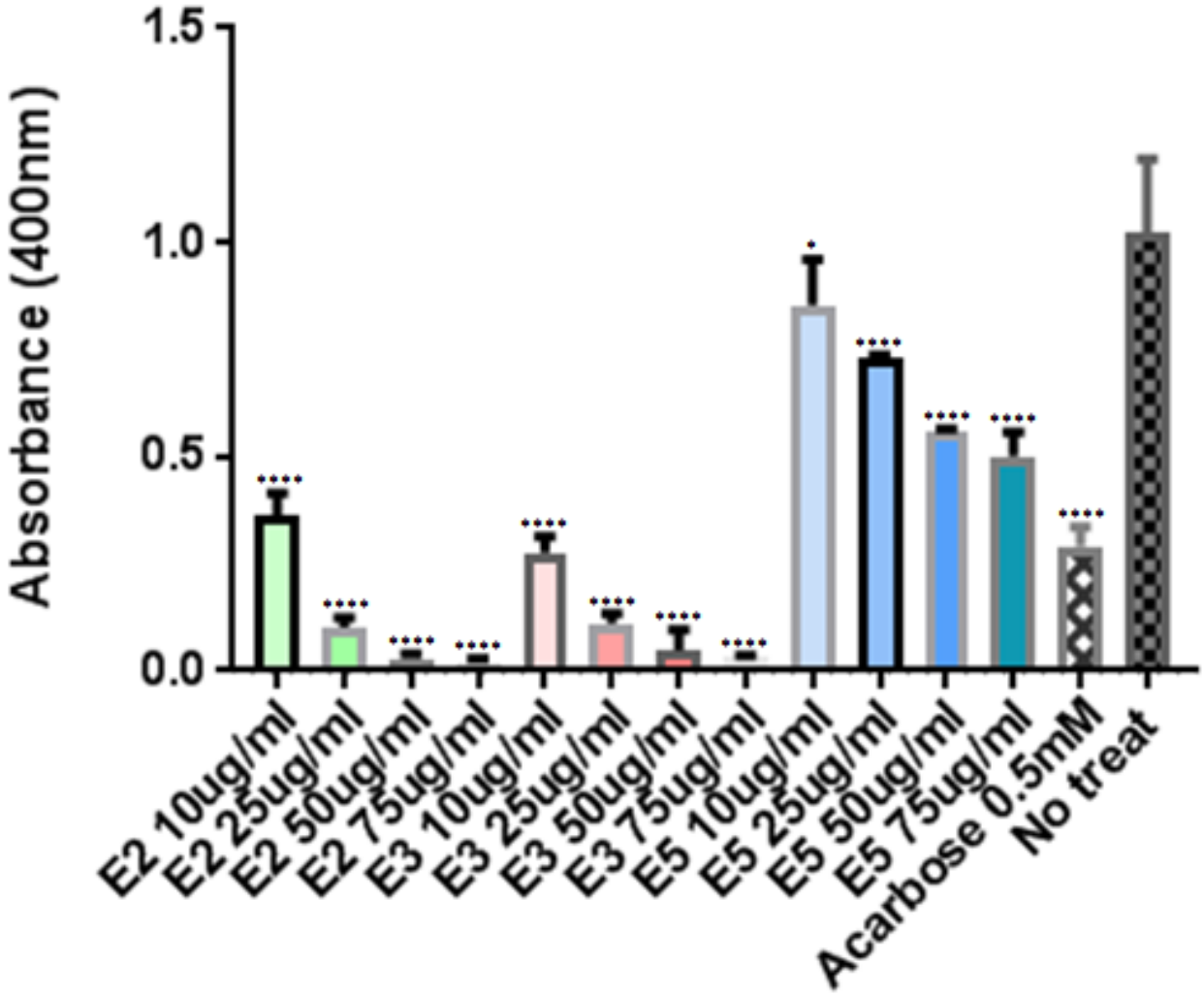


FIGURE 16: α-GLUCOSIDASE INHIBITION RESULTS OF FRACTIONS FROM THE EA PART OF *A. ORIENTALE.* * = P ≤ 0.05, **** = P ≤ 0.0001 COMPARED TO NO TREATMENT.

active constituents. This activity was observed in individual batches, as well as their combination in subsequent partitioning. Further, the initial chromatographic fractions produced in the butanol and ethyl acetate fractions demonstrated activity that informed further separation by identifying areas in which the biological activity resides.

CHAPTER III. ISOLATION OF CHEMICAL CONSTITUENTS AND BIOLOGICAL EVALUATION OF *A. ORIENTALE* RHIZOME

3.1. INTRODUCTION

The fractionation and isolation of active constituents from the butanol and ethyl acetate partitions were conducted using a variety of chromatographic techniques specific to the chemistry present within these respective parts of the plant.

3.2. EXPERIMENTAL PROCEDURES

3.2.1. GENERAL EXPERIMENTAL PROCEDURES

Chemical separation was achieved using the process of open column chromatography on both normal (silica) stationary phases, as well as reverse-phase (MCI) columns. Samples were dry-loaded in the former method using suitable solvents and eluted using 3-5 bed-volumes of solvent at various degrees of polarity in a gradient. In the latter method samples were wet-loaded using aqueous methanol, sometimes with the help of volatile organic solvents such as DCM and EA in order to achieve solubility. In both methods suitable mobile phase gradients for separation were determined by TLC to achieve separation of the constituents of interest at ~0.3 Rf.

3.2.2. SEPARATION OF THE ETHY ACETATE-SOLUBLE FRACTION

Fractionation of the ethyl acetate partition was mainly bioactivity-guided with areas of interest represented by their ability to inhibit α-glucosidase enzyme. This process started with groupings and a primary analysis of the crude fractions of the ethyl acetate partition.

This fractionation led from the EA grouping of E5 into subsequent fractionation to yield several compounds isolated for testing in the α-glucosidase assay (DIAGRAM 5).

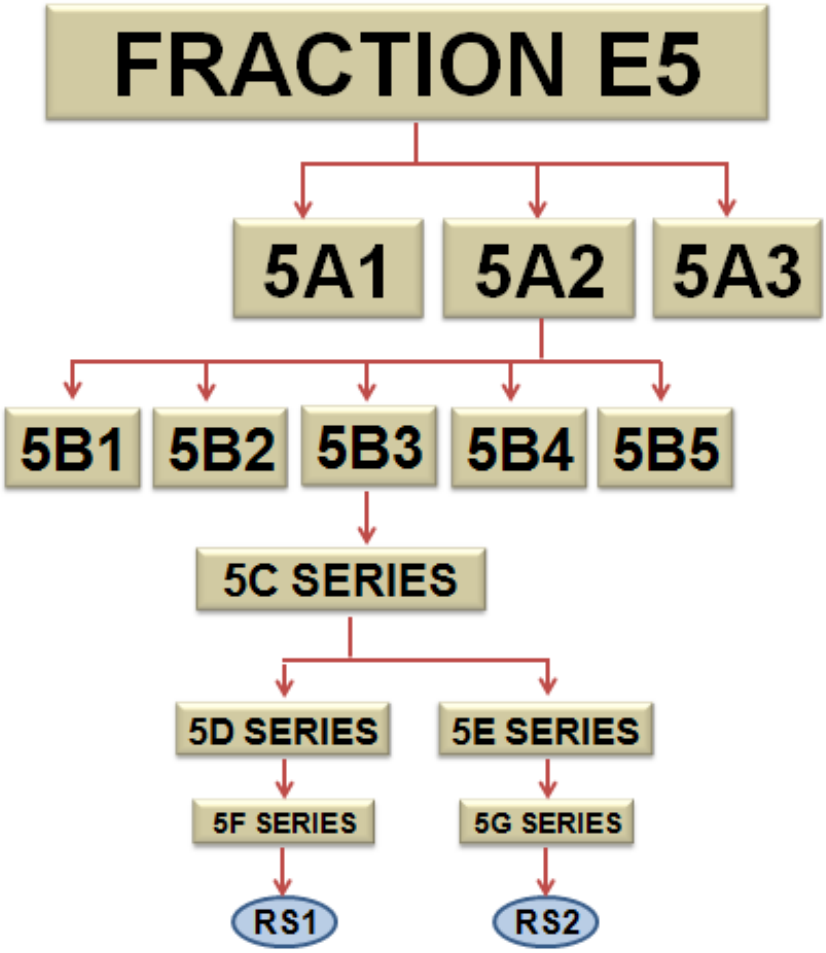


DIAGRAM 5: REPRESENTATION OF THE BASIC LAYOUT OF THE ISOLATION OF THE ETHYL ACETATE FRACTION IN AREAS OF INTEREST.

What follows are the fractionation schemes which produced pure isolates that were tested in the α-glucosidase inhibition assay using this method.

Silica columns using first a wide-range pure DCM to pure MeOH gradient in six steps, two of which were taken on and followed by a more narrow range of 95:5 to 90:10 DCM/MeOH in two steps. The biological activity was then followed up using a reverse-phase column with a 50-100% MeOH mobile phase consisting of eight steps. These fractions were then combined based on their chemical signatures on TLC into two groups of major components, put through two respective reverse-phase cleanup columns each, and produced the compounds RS1 and RS2 for identification and biological testing.

Compounds tested within this project were obtained from isolation of *A. orientale* rhizome as well as from commercially available standards. These compounds (designated RS or ZX) could be detected in the ethyl acetate fraction and they include non-ubiquitous triterpenes and sesquiterpenes which are the major compound classes of secondary metabolites within this species, which have the potential to explain observed bioactivity.

A list of all the compounds this project tested from the ethyl acetate partition, complete with their compound codes as used in this text, compound ID's, and compound class are listed in TABLE XII.

3.2.3. SEPARATION OF THE BUTANOL-SOLUBLE FRACTION

After identifying main areas of interest, the isolation on the butanol partition was mainly done blind and not guided by bioactivity. Rather, it was based on chemical analysis, specifically the TLC and HPLC patterning. This way the focus would be on isolating compounds in the areas of interest, albeit without knowing their bioactivity

beforehand. After gathering some group of compounds chosen by their quantity or characteristics suggesting a compound type of interest and attempting to purify them they were then tested to determine their bioactivity. This alternative approach is

TABLE XII: COMPOUNDS TESTED FROM THE ETHYL ACETATE-SOLUBLE FRACTION.

COMPOUND CODE	COMPOUND ID	COMPOUND CLASS
NONE	ALISOL B	TRITERPENE
NONE	ALISOL B 23-ACETATE	TRITERPENE
RS1	ALISOL A	TRITERPENE
RS2	13,17-EPOXY ALISOL A	TRITERPENE
ZX-18	25-ANHYDROXY ALISOL A	TRITERPENE
ZX-10	DAUCOSTEROL 6'-O-STEARATE	TRITERPENE
ZX-6	ALISOL O	TRITERPENE
ZX-9	ALISOL P	TRITERPENE
ZX-3	ALISOL E 23-ACETATE	TRITERPENE
ZX-17	25-0-METHYL ALISOL A	TRITERPENE
ZX-8	ALISOMOXIDE	SESQUITERPENE

sometimes taken to avoid issues tracing the activity through complex fractions during the process of bioactivity-guided fractionation, as compounds also present alongside target compounds with activity can have opposing actions or off-target interactions that can mask the activity of these targets and prevent them from being detected.

Fraction B1 was fractionated on a C-18 column using an aqueous ethanol gradient into three subfractions: C1, C2, and C3. These three fractions went on to subsequent fractionation to yield a number of compounds isolated for testing in the α-

glucosidase assay (DIAGRAM 6). What follows are fractionation schemes which produced pure isolates that were tested in the α-glucosidase inhibition assay.

Fraction C1, was subjected to open column fractionation using a silica stationary phase and a DCM/MeOH/H_20 mobile phase consisting of a five-step gradient each of which were combined and designated D1-5 (the D series). Step one was a 7:3

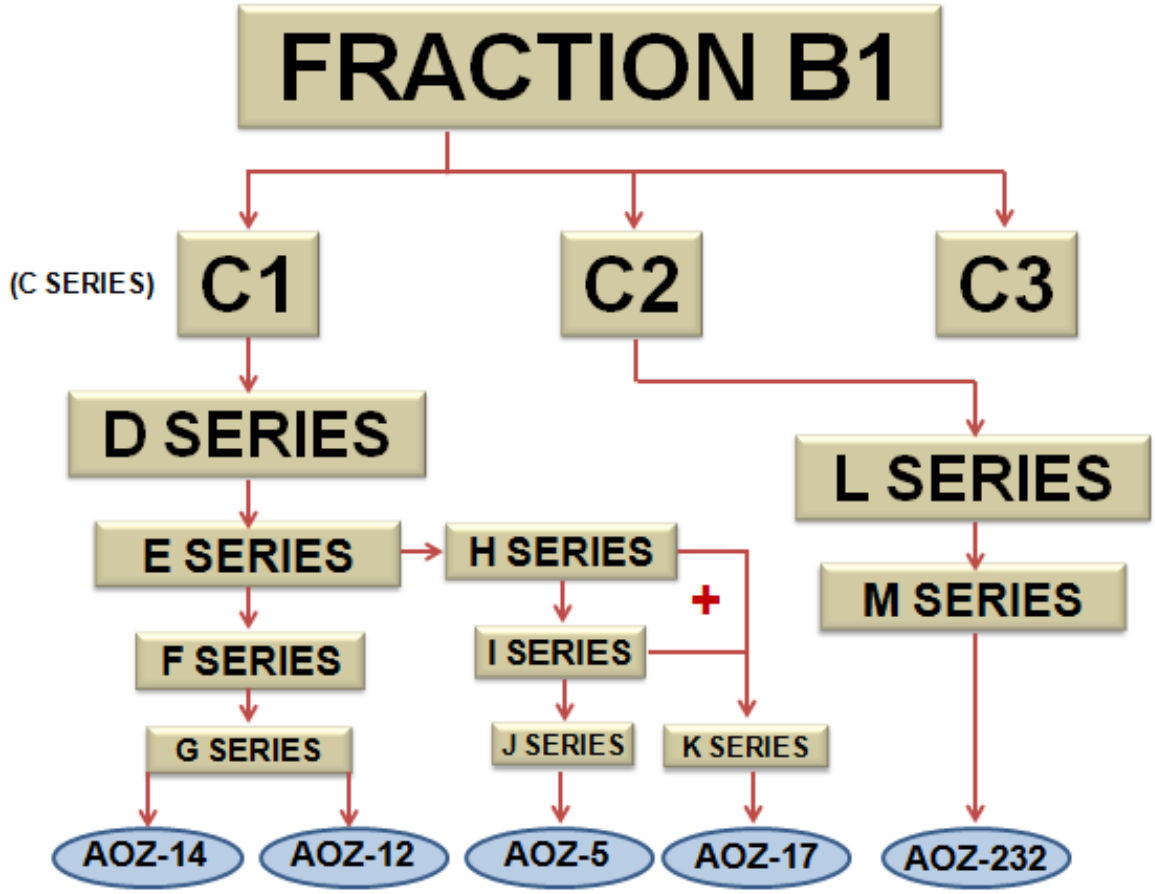


DIAGRAM 6: ISOLATION OF THE BUTANOL FRACTION

dichloromethane/methanol mix (D1), followed by 5:5 (D2), methanol (D3), 90% aqueous methanol (D4), and 70% aqueous methanol (D5) steps.

Fraction D1 was taken on to another normal-phase silica column using a DCM/MeOH mobile phase. This column used a four-step gradient of DCM/MeOH mixtures in 9:1, 8:2, 7:3, and 5:5 ratios to afford sub-fractions E1-E4 (the E series).

Further fractionation of E2 on a normal-phased silica gel column eluted with DCM/MeOH led to the F Series of F1 through F7 and subsequently G1-G10 of the G series..

Subfractions G6 and G10 within the G series afforded two compounds: AOZ-14 and AOZ-12.

Fraction E3 was separated on a sephadex LH-20 column using a H_2O/MeOH gradient to obtain the H series of fractions H1 through H3. H1 was taken on to a sephadex LH-20 column using an isocratic elution of 70% MeOH producing I1-6 or the I series. I2 was also taken on to a sephadex LH-20 column using a 100% MeOH isocratic elution, producing the J series consisting of J1-J7. Fraction J6 of the J series produced the isolate AOZ-5.

A combination of fractions from the I and J series was used going forward. Specifically, fraction I6 from the I series was combined with fractions J4, J5, and J6 from the J series. Further purification on sephadex LH-20 column using an MeOH isocratic gradient led to the isolation of AOZ-17.

Fraction C2 was separated on silica and eluted by a DCM/MeOH/H_2O mobile phase. A linear gradient starting from 9:1, followed by an 8:2, 7:3, and 5:5 DCM/MeOH, 100%, and 80% MeOH mix afforded the L Series of fractions L1 through L6.

From L1, further chromatogrphaic separation on a sephadex column using an isocratic MeOH mobile phase produced the M series of fractions, of which M9 led to the isolation of AOZ-232.

A list of all the compounds this project tested from the butanol partition, complete with their compound codes as used in this text, compound ID's, and compound class are listed in TABLE XIII below.

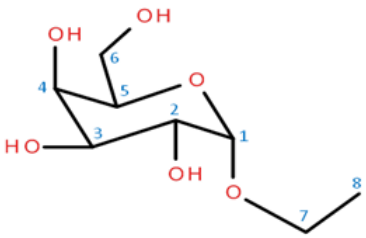
TABLE XIII: COMPOUNDS TESTED FROM THE BUTANOL-SOLUBLE FRACTION

COMPOUND CODE	COMPOUND ID	COMPOUND CLASS
AOZ-14	1-O-ETHYL-D-GALACTOPYRANOSE	MONOSACCHARIDE
AOZ-5	D-GALACTOPYRANOSE	MONOSACCHARIDE
AOZ-17	D-FRUCTOPYRANOSE	MONOSACCHARIDE
AOZ-12	CYTIDINE	NUCLEOSIDE
AOZ-232	ALISMOXIDE	SESQUITERPENE

3.3. STRUCTURAL CHARACTERIZATION OF ISOLATED COMPOUNDS

Structural characterization for the compounds isolated from the butanol and ethyl acetate fractions were primarily based on analyzing 13C NMR spectra obtained from these isolates and comparison with published data in the literature.

3.3.1. STRUCTURAL CHARACTERIZATION OF 1-O-ETHYL-D-GALACTOPYRANOSE (AOZ-14)



The 13C nmr spectrum of AOZ-14 displayed sixteen carbon signals (TABLE XIV). The most characteristic chemical shift present within the spectrum was observed to be the carbon at δ100.2 and 104.3 (C-1 α & β). This is within the known range for anomeric carbons of a sugar.

Another characteristic shift observed within the carbon spectrum were the carbon signals at δ15.4 and 15.6 (C-8 α & β). These carbon shifts suggests CH_3 moieties. Since methyl groups are normally not found in simple monosacharrides this suggests a sugar that is somewhat modified.

The eight CH shifts of δ70.3, 75.2, 71.7, 78.0, 71.2, 76.7, 72.4, and 78.3 were consistent with C-2 through C-5 (α & β) of monosaccharides. Four CH_2 signals were observed in the molecule at δ62.9, 64.7, 66.2, and 66.3. Two are assignable to C-6 (α & β) and the other two to the CH_2 (α & β) of an ethyl group.

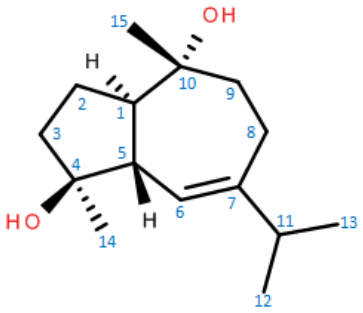
TABLE XIV: EXPERIMENTAL 13C NMR CHEMICAL SHIFTS FOR COMPOUND AOZ-14 AND A COMPARISON WITH THE LITERATURE DATA REPORTED FOR METHYL-D-GALACTOPYRANOSE.

EXPERIMENTAL DATA		LITERATURE DATA OF METHYL-D-GALACTOPYRANOSE
PEAK	CHEMICAL SHIFT IN PPM (IN METHANOL-d_4)	CHEMICAL SHIFT (IN D_20)
C-1 (α)	100.2	100.5
C-1 (β)	104.3	104.9
C-2 (α)	70.3	69.4
C-2 (β)	75.2	71.8
C-3 (α)	71.7	70.6
C-3 (β)	78.0	73.9
C-4 (α)	71.2	70.4
C-4 (β)	76.7	69.8
C-5 (α)	72.4	71.8
C-5 (β)	78.3	76.2
C-6 (α)	62.9	62.3
C-6 (β)	64.7	62.1
C-7 (α)	66.2	56.3
C-7 (β)	66.3	58.3
C-8 (α)	15.4	
C-8 (β)	15.6	

Two signals at δ15.4 and 15.6 are consistent with α & β CH_3 groups of an ethyl moiety.

The above-mentioned data for this isolate possibly belong to a new compound both for this species and generally as there is nothing in the literature to compare it to. For characterization this isolate was compared to methyl-D-galactopyranose.

3.3.2. STRUCTURAL CHARACTERIZATION OF ALISMOXIDE (AOZ-232)



Fifteen carbons were observed in AOZ-232 in the 13C NMR spectrum, corresponding to that of a sesquiterpene structure. Four chemical shifts were indicative of CH_3 groups, located at 21.8, 21.8, 18.0, and 24.0 ppm (C12-C15). These CH_3 groups must then be located somewhere off the main skeleton of either a Germacrene-type or Guaiane-type sesquiterpene. A pair of carbons at δ 125.0 and 148.9 ppm are assignable to two olefinic carbons in the molecule. It is also apparent that oxygenated functional groups are present on two carbons at δ 76.0 and 78.0. Carbons resonating between δ 21.8 and 43.6 represent aliphatic CH_2 groups. In addition, two CH carbons signals were observed at δ 50.0 and 49.9.

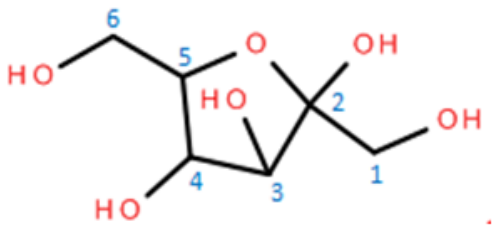
Taken together the information gleaned from the 13C spectrum make it clear that the compound possesses a sesquiterpene structure. Direct comparison with literature

values led to the identification of AOZ-232 as alismoxide.[130] A comparison of the experimental and literature values of 13C nmr data is shown in TABLE XV.

TABLE XV: EXPERIMENTAL 13C NMR CHEMICAL SHIFTS FOR COMPOUND AOZ-232 COMPARED TO THE LITERATURE DATA REPORTED FOR ALISMOXIDE.

EXPERIMENTAL DATA		LITERATURE DATA OF ALISMOXIDE[130]
PEAK	CHEMICAL SHIFT IN PPM (IN METHANOL-d_4)	CHEMICAL SHIFT (IN $CDCL_3$)
C-1	53.8	50.5
C-2	21.8	21.5
C-3	38.7	40.4
C-4	78.1	80.1
C-5	46.1	50.2
C-6	125.1	121.3
C-7	148.9	149.5
C-8	25.6	25.0
C-9	43.6	42.5
C-10	75.2	75.2
C-11	37.0	37.2
C-12	18.0	21.3
C-13	21.8	21.4
C-14	15.7	21.1
C-15	24.0	22.4

3.3.3. STRUCTURAL CHARACTERIZATION OF D-FRUCTOPYRANOSE (AOZ-17)



The 13C spectrum of this isolate contains twelve signals. Shifts of δ106.0 and 103.2 were observed (C-2 α & β) which are consistent with anomeric carbons and suggest the presence of a sugar.

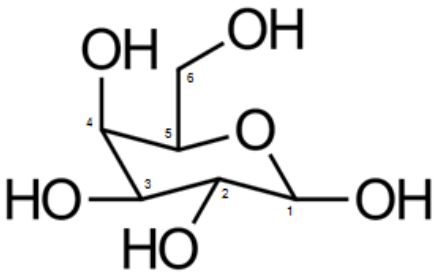
Signals at δ76.9, 66.0, 78.0, 71.3, 83.3, 71.9, 77.6, 69.5, 64.7, and 64.3 (C-1 & C-3 through C-6 α & β) are in line with that of ring-carbons and a CH_2OH moiety. The number of carbons in total suggest this is a mixture of α and β versions of a 6-carbon monosaccharide.

Comparing the shifts observed to literature data of common monosacharides yielded a match, namely with that of D-fructopyranose.[131] A direct comparison between the experimental values of AOZ-17 and a commercial sample of D-fructopyranose is found in TABLE XVI.

TABLE XVI: EXPERIMENTAL 13C NMR CHEMICAL SHIFTS FOR COMPOUND AOZ-17 COMPARED TO A COMMERCIAL SAMPLE OF D-FRUCTOPYRANOSE.

EXPERIMENTAL DATA		COMMERCIAL SAMPLE OF D-FRUCTOPYRANOSE
PEAK	CHEMICAL SHIFT IN PPM (IN METHANOL-d_4)	CHEMICAL SHIFT (IN METHANOL-d_4/D_20)
C-1 (α)	76.9	75.0
C-1 (β)	66.0	64.3
C-2 (α)	106.0	101.7
C-2 (β)	103.2	98.0
C-3 (α)	78.0	76.4
C-3 (β)	71.3	69.6
C-4 (α)	83.3	81.5
C-4 (β)	71.9	70.2
C-5 (α)	77.6	75.9
C-5 (β)	69.5	67.8
C-6 (α)	64.7	63.2
C-6 (β)	64.3	62.9

3.3.4. STRUCTURAL CHARACTERIZATION (OF D-GALACTOPYRANOSE AOZ-5)



Twelve signals are displayed in the 13C spectrum of this compound. There are characteristic shifts at δ 99.4 and 103.1 (C-1 α & β), which corresponds to an anomeric carbon. The presence of twelve carbon signals in two different proportions suggests an α & β mixture of a six-membered sugar. The shifts at δ64.1, 65.6, 71.0, 77.5, 69.2, 76.5, 71.7, 82.9, 62.5,and 64.6 (C-2 through C-6 α & β) indicate CH groups in proximity to hydroxyl groups which are consistent with members of a ring. The signals for C-6 are slightly upfield of the rest. This is in range to be a CH_2 group residing outside of the ring structure upon cyclization.

The experimental values obtained were subject to comparison with a commercial sample of D-galactopyranose. Through this comparison it was found that the chemical shifts of this this isolate match that of D-galactopyranose.[132] A direct comparison between the experimental data acquired for this isolate and that of the published data on D-galactopyranose is summarized in TABLE XVII

3.3.5. STRUCTURAL CHARACTERIZATION OF CYTIDINE (AOZ-12)

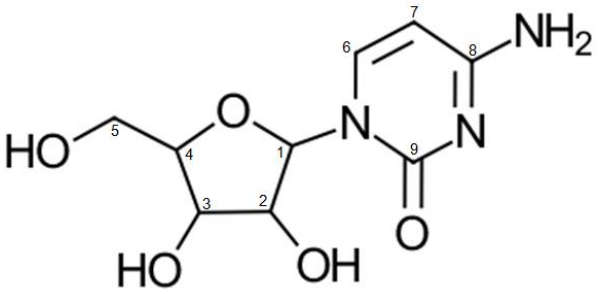


TABLE XVII: EXPERIMENTAL 13C NMR CHEMICAL SHIFTS FOR COMPOUND AOZ-5 COMPARED TO A COMMERCIAL SAMPLE OF D-GALACTOPYRANOSE.

EXPERIMENTAL DATA		COMMERCIAL SAMPLE OF D-GALACTOPYRANOSE
PEAK	CHEMICAL SHIFT IN PPM (IN METHANOL-d_4)	CHEMICAL SHIFT (IN METHANOL-d_4/D_2O)
C-1 (α)	99.4	99.2
C-1 (β)	103.1	102. 9
C-2 (α)	64.1	64.2
C-2 (β)	65.6	65.5
C-3 (α)	71.0	70.8
C-3 (β)	77.5	77.1
C-4 (α)	69.2	69.0
C-4 (β)	76.5	76.2
C-5 (α)	71.7	71.5
C-5 (β)	82.9	82.5
C-6 (α)	62.5	64.0
C-6 (β)	64.6	64.5

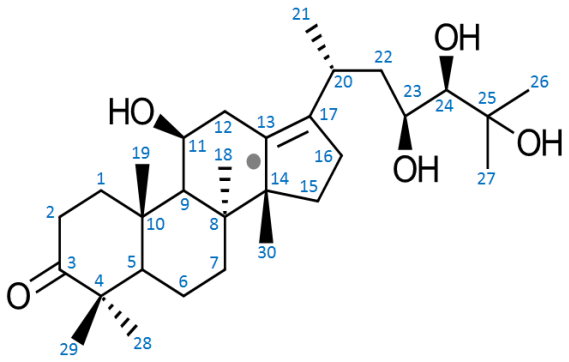
The 13C spectra of this isolate contains nine carbons. These include a shift at δ 90.8 which is within range of anomeric carbons. These shifts suggest the presence of a sugar. The four signals at δ75.5, 71.4, 86.1, and 62.4 (C-2 through C-5) are indicative of non-anomeric carbons within a sugar which would be found within a five-membered sugar. The three signals with chemical shifts of δ142.9, 152.7, and 166.5 fall within the range for aromatic or heteroaromatic carbons within a base, indicative of a nucleoside. When comparing these data to known nucleosides, the carbon number and absence of a methyl signal suggests an identity of cytosine.

A direct comparison was conducted between the chemical shifts of this isolate and the published 13C NMR data on cytosine (TABLE XVIII). The shifts between these two sets of data were found to match.[133]

TABLE XVIII: EXPERIMENTAL 13C NMR CHEMICAL SHIFTS FOR COMPOUND AOZ-12 COMPARED TO THE LITERATURE DATA REPORTED FOR CYTIDINE.

EXPERIMENTAL DATA		LITERATURE DATA FOR CYTIDINE[133]
PEAK	CHEMICAL SHIFT IN PPM (IN METHANOL-d_4)	CHEMICAL SHIFT (IN D_20)
C-1	90.8	93.0
C-2	75.5	76.7
C-3	71.4	72.0
C-4	86.1	86.5
C-5	62.4	63.4
C-6	152.7	160.4
C-7	166.5	168.9
C-8	103.0	99.0
C-9	142.9	144.3

3.3.6. STRUCTURAL CHARACTERIZATION OF ALISOL A (RS1)



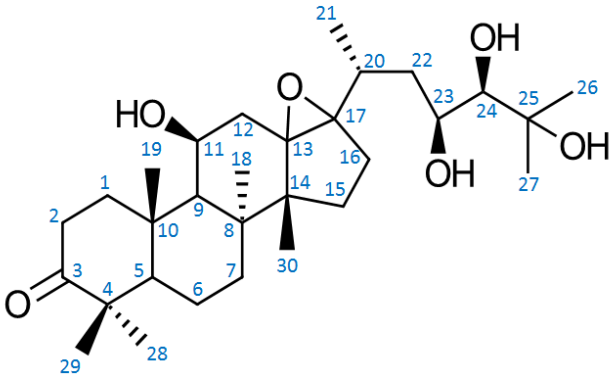
The carbon spectrum of this isolate contains 30 signals. This carbon number is consistent with a triterpene, a major compound class within this plant species with many known and published examples. The sixteen signals between δ20-40ppm suggest CH_2 and CH_3 groups within the ring structures of such compounds. Four signals at δ69.7, 70.1, 74.2, and 77.9 are consistent with carbons attached to hydroxyl groups within the structure. Characteristic shifts of δ135.9 and 137.9 of carbons C-17 and C-13 are consistent with carbons flanking a double bond. This attribute is diagnostic of the particular type of triterpene, as not all triterpenes contain this chemical feature.

Together these attributes were used for literature comparison and the identity was determined to be alisol A.[52] A direct comparison of these values is found in TABLE XIX

TABLE XIX: EXPERIMENTAL 13C NMR CHEMICAL SHIFTS FOR COMPOUND RS1 COMPARED TO THE LITERATURE DATA REPORTED FOR ALISOL A.

EXPERIMENTAL DATA		LITERATURE DATA FOR ALISOL A[130]
PEAK	CHEMICAL SHIFT IN PPM (IN $CDCl_3$)	CHEMICAL SHIFT (IN $CDCl_3$)
C-1	31.3	31.0
C-2	33.9	33.7
C-3	220.6	220.6
C-4	47.2	46.9
C-5	48.8	48.5
C-6	20.3	20.0
C-7	34.6	34.3
C-8	40.7	40.3
C-9	49.9	49.6
C-10	37.2	36.9
C-11	70.1	69.7
C-12	34.8	34.4
C-13	137.9	137.6
C-14	57.3	57.0
C-15	30.7	30.5
C-16	29.7	29.5
C-17	135.9	135.5
C-18	23.4	23.0
C-19	25.8	25.6
C-20	28.5	28.2
C-21	20.4	20.1
C-22	40.3	40.0
C-23	69.7	69.4
C-24	77.9	77.6
C-25	74.2	74.2
C-26	26.4	26.2
C-27	27.5	27.3
C-28	29.3	29.5
C-29	20.3	20.0
C-30	24.4	24.1

3.3.7. STRUCTURAL CHARACTERIZATION OF 13,17-EPOXY ALISOL A (RS2)



This isolate is very similar to the previously described isolate determined to be alisol A. Present within the spectrum were nearly identical signals for CH_2 and CH_3 groups, the carbons attached to hydroxyl groups, and a carbonyl group. The difference between this isolate and that of alisol A is the absence of the two signals for double bonded carbons. Instead, signals are observed at δ73.5 and 78.6, suggesting a modification at this point in the molecule.

One known modification in this location (C-13 and C-17) is the addition of a epoxy ring. Direct comparison between these signals and the 13C NMR data reported for 13,17-epoxy alisol A (TABLE XX) revealed a match.[52]

TABLE XX: EXPERIMENTAL 13C NMR CHEMICAL SHIFTS FOR COMPOUND RS2 COMPARED TO THE LITERATURE DATA REPORTED FOR 13,17-EPOXY ALISOL A.

EXPERIMENTAL DATA		LITERATURE DATA FOR 13,17-EPOXY ALISOL A[130]
PEAK	CHEMICAL SHIFT IN PPM (IN $CDCl_3$)	CHEMICAL SHIFT (IN $CDCl_3$)
C-1	31.0	30.9
C-2	33.7	33.5
C-3	219.9	220.1
C-4	47.1	46.9
C-5	48.9	48.7
C-6	20.2	20.1
C-7	34.8	34.7
C-8	41.0	40.3
C-9	49.0	48.9
C-10	37.1	36.9
C-11	68.5	68.4
C-12	35.5	35.3
C-13	73.5	73.3
C-14	50.3	50.0
C-15	30.7	30.6
C-16	26.6	26.5
C-17	78.6	78.5
C-18	19.5	19.2
C-19	25.8	25.7
C-20	31.0	30.7
C-21	17.7	17.5
C-22	38.9	38.7
C-23	68.8	68.6
C-24	77.2	76.9
C-25	73.8	73.6
C-26	26.7	26.6
C-27	26.9	26.7
C-28	29.8	29.6
C-29	20.1	19.9
C-30	24.9	24.6

3.3.8. α-GLUCOSIDASE INHIBITORY ACTIVITY OF THE ISOLATES

Pure compounds isolated from *A. orientale* by this study or otherwise obtained were screened for their ability to inhibit α-glucosidase enzyme function. The results of the pure compound screening and their comparison can be found in TABLE XXI

What follows is a summary of the pure compounds from the ethyl acetate and butanol-soluble fractions that were found to be active in the initial screen and subject to further testing to confirm their activity and determine their IC_{50} values.

RS1 (ALISOL A)

Alisol A, also referred to in this text as RS1, was tested in the α-glucosidase inhibition assay and the results showed significant inhibition at all three tested doses. At 150 µg/ml the compound inhibited 43% of the enzyme activity. This inhibition increased to 44 and then 57% when increasing the concentrations to 300 and 600 µg/ml. All doses were found to be statistically significant, as was the positive control 0.5 mM acarbose, indicating a working assay.

Further testing was conducted to hone in on RS1's IC_{50} value. Through a series of concentrations between 250-500 µg/ml the enzyme activity was measured and plotted. The IC_{50} was calculated to be 412.60 µg/ml (0.84 mM).

AOZ-14 (1-O-ETHYL-D-GALACTOPYRANOSE)

1-O-ethyl-D-galactopyranose (AOZ-14) was found to have α-glucosidase inhibition at all three doses of 150, 300, and 600 µg/ml. These concentrations produced 71, 81, and 78% inhibition, respectively, and had a P-Value of < 0.0001. A dose of 0.5

TABLE XXI: A CHART COMPARING THE PERCENT INHIBITIONS FOUND IN PURE COMPOUNDS ISOLATED FROM *A. ORIENTALE*. CELLS ARE HIGHLIGHTED BY PERCENT INHIBITION INDICATING THE FRACTIONS WHICH CAUSED THE GREATEST INHIBITION.

Inhibition color index
<0%
0-25%
25-50%
50-75%
75-100%

Color scheme modified from the Chandler Burning Index. Inhibition determined by comparison to no-treatment group.

ISOLATE	% INHIBITION		
	600 µg/ml	300 µg/ml	150 µg/ml
ALISOL B ACETATE	-12.22515	-4.74934	-13.58839
ALISOL A	56.816183	44.371152	43.095866
ALISMOXIDE	-3.478687	-4.164625	-1.077903
1-O-ETHYL-D-GALACTOSE	78.490936	80.842724	71.435571
D-FRUCTOSE	46.937776	29.348359	16.462518
CYTIDINE	90.752351	64.263323	61.755486
	500 µg/ml	250 µg/ml	125 µg/ml
ALISOL O	97.479564	84.945504	82.050409
25-ANHYDROXY ALISOL A	13.521798	2.7247956	-3.474114
ALISOL P	86.103542	38.555858	31.743869
DAUCOSTEROL-6-O-STEARATE	-5.347411	-7.425068	-10.38828
	100 µg/ml	50 µg/ml	25 µg/ml
13,17-EPOXY ALISOL A	45.990975	32.801111	35.022562
ALISOL E 23-ACETATE	68.136064	21.416175	27.247484
ALISOL B	10.14106	4.6130385	6.557377
D-GALACTOPYRANOSE	9.6454441	-3.964926	-2.40183
	50 µg/ml	25 µg/ml	12.5 µg/ml
25-O-METHYL ALISOL A	63.589032	15.272475	22.42277

mM acarbose was found to have a typical inhibition with a P-Value in this range as well indicating a working assay.

Using the above information, a series of additional concentrations were tested between 5 and 50 µg/ml to attempt to capture the IC_{50} of this compound, which was subsequently determined to be 104.71 µg/ml (0.50 mM).

AOZ-12 (CYTIDINE)

Cytidine (AOZ-12) was found to have notable α-glucosidase inhibition in the tests conducted at concentrations of 150, 300, and 600 µg/ml producing 62, 64, and 91% inhibitions, respectively. These were found to be statistically significant when compared to the no treatment group. The positive control acarbose was found to be in range for a working assay.

These results were expanded upon using concentrations ranging from 40-100 µg/ml. The calculated IC_{50} from these tests was 86.98 µg/ml (0.36 mM).

ZX-6 (ALISOL O)

The compound alisol O, whose compound code is ZX-6, was found to produce extremely potent α-glucosidase inhibition in these studies. Not only did these concentrations produce inhibition which rivaled the clinical drug acarbose in a statistically significant and dose-dependent manner, they did this at concentrations below any other pure compounds tested in this project. Indeed at mere concentrations of 125, 250, and 500 µg/ml this compound inhibited 82, 85, and a whopping 97% of enzyme activity. This test was considered functional based on the inhibition of the positive control, which was observed to be within range.

From here much lower concentrations were attempted to assess the IC_{50} of this compound. According to these studies, which ranged from 6.25 to 125 µg/ml, the IC_{50} was calculated as 18.38 µg/ml (0.036 mM).

ZX-9 (ALISOL P)

The compound alisol P, referred to as ZX-9 in this text, was also witnessed to produce observable α-glucosidase inhibition at rather low concentrations. The concentrations tested were 125, 250, and 500 µg/ml. At these concentrations this compound inhibited 32, 39, and 86% in a working assay.

Given these results an additional test was conducted with concentrations ranging between 250-500 µg/ml. The results of this additional test indicated an IC_{50} of 428.01 µg/ml (0.82 mM).

RS2 (13,17-EPOXY ALISOL A)

13,17-epoxy A, coded as RS2, was observed to inhibit the α-glucosidase enzyme at notably small concentrations in the tests performed. At concentrations of 25, 50, and 100 µg/ml this compound caused 35, 33, and 46% enzyme inhibition in a functional assay. These results were found to be statistically significant with a P-Value of < 0.0001 when comparing treatment groups to the no treatment group.

Subsequent studies at concentrations ranging from 75 to 250 µg/ml indicated an IC_{50} of 206.61 µg/ml (0.41 mM).

ZX-3 (ALISOL E 23-ACETATE)

Alisol E 23-acetate (ZX-3) produced abundant inhibition as well considering the concentrations in which it was tested. Specifically, it produced 27, 21, and 68% inhibition at concentrations of 25, 50, and 100 µg/ml, respectively. This was considered

to be a working assay and the results were all found to be statistically significant when compared to the no treatment group.

Although the precise IC_{50} of this isolate could not be determined due to scarcity of material the above-mentioned tests indicate an IC_{50} of < 100.00 µg/ml (< 0.19 mM).

ZX-17 (25-O-METHYL ALISOL A)

25-O-methyl alisol A, compound code ZX-17, displayed moderate to strong inhibitory activity in what is considered quite low concentrations compared to the other pure compounds tested in this project. At concentrations of 12.5 and 25 µg/ml moderate inhibition of 15 and 22%. However, at the still low concentration of 50 µg/ml, a 64% enzyme inhibition was measured. This is more potent than the positive control in what is considered a working assay with statistically significant results.

Due to lack of material an exact IC_{50} of this isolate could not be produced. However, the tests conducted suggest an IC_{50} of < 50.00 µg/ml (< 0.099 mM).

A number of isolates tested had IC_{50} values estimated to be weakly active or inactive entirely. These include the compounds D-fructopyranose, daucosterol-6-O-stearate, alisol B 23-acetate, 25-anhydroxy alisol A, alismoxide, and D-galactopyranose. A full list of the IC_{50} values determined by this project are located in TABLE XXII.

3.4. CONCLUSION

These results suggest that compounds present within the butanol and ethyl acetate fraction of *A. orientale* cause significant α-glucosidase inhibition. A number of

TABLE XXII: DETERMINED IC_{50} VALUES OF THE ISOLATES FROM *A. ORIENTALE*

COMPOUND	a-GLUCOSIDASE INHIBITION IC_{50} (mM)
ALISOL O	0.036
25-*O*-METHYLALISOL A	<0.099
ALISOL E 23-ACETATE	<0.19
CYTIDINE	0.36
13,17-EPOXYALISOL A	0.41
1-*O*-ETHYL-D-GALACTOPYRANOSE	0.50
ALISOL P	0.82
ALISOL A	0.84
ALISOL B	>0.21
D-FRUCTOPYRANOSE	1.67
DAUCOSTEROL-6-O-STEARATE	>0.58
ALISOL B 23-ACETATE	>1.17
25-ANHYDROALISOL A	>1.26
ALISMOXIDE	>2.52
D-GALACTOPYRANOSE	>5.55
ACARBOSE (POSITIVE CONTROL)	0.15

these compounds appear to be the most potent ever isolated from this species. Together these findings help to explain the activity of these partitions as a whole. Some of the isolates which showed activity were sugars such as 1-O-ethyl-D-galactopyranose, and D-fructopyranose. In addition to these sugars the nucleoside cytidine and a number of terpenes showed inhibitory activity including alisol A, 13,17-epoxy alisol A,

alisol E 23-acetate, 25-o-methyl alisol A, alisol O, and alisol P. On the other hand D-galactopyranose, alismoxide, daucosterol-6-O-stearate, 25-anhydroxy alisol A, alisol B, and alisol B 23-acetate did not show α-glucosidase inhibition activity.

CHAPTER IV. *IN VITRO* 3T3-L1 GLUCOSE UPTAKE STUDIES ON *A. ORIENTALE* RHIZOME

4.1. INTRODUCTION

The 3T3-L1 glucose uptake assay is an *in vitro* assay utilizing a clonally isolated substrain of Swiss albino (3T3) fibroblasts known as 3T3-L1 cells grown to confluence and with the help of hormonal treatments differentiated into adipocytes with functioning insulin-sensitive GLUT4 transporters capable of uptaking serum and glucose in its environment and producing triglycerides and accumulating fat. The process of uptaking glucose through GLUT4 is sensitive to the presence or absence of insulin and/or IMAs and ISAs. By using radio-labeled glucose in the presence of extracts, partitions, and fractions the insulin mimetic or sensitizing effects of these preparations can be quantified using a scintillation counter.

4.2. ANALYSIS OF EXTRACTS, PARTITIONS, AND FRACTIONS OF *A. ORIENTALE* RHIZOME

Various extracts, partitions, and fractions from *A. orientale* were tested for activity in the glucose uptake study to determine their activity and guide future isolation work.

4.2.1. EXPERIMENTAL

For 3T3-L1 differentiation cell-stocks stored at -70°C were defrosted in 37°C. A 7-8x volume of Dulbecco's Modified Eagle's Medium (DMEM) containing 10% Fetal Bovine Serum (FBS) was added and centrifuged. The supernatant was removed and replaced with 5 ml of DMEM containing 10% FBS and incubated at 37°C and 5% CO_2.

Once cells reached 80% confluence cells were washed with Phosphate Buffered Saline (PBS). The PBS was then removed and 3 ml of trypsin was added. Flasks were rotated and gently tapped for ~1 min to dislodge cells. Cells were then split in a 1:4-6 ratio using 10% calf serum (CS) and were plated in either T-75 flasks for further propagation or plated in 12-well plates in preparation for assay and incubated at 37°C at 5%CO_2. Flasks being prepared for assay were grown to confluence and re-fed a 10% CS media and incubated for an additional 2 days to ensure 100% confluency (DAY 0).

A number of solutions were needed for cell differentiation, as well as the assay itself. A fibroblast media was made using DMEM (1 L), CS (100 ml), glutamine (10 ml), and pen/strep mix (10 ml), filtered and stored at 4°C. Differentiation media was concocted using DMEM (1 L), FBS (10%), insulin (1 µg/ml), dexamethasone (0.25 µM), and IBMX (0.5 mM) and filtered. Insulin media contained DMEM (1L), FBS (10%), and insulin (1 µg/ml) and was filtered. Adipocyte media contained DMEM (1 L), FBS (100 ml), glutamine (10 ml), and pen/strep mix (10 ml) and was filtered and stored at 4°C. Insulin stock was made by dissolving HCL (0.01 M) as a stock (167 µM), filtering, and then stocking aliquots at -20°C. A working stock was then made by a 1:1000 dilution, which was stored at 4°C and used within 30 days. Dexamethasone (2.5 mM) was made by dissolving in ethanol and filtering, and was stored at 20°C as a stock solution (shelf-life of 1 year). A working stock was then made by a 1:10,000 dilution. An IBMX stock was made by dissolving 55.6 mg in 1ml KOH (0.35 M), and filtering. This stock was stored at -20°C, and was aliquoted to be only thawed from frozen once. A working solution was then made by a 1:500 dilution. Glucose-free assay media was made by using glucose-free DMEM, FBS (0.5%), and Hyrdoxyethylpiperazine Ethane Sulfonic

Acid (HEPES buffer) (25 mM) (pH 7.4). Glucose assay media contained glucose-free DMEM, FBS (0.5%), HEPES buffer (25 mM) (pH 7.4), and glucose (5 mM). The isotope stock contained DMEM and 0.2 µCi of radiolabelled glucose (^{14}C-2DG). Termination stock contained DMEM and 2 –Deoxy-D-Glucose (2DG) (200 mM).

After 2 days (DAY 2), media was removed and changed to differentiation media. After 3 days (DAY 5), the media was changed to an insulin media. After 2 days (DAY 7), the media was acidic (orange or yellow in color) and noticeably viscous in successfully differentiated cells. Insulin media was then discontinued and changed to adipocyte media. Adipocyte media was changed every 2-3 days. Adipocytes were ready for assay 5-14 days after switching to adipocyte media (DAY 12-19).

For the assay glucose-free and glucose assay media was warmed to 37°C in a water bath. The media was then removed from 12-well plates and washed 2x with 1 ml/well glucose-free assay media, aspirating completely between each wash. Glucose assay media (1 ml/well) was then added and incubated for 2.5 hr at 37°C and 5% CO_2. Cells were washed 2x with 1 ml/well warm glucose free media, aspirating completely between each wash.

Treatments being tested in the 3T3-L1 glucose uptake assay were given in triplicate. These triplicate treatments were arranged vertically along numerical columns (e.g. wells A1, B1, and C1) in order to minimize the effects of differences in wells exposed to more or less oxygen and evaporation. All treatments and preparations from this point of the assay onward were done in the same pattern and order to maintain minimal variation in treatment times. Treatment groups consisted of different plant

extracts, partitions, and fractions. Testing often also included different doses of the same sample to evaluate their cellular effects at different concentrations. All assays run, regardless of their size or composition, included both a no treatment group in triplicate, as well as a positive control group in triplicate that contained 100 nM insulin (final concentration). Once cells were glucose starved using the glucose assay media and washed, 0.5 ml of glucose free media containing treatments and controls were added to each well and incubated for 0.5 hr at 37°C and 5% CO_2.

Plates were then transferred to the radioactive work area and 0.2 µCi (50 µl) of ^{3}H-2-deoxyglucose (2-DG) stock was added to each well. Plates were incubated at room temperature for 2.5-5 min. The reaction was terminated by addition of 50 µl of 200 mM non-radioactive 2-DG to all wells and placing the plates on ice. The addition of termination stock was in the same pattern as the addition of isotope stock to ensure each well had a roughly equal time in the presence of the isotope.

Plates were then placed on ice, washed with cold PBS 3x, and aspirated completely between each wash. Deionized water (0.5 ml) was added to each well and the contents were homogenized using the back of a 200 µl pipette tip. This homogenate was then transferred to scintillation vials containing 5 ml of scintillation fluid. Each set of triplicate treatments were then vortexed and loaded next to one another in a scintillation rack and loaded into the machine for counting. Counting was done immediately, as well as the next morning for accuracy and to account for any homogeneity lacking in the samples.

Data from the 3T3-L1 glucose uptake assay initially comes in the form of a readout from a Beckman Coulter LS6500 Multi Purpose Scintillation Counter (Beckman Coulter, Inc., Brea, CA). This machine measures the beta-particles radiated from the samples as a function of their concentration of tritium radiolabeled glucose. The units of measure are counts per minute (CPM). The machine counts the CPM of each scintillation vial in series producing a readout consisting of position, time, and CPM.

After a cursory look over of the experimental results CPM values in the raw data printout from the scintillation counter are transferred to excel for further data analysis. The three CPM values corresponding to the triplicates within a treatment group are arranged horizontally on the same row. At the end of each row is a mean column for each treatment group, which calculates the mean average of the three triplicate well values using an excel formula function. These values are then automatically calculated based on the values within each row's triplicate values, and changes automatically once programmed.

A rudimentary 2-D cluster graph was produced in excel's graphing function by using the mean column absorption values plotted against their concentrations.

For in-depth analysis of the results of the 3T3-L1 glucose-uptake assay GraphPad Prism 6 was employed. This software allows a much more robust working of the data, providing more information relevant to the statistical analysis of data as well as more functionality with respect to its presentation.

The specific analysis done on data from the glucose uptake assay is a One-Way ANOVA Dunnette's Multiple Comparison analysis. This allows the comparison between

multiple test groups, be it different extracts, partitions, fractions, different doses of the same extract, etc. and the positive control against the no treatment group. P-values are then calculated to determine the probability that the groups represent statistically significant phenomena.

This data, along with the calculated % error values, are then displayed in a comprehensive bar graph allowing for the visualization of the most important aspects of the data analysis of the raw data.

4.2.2. RESULTS

Three plant species within the herbal complex Liuwei Dihuang Wan suspected of causing increased glucose uptake from the literature were chosen for study in the 3T3-L1 glucose uptake experiment. These species were *Alisma orientale*, *Cornus officinalis*, and *Schisandra chinensis*. Decoctions were made from each species and tested. A mixture of these species was also tested in case of synergy between species (FIGURE 17).

A. orientale, C. officinalis, and *S. chinensis* were tested at three doses of 0.01, 0.1, and 1.0 mg/ml final concentration. The results of this assay indicated a 1.9 fold increase in 2-DG uptake between the basal (no treatment) group and the positive control of 100 nm insulin. Statistical analysis indicated this was a working assay with a P-value between basal and insulin of < 0.0001. Numerous other groups showed activity in this assay as well.

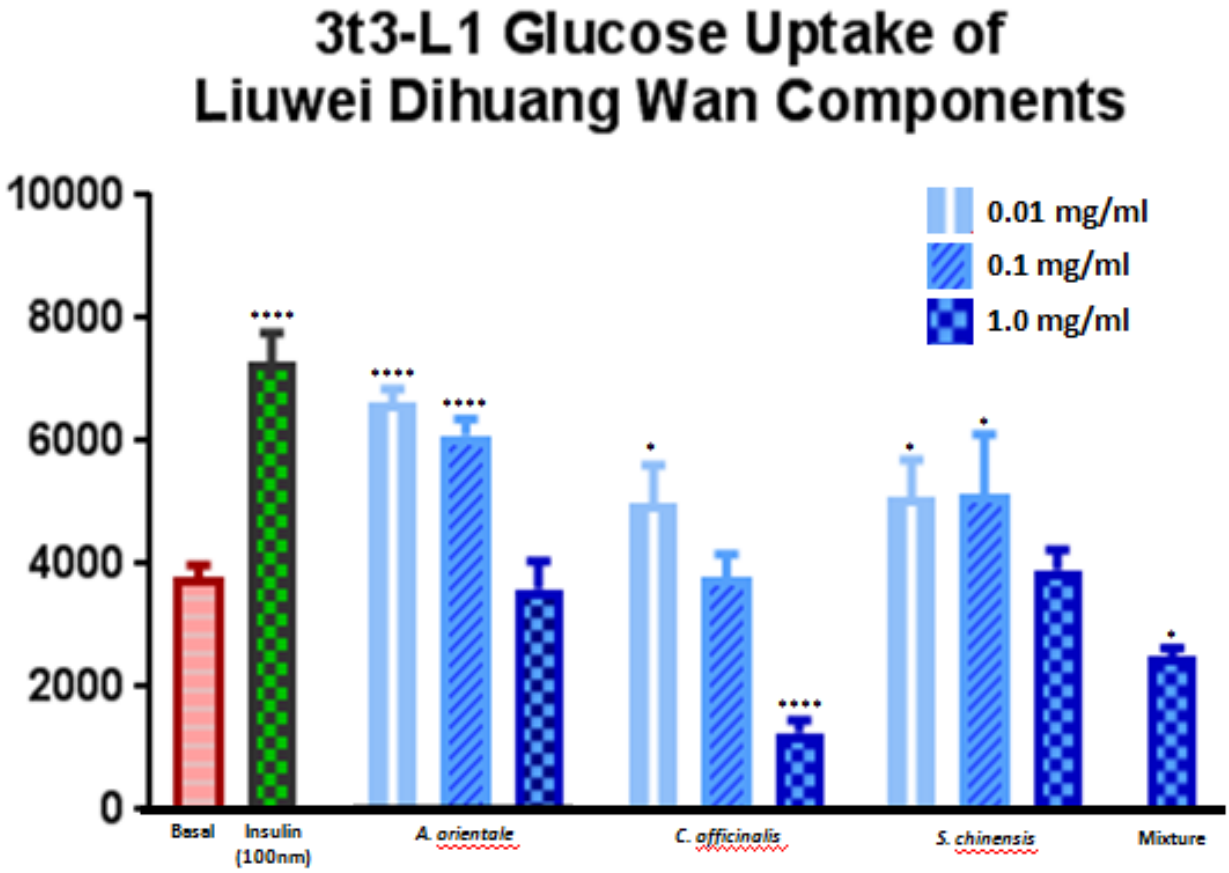


FIGURE 17: 3T3-L1 GLUCOSE UPTAKE RESULTS FOR DECOCTIONS OF THE SPECIES *A. ORIENTALE*, *C. OFFICINALIS*, AND *S. CHINENSIS* AS WELL AS A MIXTURE OF DECOCTIONS FROM ALL THREE SPECIES. NS (NO MARK) = P > 0.05, * = P ≤ 0.05, **** = P ≤ 0.0001

A. orientale showed 1.7 and 1.6 fold increases over basal at the two smallest doses of 0.01 and 0.1 mg/ml. Both of these results were considered statistically significant with P values < 0.0001. The high dose of 1.0 mg/ml of *A. orientale* showed no impact on glucose uptake as evidenced by amounts of 2-DG uptake.

C. officinalis showed a 1.3 fold increase over the no treatment group at a dose of 0.01 mg/ml. This result had a P value of < 0.05. No increase of 2-DG uptake was

observed with a dose of 0.1 mg/ml. A 66.4% decrease in glucose uptake was observed at a dose of 1.0 mg/ml which was found to be statistically significant with a < 0.0001 P-value.

S. chinensis saw a slight 1.3 and 1.4 increases in glucose uptake at the two smallest doses of 0.01 and 0.1 mg/ml. These increases were minor but significant with P-values of < 0.05. The high dose of 1.0 mg/ml did not show an increase in 2-DG uptake.

A 1 mg/ml mixture of decoctions from the three species showed a slight but significant 32.6% decrease in 2-DG uptake with a P-value < 0.05.

Based on these results *A. orientale* was chosen for further study. A total EtOH extract was tested along with partitions of EA, BuOH, and H_2O. A fifth group was then made by re-combining the partitions designated group B. Each group was tested at concentrations of 0.01 and 0.1 mg/ml (FIGURE 18).

The assay witnessed a statistically significant 2.8 fold increase between the no treatment group and 100 nm insulin with a P-value < 0.0001. These results indicate a functional assay.

The total aqueous ethanol extract of *A. orientale* did not produce noticeable changes in 2-DG uptake at either dose.

Likewise, the butanol partition of *A. orientale* failed to stimulate 2-DG uptake at both concentrations.

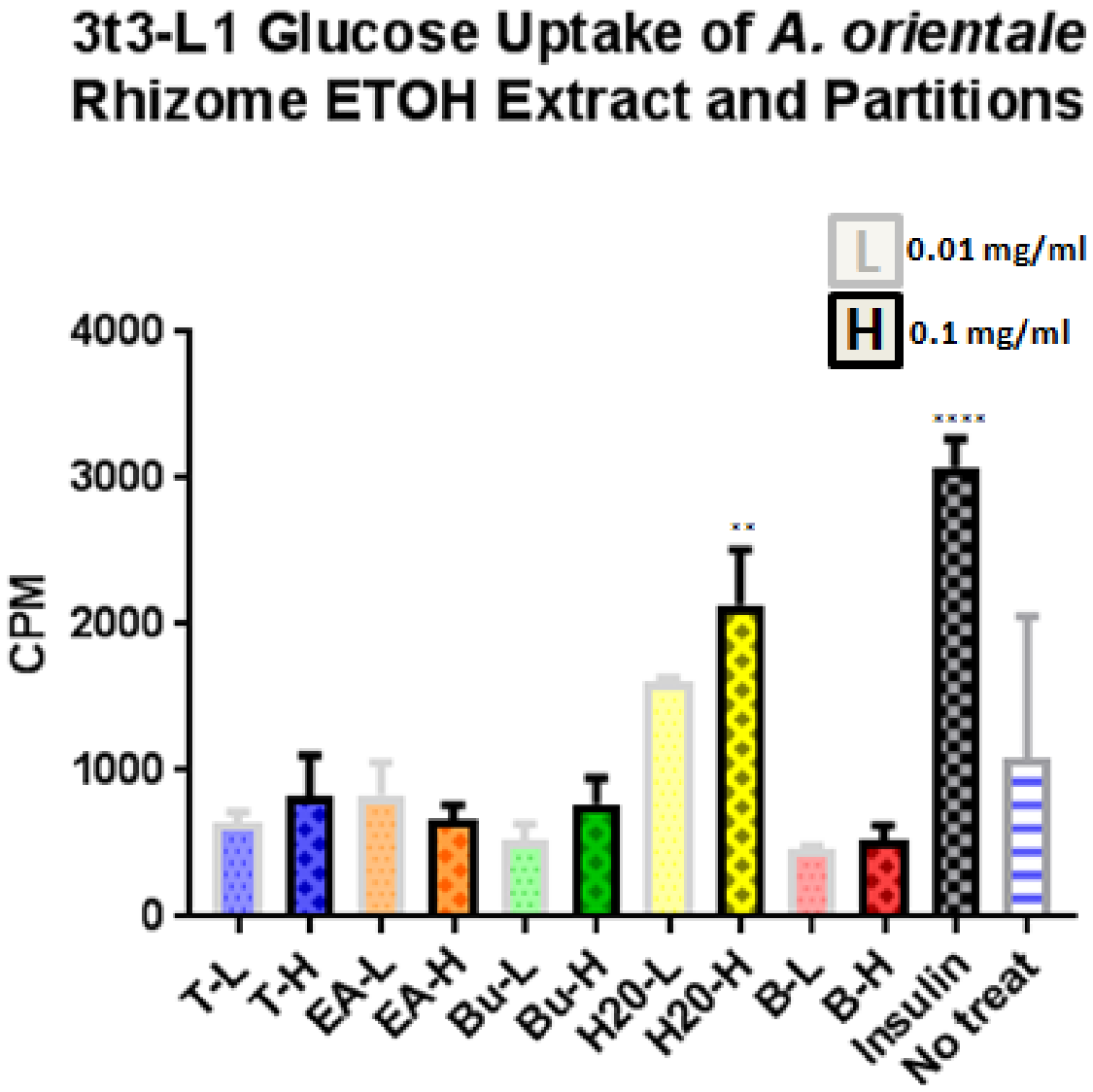


FIGURE 18: 2-DG UPTAKE INDUCTION OF THE TOTAL EXTRACT (T) AND PARTITIONS (EA, BU, AND H_2O) OF *A. ORIENTALE* AS WELL AS THE PARTITIONS PUT BACK TOGETHER (B) AFTER SEPARATION. NS (NO MARK) = $P > 0.05$, * = $P \leq 0.05$, ** = $P \leq 0.01$, **** = $P \leq 0.0001$

The H_2O partition of *A. orientale* displayed 2-DG uptake induction at both doses. Statistical analysis revealed that the 1.5 fold increase of the smaller dose of 0.01 mg/ml

was not statistically significant. The high dose of 0.1 mg/ml, however, produced a 2.0 fold increase in 2-DG uptake that was statistically significant with a P-value of < 0.001.

The test group consisting of a re-combination of partitions produced from the total extract of *A. orientale* showed a moderate albeit insignificant decrease in 2-DG uptake of 56 and 52% at doses of 0.01 and 0.1 mg/ml, respectively.

Several preparations of *A. orientale* were then tested. These included the original (old) decoctions made a few months earlier and stored at -20 C and new decoctions and an ETOH extract at doses of 0.01, 0.1, and 1.0 mg/ml (FIGURE 19).

There was a 6.7 fold increase in 2-DG uptake between the basal group and the group containing 100nm insulin. Multiple comparison between these groups indicates this is a working assay with P-value of < 0.0001.

A substantial increase in 2-DG uptake was observed for all concentrations of the old decoction. At the lower doses of 0.01 and 0.1 mg/ml the old decoction produced a 3.6 and 3.0 fold increase in uptake, respectively. The P-value for these tests were both < 0.0001. The highest dose for this treatment group of 1.0 mg/ml produced a 2.5 fold increase and had a P-value of < 0.01.

The new decoction produced a 2.5 fold increase in 2-DG uptake at the lowest dose of 0.01 mg/ml. This finding was considered significant and had a P-value of < 0.01. Higher doses of 0.1 and 1.0 mg/ml produced no detectable changes in 2-DG uptake.

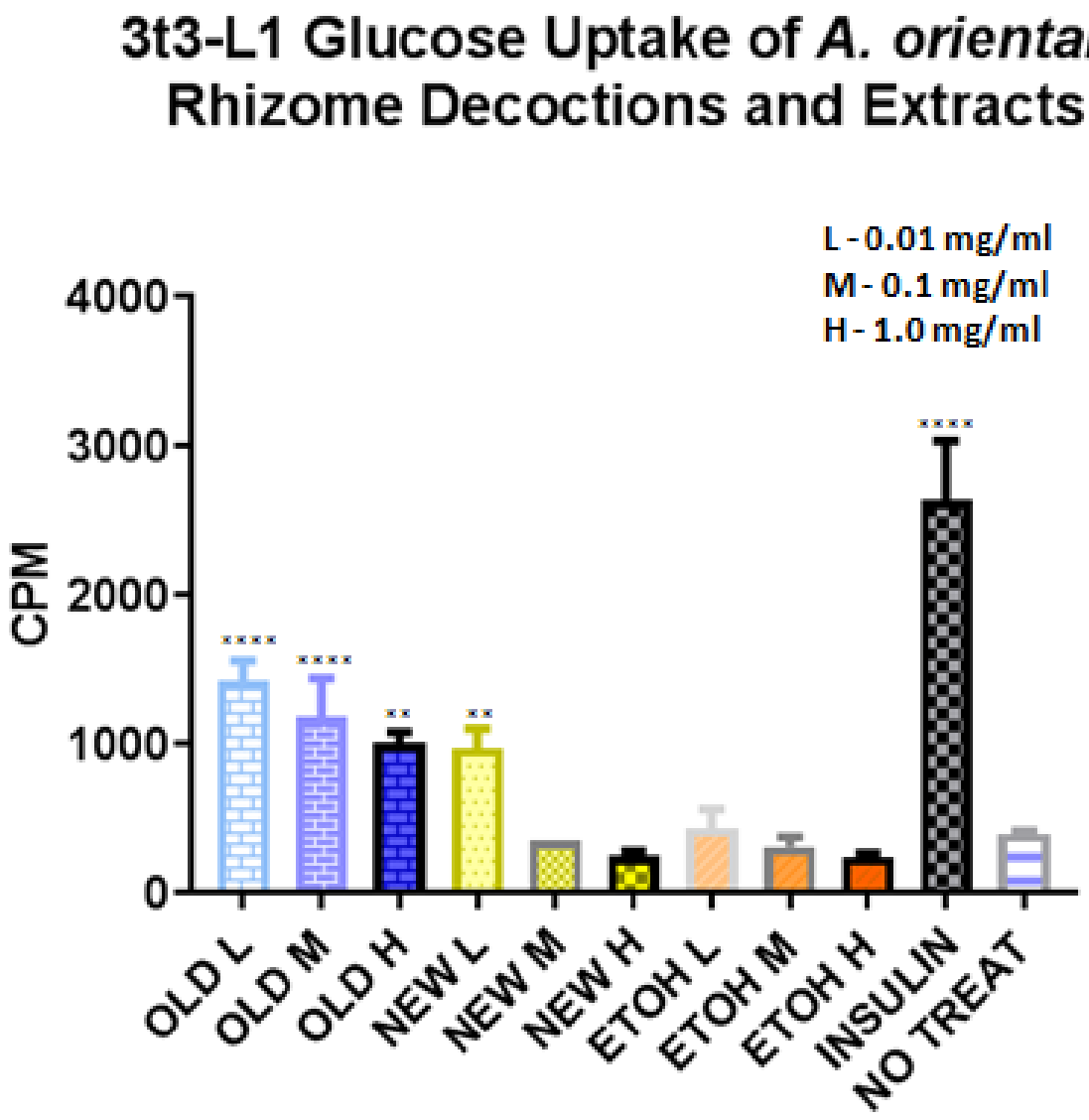


FIGURE 19: 2-DG UPTAKE INDUCTION OF NEW AND OLD DECOCTIONS AND AN ETOH EXTRACT OF *A. ORIENTALE*. NS (NO MARK) = P > 0.05, * = P ≤ 0.05, ** = P ≤ 0.01, **** = P ≤ 0.0001

Aqueous ethanol extracts of *A. orientale* failed to produce changes in 2-DG uptake at 0.01, 0.1, and 1.0 mg/ml.

Numerous preparation techniques were employed on *A.* orientale material and tested for their 2-DG uptake activity modulation (FIGURE 20).

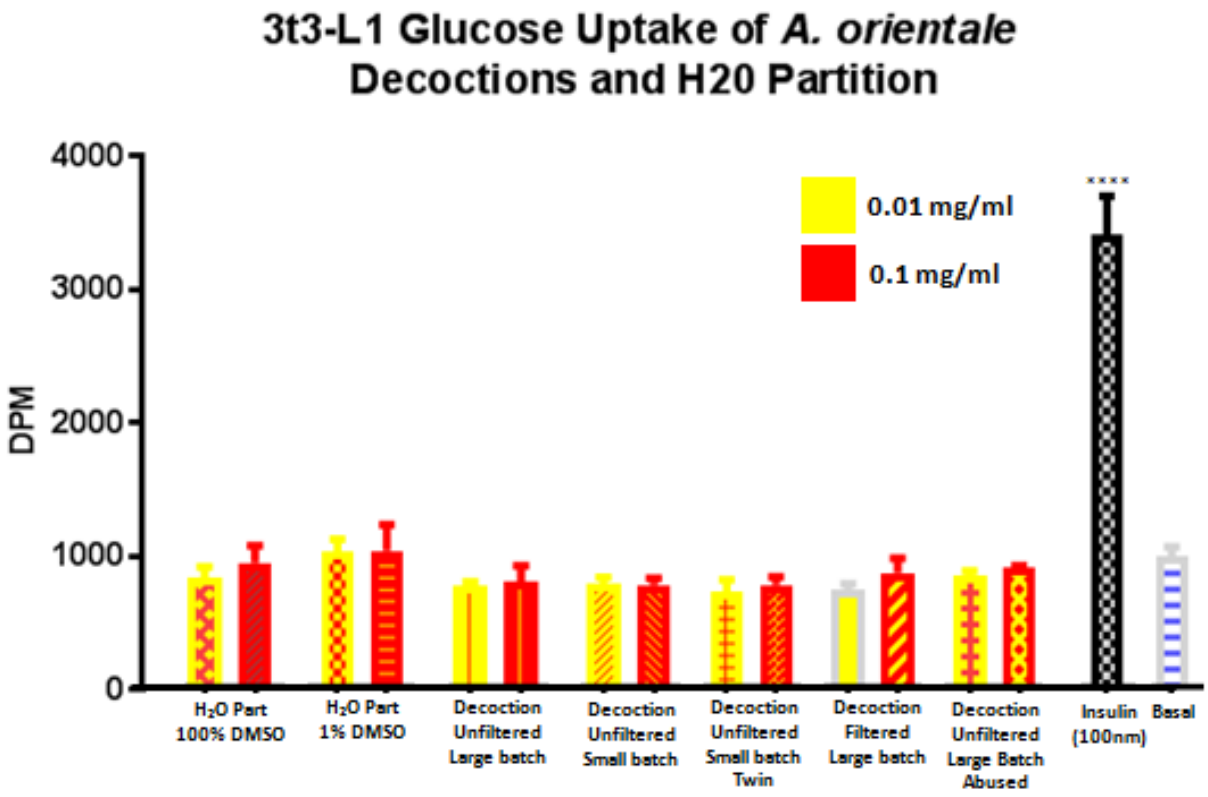


FIGURE 20: 3T3-L1 2-DG UPTAKE INDUCTION OF DIFFERENT PREPARATIONS OF *A. ORIENTALE*. NS (NO MARK) = P > 0.05, **** = P ≤ 0.0001

These included H_2O partitions at 0.01 and 0.1 mg/ml brought up in 100% and 1% DMSO (Final concentration 10% and 0.1%), respectively. Various decoctions were also

employed at these concentrations from different batches some of which were unfiltered and some of which were filtered. Additionally, what was termed an 'abused batch' was tested which consisted of an unfiltered decoction which was submitted to unnecessary and lengthy sonication and deprived of refrigeration per normal procedure.

The assay produced a 3.4 fold increase in 2-DG uptake between the 100 nm insulin positive control and no treatment groups. The difference between these groups had a P-value of < 0.0001 and indicates a functioning assay.

The H_2O partitions showed normal 2-DG uptake both in 100% and 1% DMSO at concentrations of 0.01 and 0.1 mg/ml. This is in contrast to the ~2000 CPM (2-fold increase) seen by these samples in their previous test.

The decoctions showed no 2-DG uptake induction in either the large or small batches, with or without filtering and/or abuse at concentrations of 0.01 and 0.1 mg/ml.

4.2.3. CONCLUSION

Results from the previous 3T3-L1 2-DG uptake studies on the extracts, partitions, and fractions paint a complex picture of the plant's glucose-uptake potential. On the one hand this species has demonstrated undeniable activity as witnessed by the numerous dose-dependent, statistically significant positive results. However, on the other hand, this material and its preparations have also shown the ability to withhold this activity on numerous occasions for currently unknown reasons. Some possible reasons for the discrepancies in α-glucosidase inhibitory activity within and between samples include variations in preparation of samples and stability of active components within

samples. Additionally, components causing off-target interactions and/or toxic compounds and their presence, possibly also affected by preparation or stability, could also be contributing to these variances. Despite this conflicting data the fact remains that further studies are warranted to identify active principles which can help explain the activity witnessed and provide leads for pharmaceutical development and the standardization of preparations which use this plant for that purpose.

4.3. ANALYSIS OF PURE COMPOUNDS OF *A. ORIENTALE* RHIZOME

Pure compounds isolated from *A. orientale* were tested in 3T3-L1 cells for glucose uptake induction potential to detect the possible presence of IMAs.

4.3.1. EXPERIMENTAL

3T3-L1 cells were grown from cell-stock stored at -70°C were defrosted in 37°C. 7-8x volume of DMEM containing 10% FBS was added and centrifuged. The supernatant was removed and replaced with 5 ml of DMEM containing 10% FBS and incubated at 37°C and 5% CO_2.

Cells were incubated with regular media changes until 80% confluent. Cells were then washed twice with PBS and trypsin was added. Cells were then split and re-suspended in DMEM and grown until 70% confluent. The media was replaced with a DMEM/FBS media containing 1 mM dexamethosone, 0.5 mM IBMX, and 10 µg/ml insulin and incubated for 48 hours. The media was changed to an adipocyte

maintenance medium containing DMEM/FBS and 10 µg/ml insulin and replaced every 48 hours for 10 days until fully differentiated as evidenced by lipid droplet formation.

Cells were seeded at $1x10^6$ cells/ml into 48-well plates and washed twice with PBS and fasted in KRH for 4 hours. Test groups consisted of a blank KRH control set, test samples of 10 µM, and a 1 mM metformin positive control. Once treated cells were incubated for 30 min and each well was given media containing 1:1 DMEM:KRH and 11 mM glucose (final concentration). Glucose content in the media was measured four hours later at 505 nm using a Synergy H1 Hybrid Multi-Mode Reader Spectrophotometer and compared with the original 11 mM glucose starting concentration. Decrease in glucose media concentration indicated glucose uptake induction and was compared to the positive and negative controls.

In order to test the glucose uptake potential of pure compounds isolated from *A. orientale* the following were prepared in 10 µM concentrations: 13,17-epoxy alisol A, alismoxide, and alisol B-23-acetate.

4.3.2. RESULTS

The screening test had a measured positive control (1 mM Metformin) of 0.83 mmol/l. The results of the screen indicated observable glucose consumption in all test groups. Alismoxide had the weakest activity of the compounds tested with a 0.11 mmol/l sugar consumption. This was followed by the compound 13,17-epoxy-alisol A which had a consumption of 0.28 mmol/l. The strongest activity belonged to that of alisol B 23-acetate, which caused a glucose consumption of 0.48 mmol/l (FIGURE 21).

Subsequent studies on alisol B 23-acetate revealed that at a dose of 25 µM this isolate's glucose consumption could be more than tripled from a concentration of 10 µM and displayed dose-dependent activity (FIGURE 22).

These findings may contradict previous reporting and suggest possible IMA from this species in alisol B 23-acetate.

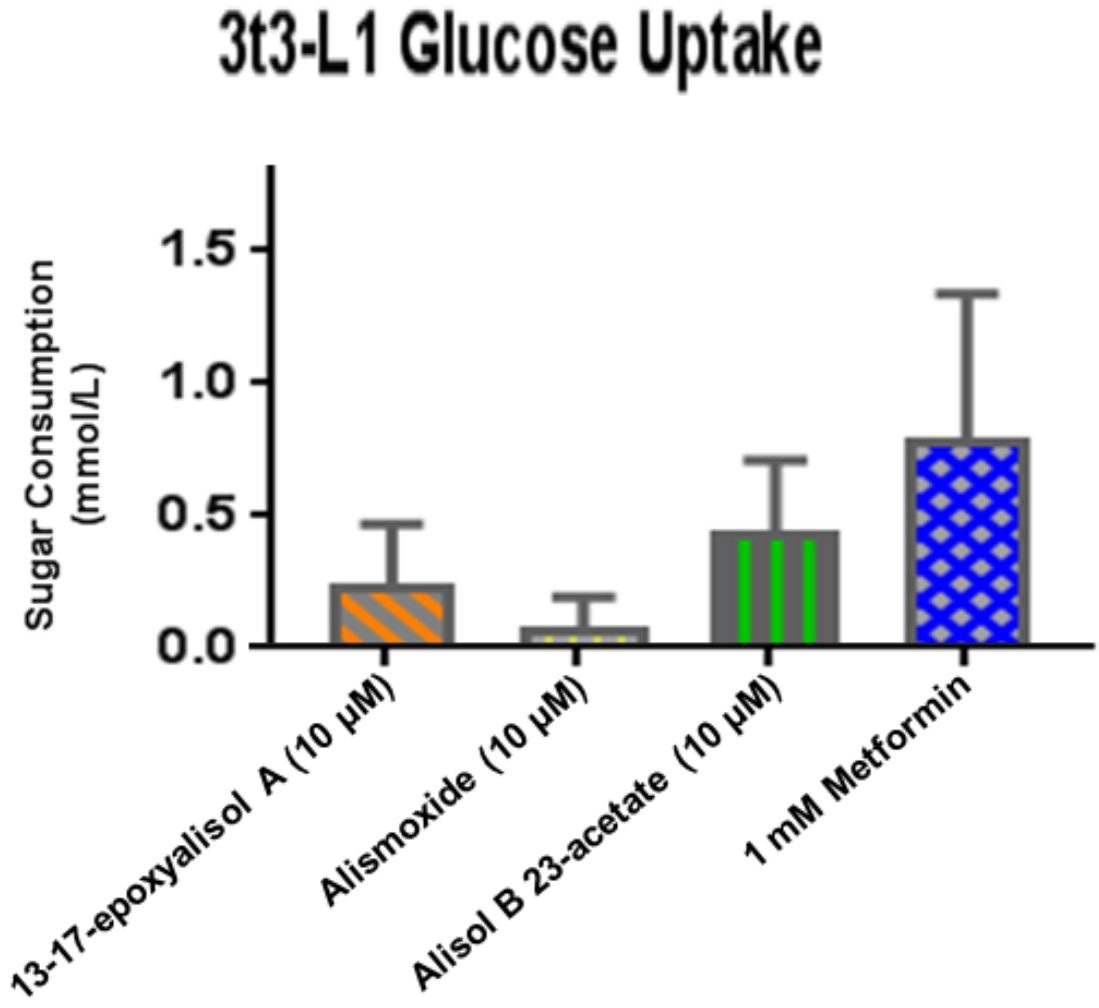


FIGURE 21: 3T3-L1 GLUCOSE UPTAKE OF PURE COMPOUNDS FROM *A. ORIENTALE*.

4.3.3. CONCLUSION

Although these tests are far from conclusive and may not be statistically significant these findings indicate that isolates from the ethyl acetate and butanol partitions of *A. orientale* possibly cause weak (in the case of alismoxide) to moderate (in the case of 13,17-epoxy alisol A and alisol B 23-acetate) glucose uptake induction. Alisol B 23-acetate produced the strongest activity and even may approach that of the

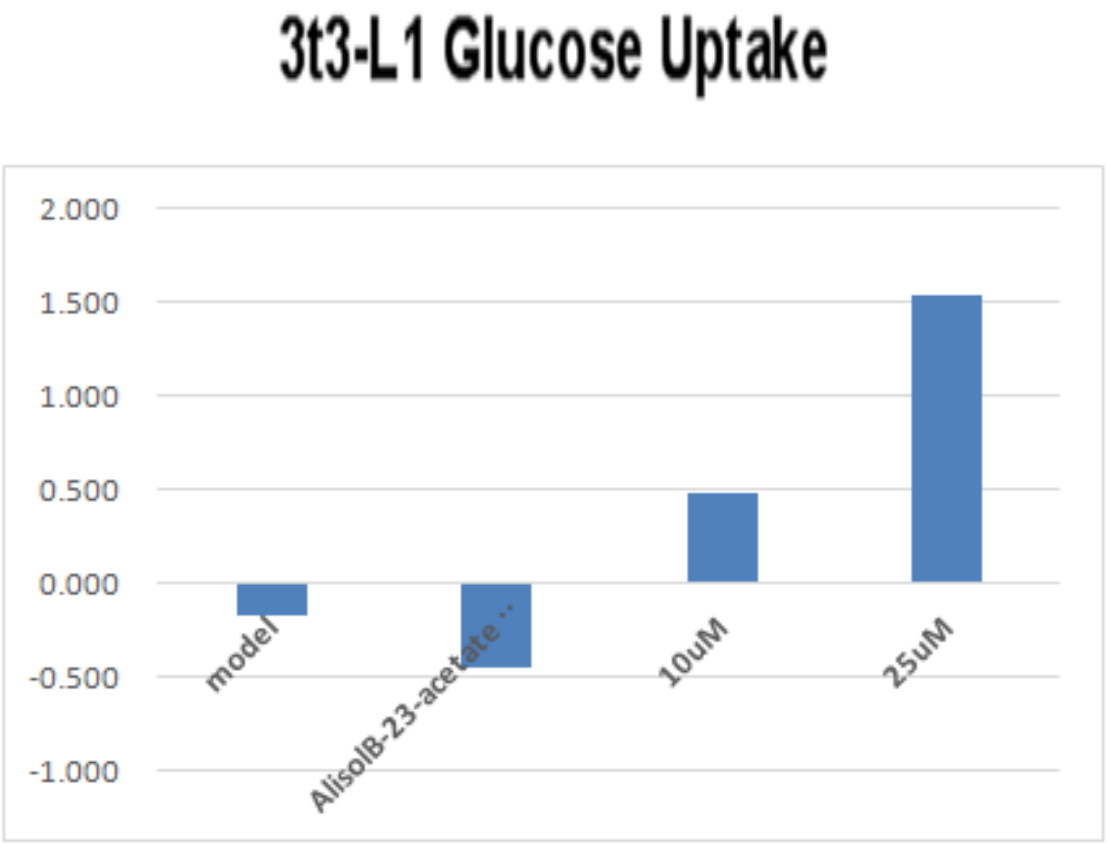


FIGURE 22: 3T3-L1 GLUCOSE UPTAKE OF ALISOL B 23-ACETATE FROM *A. ORIENTALE*. MODEL EQUALS UPTAKE FROM UNDIFFERNTIATED FIBROBLASTS.

clinical drug Metformin, although the IC_{50} was not ultimately determined so a direct comparison cannot be made and additional testing is needed. Regardless, the activity observed was much more robust and promising than previously reported for this compound and are therefore worth noting.

4.4. GLYCOGEN SYNTHESIS STUDIES ON FRACTIONS OF *A. ORIENTALE* RHIZOME

4.4.1. INTRODUCTION

Glycogen is the main form in which glucose is stored and then expended to buffer blood glucose levels. A variety of tissues are able to synthesize glycogen, including the kidney, heart, brain, and muscle, but the main producer of glycogen is the liver, which can be composed of up to 10% glycogen by weight. The buffering action of glycogen is a tightly regulated process with diametrically opposed processes known as glycogenesis (the production of glycogen) and glycogenolysis (the breakdown of glycogen into glucose). Insulin is the primary stimulator of glycogenesis (it also inhibits glycogenolysis). By measuring glycogen, which differentiated 3T3-L1 cells are able to produce in response to insulin,[134] in the presence of extracts, partitions, and fractions one can determine the insulin mimetic potential of those fractions and detect the presence of IMAs.

4.4.2. EXPERIMENTAL

An aqueous extract and crude fractions of *A. orientale* were tested for stimulation of glycogen synthesis. These included a 95% ETOH extract, two reverse phase polymeric resin (XAD) column fractions (one H_20 and one MeOH), and six fractions from a silica column of the EA partition (referred to as E-1 through E-6) all at concentrations of 0.01 and 0.1 mg/ml.

3T3-L1 cells were prepared and differentiated as described in section 4.2.1. Once differentiated cells were serum starved for 2.5 hr in DMEM containing 5 mM glucose, 0.5% FBS, and 10 mM HEPES buffer (pH 7.4). Insulin (100 nm) was then added to half the wells, and the cells were returned to the incubator for 30 min. Plates were then washed 3x with PBS on ice, 1 ml PBS was added to each well, and the cells were scraped. Cells (500 µl) were added to PBS (500 µl) and Betafluor (4 ml), shaken, and left overnight to extract the lipids. The next day the top 3ml were removed and counted using a scintillation counter.

A separate 500 µl of cells was added to 0.5ml of 60% KOH along with 500 µl of PBS for two blank tubes. KOH (30%, 200 µl) was added to all tubes and heated to 100°C on a heating block for 15 min and then brought down to 85°C for an additional 15 min. Tubes were removed and pure ethanol (2.5 ml) was added to each tube and vortexed gently but thoroughly turning the solution cloudy. Next the tubes were heated to 85°C for 30 min followed by a 15 min ice bath and spun at 3500 rpm for 10 min at 4°C. The ethanol was then aspirated and the contents were washed 2x with ice cold ethanol (70%) and spun after each wash as above. Cells were then aspirated completely and left to dry. The next day DD water (1 ml) was added to each tube, shaken at 37°C for 30 min (being vortexed every 15 min), and transferred to 20 ml scintillation vials with 9 ml of Ecolight scintillation solution for counting.

Together these procedures output a measure of the total CPM added into cells and the CPM that has moved into adipocytes for conversion into lipid or glycogen.

4.4.3. RESULTS

The results of the 100 nm insulin positive control group and no treatment group, albeit somewhat different than expected (or wanted), fall in range for a working assay.

The results of this assay indicate a 4.2 fold increase between basal and 100 nm insulin groups (FIGURE 23).

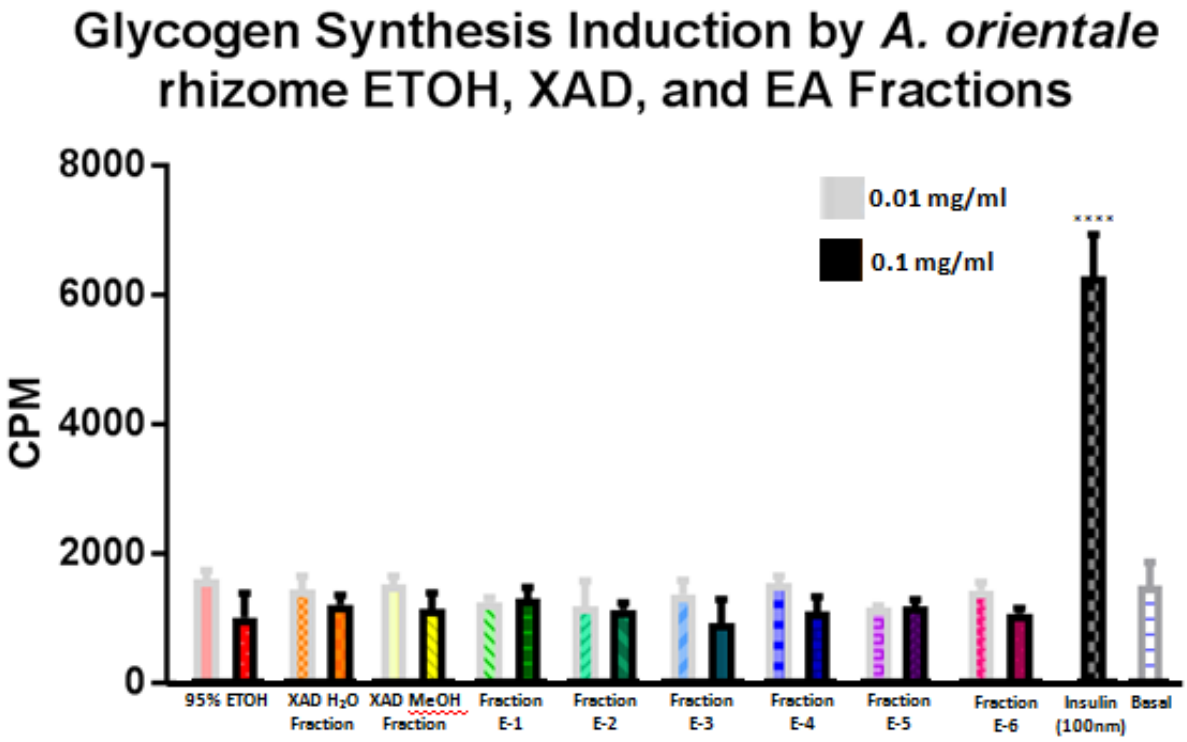


FIGURE 23: RAW DATA FROM THE GLYCOGEN SYNTHESIS ASSAY IN EXCEL XAD = REVERSE-PHASE RESIN COLUMN. NS = $P > 0.05$, **** = $P \leq 0.0001$

Although this is a statistically significant increase with a P value of ≤ 0.0001 the results indicate a high basal (no treatment) value as a function of glycogen synthesis activity within this group. This mirrors the fold normally found using the glucose uptake assay but was expected to be higher in this assay. Because of a higher basal level than anticipated it was not.

Although there is some movement within and between treatment groups tested in this assay the fractions, partitions, and extracts analyzed in this test did not demonstrate the ability to induce glycogen synthesis and were not statistically significant.

4.4.4. CONCLUSION

These experimental results indicate that none of the extracts, partitions, or fractions tested produced glycogen synthesis induction in this assay. In addition, although the experimental controls are considered within range of a working assay, our hopes to establish a more sensitive assay for our screening purposes were not realized.

CHAPTER VI. GLUCOSE GUT ABSORPTION ASSAY STUDIES ON *A. ORIENTALE* RHIZOME

This assay was performed following the protocols of Kessleer et al, 1978, Chan et al, 2007, and Hopfer et al 1973.

6.1. INTRODUCTION

Glucose gut absorption is an important mechanism by which free glucose present in the diet as well as glucose freed from the breakdown of complex carbohydrates enters the blood stream through the small intestine. This occurs in the brush border membrane within the microvilli mainly via the membrane-bound transporters Sodium/D-glucose Cotransporter 1 (SGLT1) and Glucose Transporter 2 (GLUT2). These transporters mediate transcellular movements of glucose across the brush border membrane in the case of SGLT1, and through enterocytes and into the bloodstream in the case of GLUT2 (DIAGRAM 7).

By studying the effects of extracts, partitions, fractions, and pure compounds on this process we can discover novel bioactive molecules which have antihyperglycemic activity via their ability to disrupt these transporters and inhibit postprandial blood glucose spikes.

6.2. EXPERIMENTAL

Double-layered Brush Border Membrane Vesicles (BBMVs) were used in this study as an ex-vivo representation of the biological process of glucose gut absorption. These BBMVs were prepared using magnesium precipitation and centrifugation. By

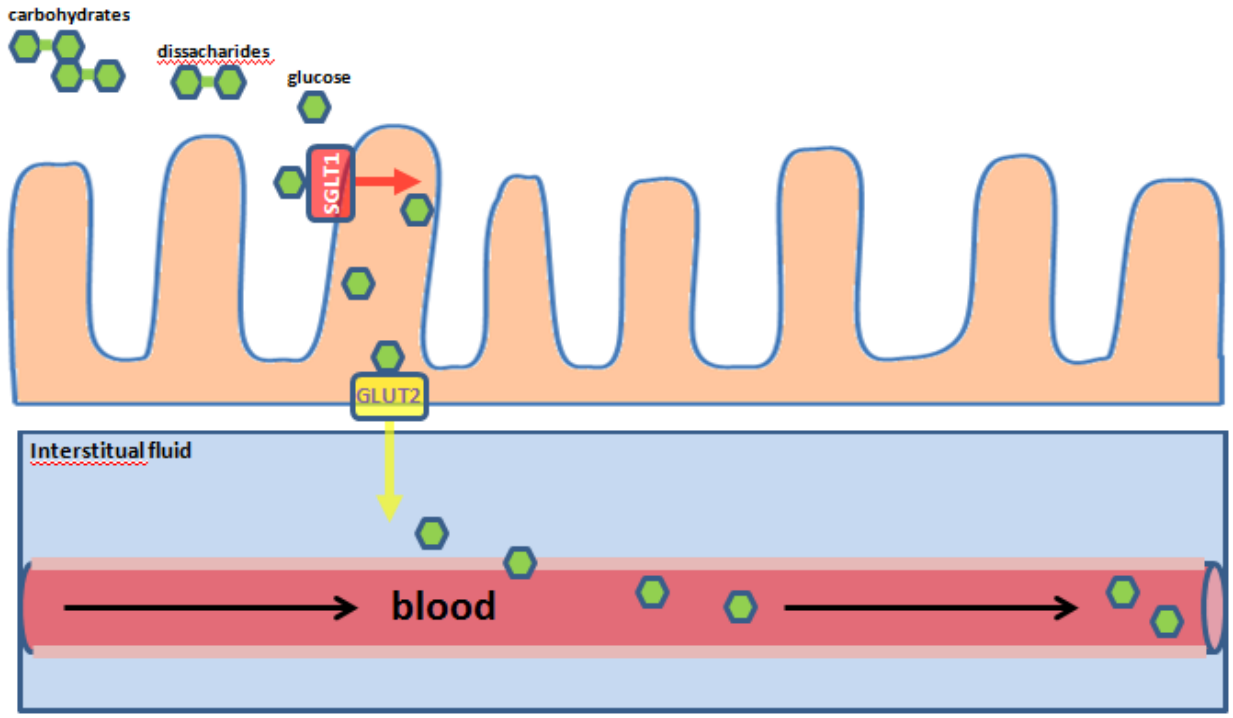


DIAGRAM 7: GLUCOSE GUT ABSORPTION VIA SGLT1 AND GLUT2 TRANSPORTERS.

adding D-[2-^{3}H], a radiolabeled glucose to this model and then quantifying accumulation via a scintillation counter, glucose absorption can be assessed.

BBMVs were supplied using adult white rabbits from New Zealand by the Laboratory Animal Services Centre, The Chinese University of Hong Kong.

Standard chemicals including Potassium chloride (KCl), D-mannitol, Magnesium chloride ($MgCl_2$), Magnesium sulfate ($MgSO_4$), Sodium thiocyanate (NaSCN), 2-amino-2-(hydroxymethyl)-1,3-propanediol (Tris), HEPES buffer, Sodium chloride (NaCl), Glucose, Phloridzin dehydrate, Tween 80 were purchased from Sigma-Aldrich Co. (St. Louis, MO). Chemicals for the radioactive portion of the assay such as D-[2-^{3}H] glucose

and Ultima Gold^{TM} liquid scintillation cocktail were purchased from PerkinElmer (HK) Ltd.

Buffer 1 contained 10 mM mannitol and 2 mM HEPES-Tris buffer at pH 7.1. Buffer 2 consisted of 100 mM mannitol, 0.1 mM $MgSO_4$, and 2 mM HEPES-Tris buffer at pH 7.4. Buffer 3 was a solution of 300 mM mannitol, 0.1 mM $MgSO_4$, and 10 mM HEPES-Tris buffer at pH 7.4. A 2-DG solution was prepared of 40 µCi/ml D-[2-^{3}H], 0.4 mM glucose, 400 mM NaSCN, 400 mM mannitol, and 40 mM HEPES-Tris buffer at pH 7.4. Additionally, a stop-wash buffer was made of 200 mM NaCl, 10 mM HEPES-Tris buffer, and 250 µM phlorizin, pH 7.4.

Rabbits were sacrificed and the small intestine was removed from the pyloric sphincter to the ileocecal junction. The intestine was then immediately placed in 200 ml ice-cold 154 mM KCl and carefully cleaned by removing mesentery and fat as well as any intestinal contents. The intestines were then cut longitudinally, cleaned thoroughly using a KCL solution, and blotted dry using paper towels. Intestines were weighed and stored using an aluminum foil wrap and liquid nitrogen. Storage was at -80°C and had a maximum storage life of 1 week.

Intestines were thawed in 200 ml buffer 1, cut into small pieces, and mechanically vibrated for 1 min. The slurry was then filtered to remove solids such as muscle and connective tissue and the filtrate was made up to 300 ml using this same buffer. Magnesium chloride (0.61 g) was added and stirred at room temperature for 20 min to precipitate contents such as nuclei, mitochondria, basolateral membranes, and miscellaneous cell debris and centrifuged at 4°C, 3800 xg for 15 minutes. The

supernatant was removed and centrifuged a second time at 4°C, 43000 xg for 30 minutes. The pellet from this centrifugation was collected and re-suspended in 5 ml buffer 2 and centrifuged at 4°C, 43000 xg for 40 minutes. Again the supernatant was removed and the pellet was carefully collected. This pellet was re-suspended in 1 ml buffer 3. The volume of the buffer plus pellet was measured and the volume was then doubled using this same buffer. This solution was then passed through a 25-gauge needle five times to form vesicles and aliquoted in volumes of 0.26 ml and frozen in liquid nitrogen.

Dried and weighed samples were brought up in 2% methanol (in the case of alismol) and 0.6% tween 80 (in the case of compounds 2-6) to make working stocks of 10 mM for the assay.

One 0.26 ml aliquot of BBMV cells previously frozen were thawed and re-suspended in 0.44 ml buffer 3. This suspension (20 µl) was mixed with 30 µl of the treatment groups and 10 µl radiolabeled 2-DG solution.

Once added these solutions were allowed to incubate at room temperature for 20 seconds. The reaction was then immediately stopped with the addition of 1 ml of ice-cold stop-wash buffer. BBMVs were then filtered using a pre-wetted 0.45 µm nitrocellulose filter (Merck Millipore, Darmstadt, Germany), and washed five times with 1 ml of ice-cold stop-wash buffer.

The filters containing the washed BBMVs were placed in separate scintillation vials and 3 ml Ultima Gold^{TM} was added to each vial. Vials were then measured using a

Beckman Coulter LS6500 Multi Purpose Liquid Scintillation Counter (Beckman Coulter, Inc., Brea, CA).

Statistical analysis was performed using the Student's t-test comparing the difference between control/solvent control and test group and presented as mean ± standard deviation. $P < 0.05$ was considered statistically significant.

6.3. RESULTS

The compounds tested in the glucose absorption assay include alismol, alisol A, alisol E 23-acetate, 13,17-epoxy alisol A, alisol B 23-acetate, and alismoxide. Phloridzin dehydrate was used as positive control, showing a 70% inhibition at a 50 µM concentration. All tested samples displayed moderate inhibitory effect (20-30% inhibition) on BBMV glucose uptake at 50 and 500 µM concentrations (FIGURE 24) when compared to the Tween 80 control.

Among these results were alisol A, 13,17-epoxyalisol A, and alismoxide which at 50 and 500 µM displayed a statistically significant ~30% inhibition of glucose uptake into BBMVs, presumably because of competitive inhibition or interference with transporter functionality.

6.4. CONCLUSION

These results indicate the possible presence of compounds (namely alisol A, 13,17-epoxy alisol A, and alismoxide) that inhibit the absorption of glucose through

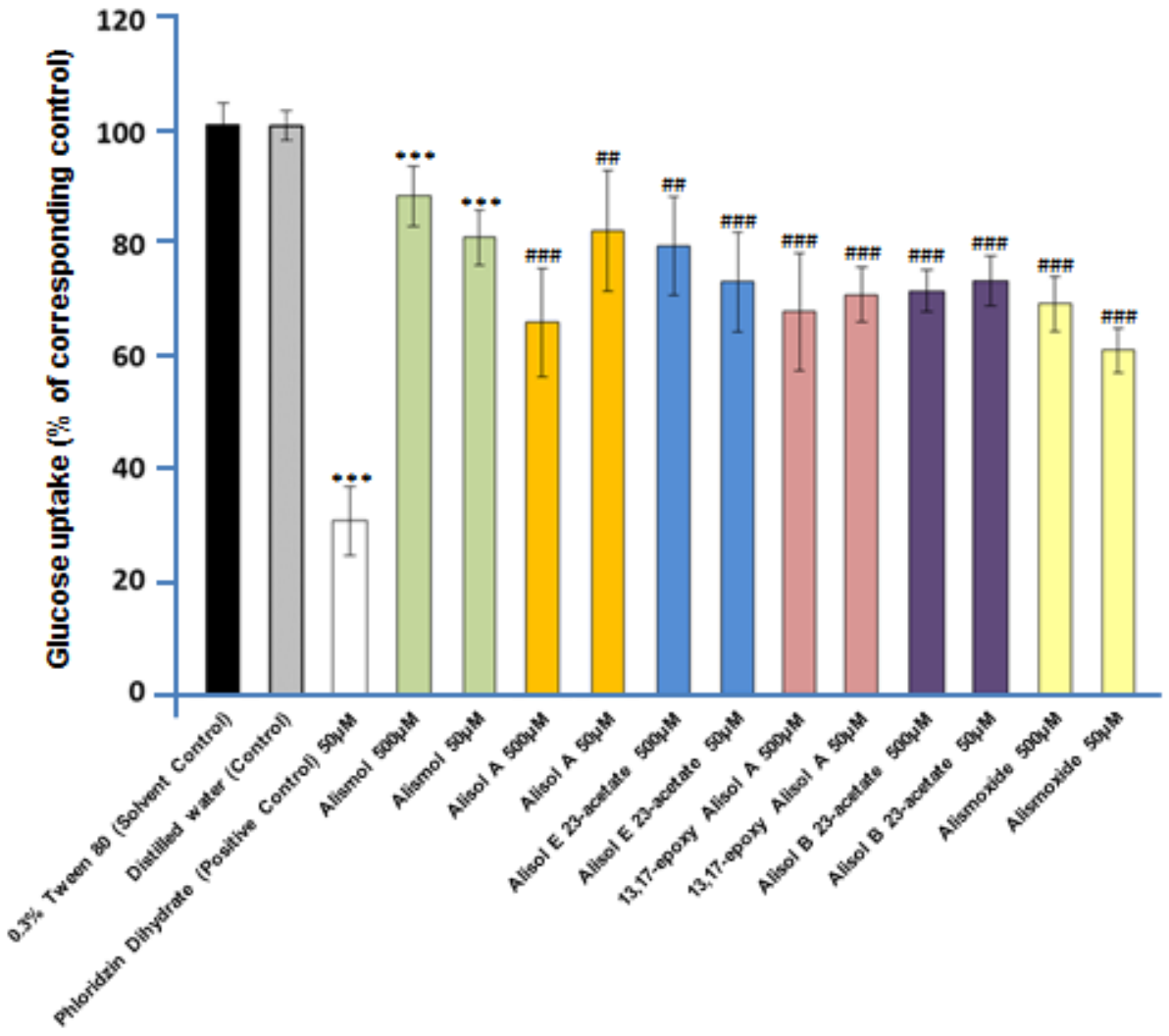


FIGURE 24. GLUCOSE GUT ABSORPTION FROM PURE COMPOUNDS OF *A. ORIENTALE*. *** DENOTES $P < 0.001$ WHEN COMPARED WITH DISTILLED WATER (CONTROL) GROUP; $^{##}$ DENOTES $P < 0.01$ AND $^{###}$ DENOTES $P < 0.001$ WHEN COMPARED WITH 0.3% TWEEN 80 (SOLVENT CONTROL) GROUP.

BBMV membranes. These activities, albeit moderate and inconclusive, could contribute to the antidiabetic action of the plant as a whole.

CHAPTER VII. ANIMAL STUDIES ON *A. ORIENTALE* RHIZOME

An animal study using a high-fat diet and streptozotocin-treated rats was used to assess the antidiabetic potential of *A. orientale in vivo.*

7.1. INTRODUCTION

High-fat diet and streptozotocin-treated rats are a standard diabetic animal model used to model the physiology found in late-stage type 2 diabetics, namely insulin resistance and the destruction of islet cells in the pancreas and the cessation of insulin production. This leads to a hyperglycemic state. By countercurrent treatment with preparations of *A. orientale* in comparison with the currently used antidiabetic drug metformin the antidiabetic potential of *A. orientale* in animal models, an important step in the development of drugs, can be assessed.

7.2. EXPERIMENTAL

Rats were housed in controlled conditions in a 12/12 hr light-dark cycle, at a temperature of 24±1°C, 60±10% relative humidity, allowed water ad libitum, and quarantined for one week.

After a one week quarantine the rats were divided into two groups: Normal diet (12 rats) and high-fat diet (70 rats). The high-fat diet consisted of 69% (w/w) normal diet feed with the addition of 15% sugar, 10% lard, 5% egg yolk powder, 0.5% cholesterol, and 0.5% bile salt.

The test group rats fed high-fat diets were subject to limited movement and thus physical activity by being placed in cages that allowed only enough space to move around to eat and drink for 6 weeks.

After the test group endured 6 weeks of high-fat diet and limited mobility they received an intraperitoneal injection of 30 mg/kg streptozoticin in citrate buffer (pH 4.5). Blood samples were taken from mildly-anesthesized rats from their orbital venous plexus and centrifuged at 3000 rpm for 15 minutes. The separated serum was then tested to determine the blood glucose level. Rats with > 11.1 mM blood glucose levels were used for the study (totaling 60 rats).

The 72 rats were divided into 6 groups, 12 rats each. These groups consisted of a normal diet/normal exercise/non-diabetic (not treated with streptozotocin) group given 10 ml/kg/day distilled water, and 5 groups of high-fat diet/reduced exercise/diabetic (streptozotocin-treated) rats. One group was left as is for a diabetic no-treatment control and given 10 ml/kg/day water. Three groups were given the ethyl acetate fraction from a 95% EtOH extract of an authenticated batch of *A. orientale* rhizome shown to be active in the 3T3-L1 glucose uptake and α-glucosidase inhibition assays in water by gavage in doses of 20, 50, and 100 mg/kg (equivalent to 0.4, 1.0, and 2.0 g dried plant/kg). These doses were chosen based on the dosage of 6-10 g dried plant commonly practiced by herbal doctors and recommended in the Chinese Pharmacopoeia.[135] Assuming a surface area factor of 6.2 and an average human body weight of 60 kg a human dose of 0.1 g/kg is equivalent to 0.62 g/kg. Since the experimental doses are 0.4, 1.0, and 2.0 g/kg they cover the range of human doses. The sixth group was given 100 mg/kg metformin in water by gavage as a positive

control. All treatments were given once daily for 4 weeks. For the diabetic and non-diabetic no treatment groups distilled water gavages were administered.

Rats were assessed daily for survival as well as general signs of health including mobility, body weight, mental activity, water and food intake, urine output, and fur quality. The study was not blinded.

Blood glucose levels were tested twice after administration of treatment, once in the second, and once in the fourth week. These samples were taken under mild anesthesia from the orbital venous plexus. Blood samples were then centrifuged at 3000 rpm for 15 minutes, and the serum was tested for glucose concentration using a glucose oxidase kit.

Rats were overnight-fasted and administered 0.5 U/kg insulin intraperitonially. Blood collected from mildly-anesthetized rats from the orbital venous plexus were then tested for serum glucose concentrations after centrifugation at 3000 rpm for 15 minutes using a glucose oxidase test kit at 30, 60, and 120 min after insulin administration.

After 4 weeks the rats are given one last treatment (where applicable) and anesthetized using 50 mg/kg sodium pentobarbital. Bloods samples were then collected abdominally through the aortic artery. Blood samples were centrifuged at 3000 rpm for 15 minutes to separate serum. Commercial kits, used in accordance with the manufacturer's directions, were used to measure blood serum concentrations of insulin, HbA1c, total (TC) as well as high (HDL-C) and low-density lipoprotene cholesterol (LDL-C), triglyceride (TG), malondialdehyde (MDA), superoxide dismutase

(SOD), glutathione peroxidase (GSH-Px), tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), and c-reactive protein (CRP).

Pancreatic tissue was collected after anesthetization for analysis. Samples were fixed in neutral formalin for 48 hr, and dehydrated with ascending grades of alcohol for preservation. Samples were then cleared in benzene and embedded in paraffin wax. Microtome sections (3 μm thick) were taken and stained with hematoxylin and eosin (HE staining) for analysis using a light microscope.

Samples were tested for insulin receptor substrate 1 (IRS-1) and protein kinase B (AKt) using western blot analysis.

7.3. RESULTS

During the initial 6-week diet phase rats fed a high-fat diet were observed to have a significant increase in body weight and tarnished fur. After the injection of streptozotocin these rats showed symptoms characteristic of diabetes such as polydispsia, polyuria, and a strong, thickened urine smell.

At week 2 after treatment no statistically significant differences in blood glucose was observed between treatment groups and the no treatment diabetic control. At week 4 after treatment, however, statistically significant and dose-dependent decreases in blood glucose levels were seen in those receiving *A. orientale* ethyl acetate fraction or metformin when compared with the diabetic control. The 100 mg/kg dose of *A. orientale* ethyl acetate fraction caused a 35% decrease in blood glucose when compared to the diabetic no treatment group.

Rats in all diabetic groups had a general elevated glucose level overall. 30 min after treatment with insulin blood glucose levels dropped but then returned to elevated levels after 2 hrs. Groups treated with *A. orientale* ethyl acetate fractions, however, showed dose-dependent decreases in blood glucose levels when compared to the diabetic no treatment group (FIGURE 25).

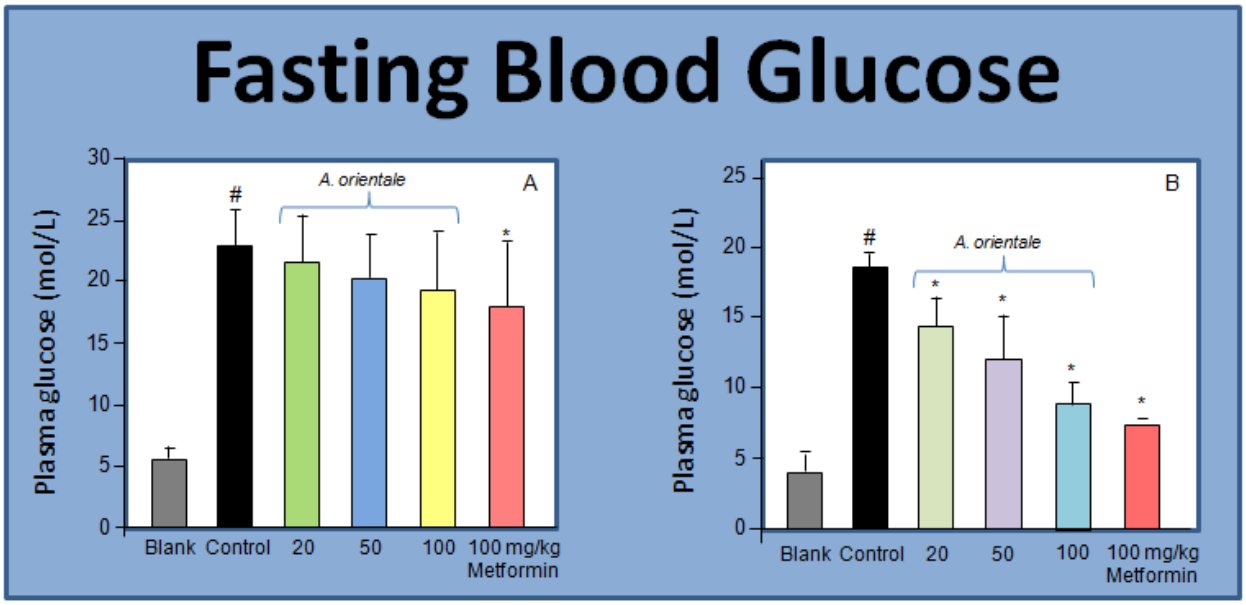


FIGURE 25: FASTING BLOOD GLUCOSE LEVELS AT WEEK 2 (A) AND WEEK 4 (B) OF TYPE 2 DIABETIC RATS TREATED WITH EA PART OF *A. ORIENTALE.* (*X* ± *S, N = 12).* MET: METFORMIN. # P < 0.05 VS BLANK GROUP; * P < 0.05 VS CONTROL GROUP.

The *A. orientale* group (100 mg/kg) showed a 12% decrease when compared to the diabetic no treatment group. The 100 mg/kg metformin group lowered the blood glucose by 15% when compared with the diabetic no treatment group.

Serum insulin levels were observed to be decreased in both the *A. orientale* (100 mg/kg) treated and metformin treated groups compared to the diabetic no treatment group. The *A. orientale* (100 mg/kg) and metformin groups caused a 34% and 36% decrease in serum insulin levels, respectively (FIGURE 26).

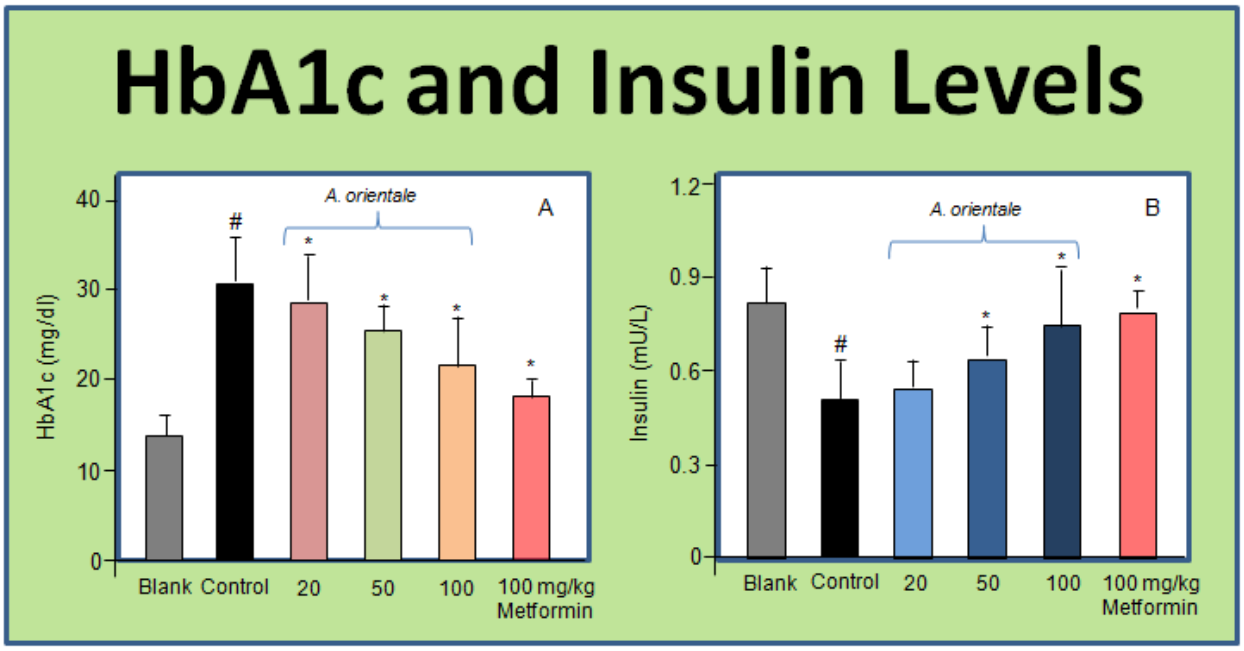


FIGURE 26: INSULIN (A) AND HBA1C (B) LEVELS IN TYPE 2 DIABETIC RATS TREATED WITH EA PART OF *A. ORIENTALE.* (*X* ± *S, N = 12).* MET: METFORMIN. # P < 0.05 VS BLANK GROUP; * P < 0.05 VS CONTROL GROUP.

HbA1c levels were found to be decreased in a dose-dependent manner within the *A. orientale* treatment groups when compared with the diabetic no treatment group. The *A. orientale* (100 mg/kg) group caused a 10% reduction when compared with the diabetic no treatment group. Metformin caused a 12% reduction (FIGURE 35).

TC was reduced by 25% in the metformin treated group. No difference was found in the groups treated with *A. orientale.* TG content was not significantly different in 20 mg/kg and 50 mg/kg when compared to the diabetic control group, but 100 mg/kg *A. orientale* and metformin reduced the serum TG content by 20% and 24%, respectively. HDL-C was not affected in a statistically significant manner when compared to the diabetic no treatment group with 20 mg/kg and 50 mg/kg *A. orientale* ethyl acetate fraction. *A. orientale* (100 mg/kg) and metformin increased HDL-C by 15% and 24%, respectively. Concentrations of 20 mg/kg and 50 mg/kg *A. orientale* had no effect on LDL-C. *A. orientale* (100 mg/kg) and metformin decreased LDL-C by 23% and 29%, respectively (FIGURE 27).

MDA was decreased by 14% with *A. orientale* (100 mg/kg) and was accompanied by a 23% increase in the antioxidant enzyme glutathione GSH-Px as well as a 24% increase in SOD.

The inflammatory markers TNF-α, lL-6, and CRP were statistically altered in groups treated with 50 mg/kg and 100 mg/kg *A. orientale*. TNF-α was reduced 32% and 44% and the IL-6 was lowered by 17% and 19% with 50 mg/kg and 100 mg/kg doses, respectively, when compared to the diabetic no treatment group. *A. orientale* (100 mg/kg) reduced CRP by 24%.

Microscopic analysis of histological changes on HE stained pancreatic tissue showed islet β-cell damage in the diabetic no treatment group. These damages appeared to be significantly prevented with respect to cellular integrity of the β-cells in the groups treated with *A. orientale.*

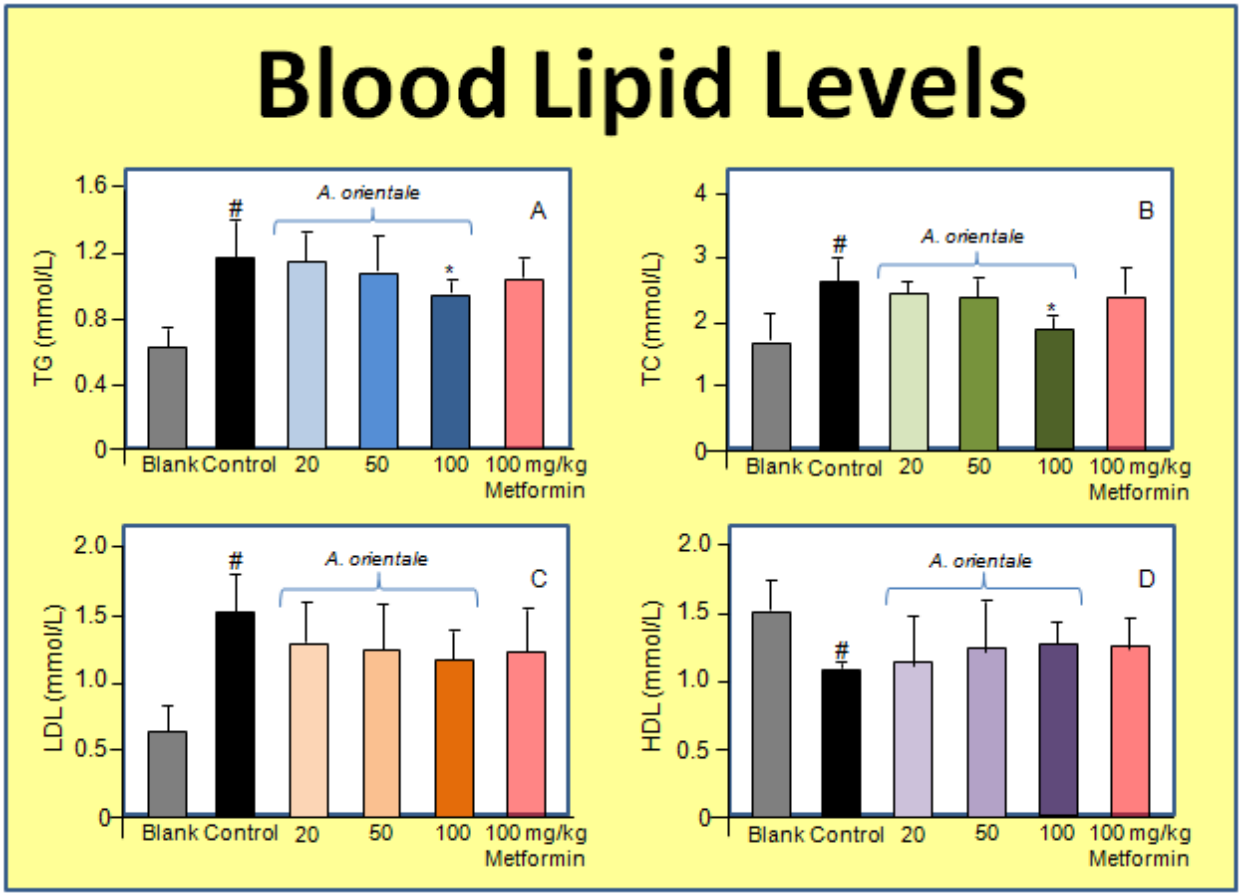


FIGURE 27: BLOOD LIPID EFFECTS OF EA PART *A. ORIENTALE*-TREATED TYPE 2 DIABETIC RATS. (*X* ± *S, N = 12).* A = TG LEVELS, B = TC LEVELS, C = LDL LEVELS, D = HDL LEVELS. MET: METFORMIN. # P < 0.05 VS BLANK GROUP; * P < 0.05 VS CONTROL GROUP.

Western blot analysis revealed a decrease in IRS-1 and AKt in the diabetic no treatment group. Treatment with *A. orientale* was accompanied by restored phosphorylated IRS-1 and AKt levels (FIGURE 28).

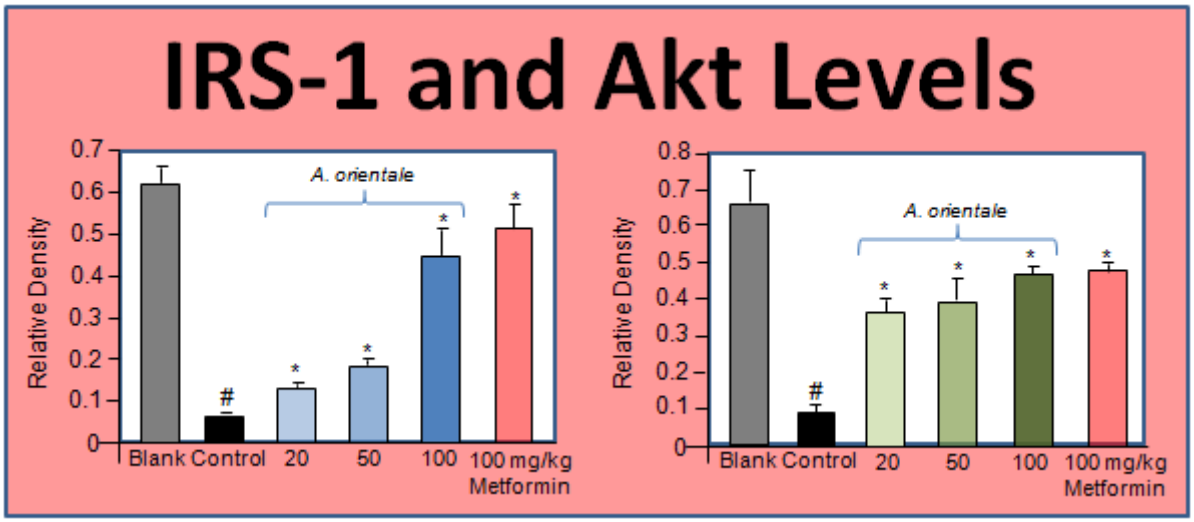


FIGURE 28: WESTERN BLOT ANALYSIS OF SAMPLES TAKEN FROM TYPE 2 DIABETIC RATS TREATED WITH EA PART OF *A. ORIENTALE* TO DETERMINE IRS-1 (A) AND AKT (B) LEVELS.

A detailed description of the rationale, methodology, results, analysis, and conclusions including these results and more can be found in Cao et al, 2017.[136]

7.4. CONCLUSION

These findings suggest that ethyl acetate partitions of *A. orientale* cause a statistically significant and dose-dependent hypoglycemic effect in streptozotocin-

induced diabetic rats four weeks after treatment. Additionally, serum insulin and HbA1C levels were decreased in treatment groups containing *A. orientale*. No difference in total cholesterol were observed. At lower doses of *A. orientale* no changes in triglycerides of HDL-C could be discerned. However, in the highest dose of 100 mg/kg TG was reduced and HDL-C was increased and was comparable to the metformin group. Treatment groups containing *A. orientale* also saw appreciable decreases in malondialdehyde, TNF-α, and lL-6 levels and increases in glutathione-peroxidase and superoxide dismutase levels. Western-blot analysis revealed that Receptor Substrate 1 and Protein Kinase B decreases found in the no treatment group were restored in groups containing *A. orientale*. Together these findings indicate a range of antidiabetic activities caused by the ethyl acetate part of *A. orientale* that could be useful in the treatment of T2DM and possibly T1DM.

CHAPTER VII. CONCLUSIONS

Through the course of this project the connection between *A. orientale* and its observed antidiabetic activity were considered in depth. Through literature studies and indeed our own initial experiments this plant species displayed promising activity in a number of mechanisms useful for the treatment of these diseases. These took the form of *in vitro* and *in vivo* experiments in the areas of α-glucosidase, glucose uptake, glucose gut absorption, and hypoglycemic studies. Our group rationalized the separation of the plant material in two ways: One was for the parsing of different and sometimes opposing actions caused by the various components of the plant. For instance, the plant may contain some glucose uptake inhibitors as well as inducers and the presence of one in the sample may mask the other and therefore the true medicinal potential of this species. Additionally, previous to this project the specific antidiabetic principles which were causing these activities were largely unknown. Our aim was also to help in the identification of active principles for three purposes: a) To provide drug leads for the development of clinical drugs, b) to help justify the use of this plant traditionally, and c) to provide information for the standardization of formulations which utilize this drug for antidiabetic purposes.

The project obtained material which it subsequently authenticated, extracted, and partitioned. The ethyl acetate partition was then tested *in vivo* for hypoglycemic activity, as well as *in vitro* for α-glucosidase inhibition and glucose uptake induction to determine activity, the first tests of their kind to be reported in the literature.

These studies were followed by crude fractionation to determine the areas in which the antidiabetic principles resided as indicated by its α-glucosidase activity. The results revealed activity residing in the butanol fractions B1 and B3 and in the ethyl acetate fractions E2, E3, and E5.

Fractionation in these areas was conducted to obtain pure compounds which could be tested for activity which resulted in the compounds 1-O-ethyl-D-galactopyranose (possibly a new compound), cytidine, alismoxide, D-fructopyranose, alisol A, and 13,17-epoxyalisol A. Additionally, compounds previously isolated from this plant as well as compounds available for purchase were gathered. Together these compounds representing areas of interest within the plant we tested to determine their biological activity relevant to diabetes by proxy of the series of assays the project employed including the α-glucosidase assay, as well as the glucose uptake and BBMV assay.

The BBMV assay resulted in the identification of the component alisol A and 13,17-epoxy alisol A from the ethyl acetate partition as a possible glucose gut absorption inhibitors. At a concentration of 500 µM alisol A and 13,17-epoxy alisol A inhibited glucose absorption across the brush border membrane by ~30%. Variable mild activity was seen in the other compounds tested.

The glucose uptake assay was able to determine several active components as well. Through these studies it was demonstrated that 10 µM alismoxide of the butanol fraction caused a slight increase in glucose uptake *in vitro*. The compound 13,17-epoxy alisol A of the ethyl acetate fraction caused a more prominent increase in glucose

uptake at a concentration of 10 µM. The strongest activity was seen from alisol B 23-acetate of the ethyl acetate fraction, which at concentrations of 10 and 25 µM displayed robust glucose uptake potential.

The α-glucosidase assays identified numerous compounds from both the butanol and ethyl acetate partitions as causing significant activity. Some of the active components of the butanol fraction were sugars such as 1-O-ethyl-D-galactopyranose, and D-fructopyranose. The compound cytidine from the butanol fraction was found to have a significant α-glucosidase inhibition potential with an IC_{50} of 0.36 mM and was the most potent isolate from this partition. From the ethyl acetate partition the compounds alisol A, alisol P, and daucosterol-6-O-stearate displayed moderate activity with IC_{50}'s between 0.5 and 1.0 mM. Compounds from this partition with IC_{50}'s between 0.5 mM and 0.15 mM (that of the positive control and clinical drug acarbose) included 13,17-epoxy alisol A, and alisol E 23-acetate. Two compounds from the ethyl acetate partition produced stronger inhibition than acarbose: Alisol O and 25-O-methyl alisol, the latter of which had an IC_{50} of < 0.099 mM and the former which was demonstrated to be a full five times more potent than acarbose with an IC_{50} of 0.03 mM making for the identification of two α-glucosidase inhibitors likely more active than anything ever isolated from this species, albeit still in the middle µM range.

Some of the isolates identified in these assays displaying measurable bioactivity in the α-glucosidase inhibition assay were sugars present within the butanol fraction of *A. orientale* and are not of interest for the development of new antidiabetic drugs. They are, however, notable as they likely contribute to and complicate whole-extract and crude fraction assessments. Others may in fact resemble sugars in ways that allow

them to become competitive inhibitors. It's even possible that some components cause both α-glucosidase inhibition and glucose gut absorption inhibition because of this resemblance.

Still yet some compounds this project identified as having activity in one or more of the assays such as alisol A, 13,17-epoxy alisol A, alisol B 23-acetate, cytidine, 25-O-methyl alisol A, and alisol O have interesting and possibly useful structures that warrant further study for the development of new antidiabetic agents for use in the treatment of diabetes and its complications, possibly in conjunction with current therapies.

These same compounds are also noteworthy for the standardization of formulas containing this species for use in the treatment of diabetes and its complications. However, one important finding from our work is that some compound's activities stretch across multiple assays such as the compound 13,17-epoxy alisol A from the ethyl acetate fraction, which was among the most active α-glucosidase inhibitors as well as the among the most active glucose uptake inhibitors found in this project. Others were found to be mechanism specific having activity in some assays but not others. Therefore, the standardization of antidiabetic preparations which utilize this species may depend on what mechanism of action is being sought.

One such case is that of alisol B 23-acetate, which preparations of *A. orientale* are currently authenticated and standardized for.[15] On the one hand this compound showed potent glucose uptake induction in our tests. On the other hand it performed quite poorly in the α-glucosidase inhibition test. These results indicate that alisol B 23-acetate may be an appropriate marker for standardization with respect to being used for

glucose-uptake activity but would be an inappropriate marker for predicting antidiabetic activity in preparations containing this species for use as an α-glucosidase inhibition agent. Because of the conflicting role the project found between the antidiabetic potentials of this compound in different mechanisms and because alisol B 23-acetate concentration is known to vary between plants within and between regions, [137-138] harvesting considerations should be further studied to optimize the production of material from *A. orientale* in a manner that is cognizant of the specific antidiabetic activity being sought.

We would be remiss to not mention that there were some issues with the theoretical approach of this project, as well as with the methodology and results of their analyses. For instance, diabetes mellitus is a recently discovered disease. This raises some doubt as to the usefulness of a long history of use in the treatment of this disease, especially since these associations have largely been made retroactively by comparison between their known symptoms and how they may fit into TCM theory. To compound this concern, it is doubtful some of the displayed actions of this plant (e.g. α-glucosidase inhibition and hypoglycemia more broadly) would have been considered beneficial at a time when the dangers of high blood sugar were not yet understood and food was scarce. In addition to these theoretical concerns there were problems with the methodology and results. For instance, the concentrations which were tested and showed activity in these assays were at relatively high concentrations. Although these concentrations are clinically achievable they beg the question of whether solubility issues could be encountered and whether any cytotoxicity as a result of these concentrations could occur. Additionally, with respect to the animal study, methodology

such as a lack of blinding, and results such as the weight of the rats throughout the study were not included. When it comes to the α-glucosidase inhibition assay none of the samples (e.g. fractions or pure compounds) were tested against a second enzyme leaving open the possibility that these displayed inhibitions aren't enzyme specific and therefore of interest for future study or use in standardization. These omissions could have important implications for the conclusions we draw from this work. Another issue which is encountered with the testing of complex mixtures such as impure fractions, partitions, and extracts, is the stability of individual components within these mixtures and their effect on activity. These mixtures must be qualitatively and quantitatively analyzed and standardized for their results to be applied to other samples or repeated themselves. The inability for some of our tests to be duplicated, such as during the glucose uptake assay, are a testament to the reality and importance of this problem.

Given unlimited time, money, and materials, these issues would be addressed directly. In the case of concentrations deemed problematically high, these samples could be retested at lower doses, solubility could be analyzed carefully, and IC50's for all compounds could be determined. With respect to cross-analysis more studies could be conducted to determine if inhibition is enzyme-specific, what sub-type of enzyme inhibition (e.g. maltase or sucrose) it belongs to.

Additionally, more research should be conducted on the structure/activity relationship of actives to assess the possible role of analogs in the development of more potent compounds which use their general structure.

In the case of *in viv*o studies it would be pertinent to conduct additional studies which are properly blinded to prevent biases in evaluation, and to record weights of the animals throughout the study as these can provide important clues for the mechanism of action which treatments are acting.

Although notoriously hard to source, this area of research would benefit from single-compound *in vivo* experiments, including with compounds which have been the focus of our *in vitro* studies. For instance, it would be interesting to further study alisol O, which was the most active compound identified in this project, or 13,17-epoxy alisol A which displayed activity in all three assays we conducted *in vivo* to see if they cause a measurable effects in animals. Likewise, given the current focus of alisol B acetate as a marker compound for preparations of *A. orientale* in standardization and authentication and our findings of its spurious activity across mechanisms, it would be interesting to see what effect it has *in vivo* as well.

Another interesting area that could be explored is that of synergy between components of this species. It is possible that the fractions, partitions, or extracts of the plant have stronger antidiabetic activity than the sum of its component parts. Through carefully designed experiments and their subsequent analysis using a pool of known active components from this species this rarely studied phenomenon could be detected and studied.

Although this plant has a long history of use without a reputation of being toxic the toxicity of this plant species and its isolates are largely unknown. As this is an important consideration for the standardization of material as well as the development of

new drugs, these studies should be conducted to assure the safety of preparations of this plant, as well as any products derived from it.

It is the hope of our team that the efforts of the present investigation on the antidiabetic potential of *A. orientale* have helped to justify the use of this plant traditionally, solidified its importance for further study, yielded information useful for the development of new drugs and the standardization of herbal products, and has had an overall impact of helping future researchers in their endeavor of battling this devastating disease.

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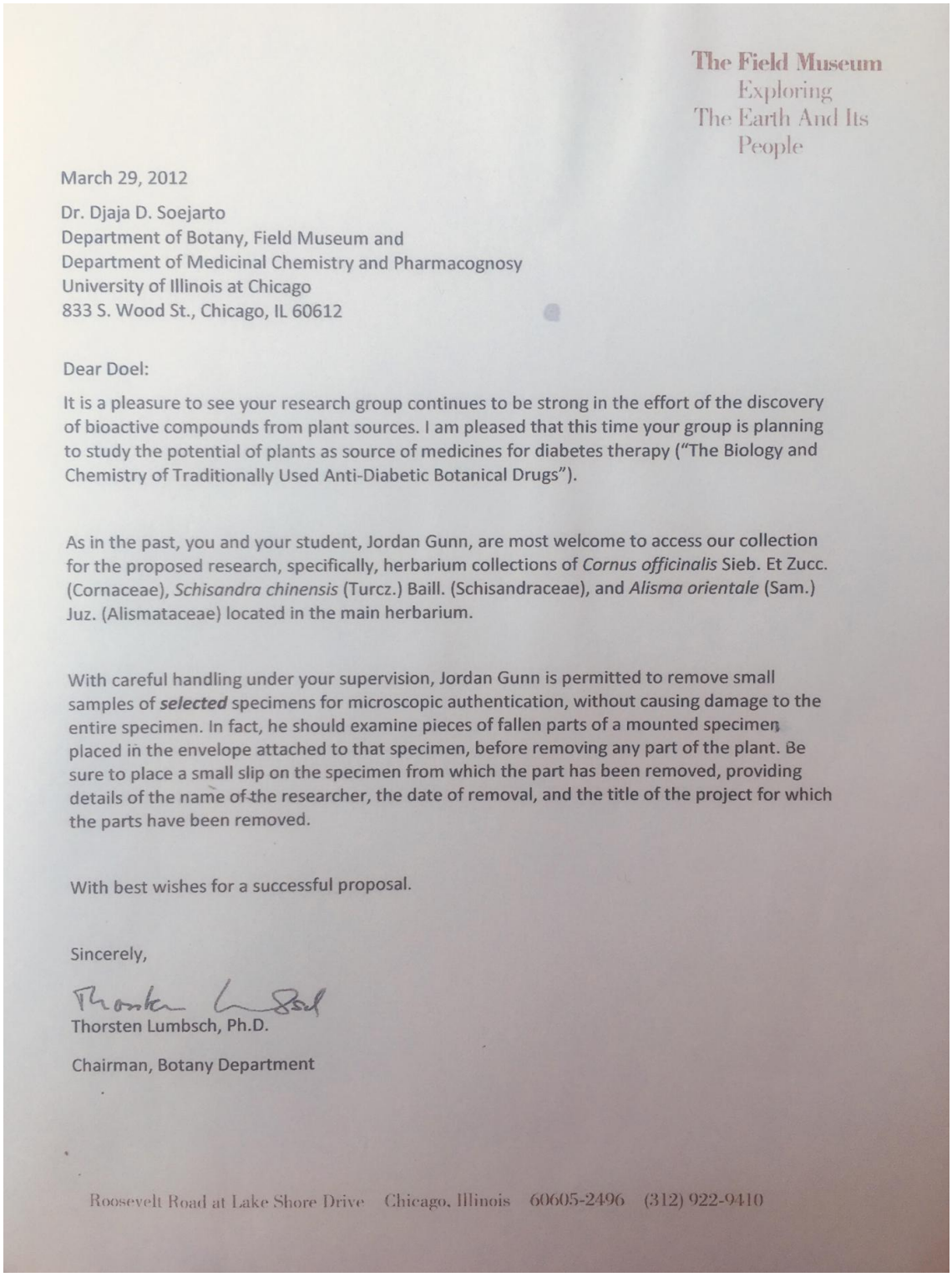
APPENDICES

APPENDIX A. COLLECTION PERMIT OF SPECIMEN WALTER KOELZ-4202: FM 149316



The Field Museum
Exploring
The Earth And Its
People

March 29, 2012

Dr. Djaja D. Soejarto
Department of Botany, Field Museum and
Department of Medicinal Chemistry and Pharmacognosy
University of Illinois at Chicago
833 S. Wood St., Chicago, IL 60612

Dear Doel:

It is a pleasure to see your research group continues to be strong in the effort of the discovery of bioactive compounds from plant sources. I am pleased that this time your group is planning to study the potential of plants as source of medicines for diabetes therapy ("The Biology and Chemistry of Traditionally Used Anti-Diabetic Botanical Drugs").

As in the past, you and your student, Jordan Gunn, are most welcome to access our collection for the proposed research, specifically, herbarium collections of *Cornus officinalis* Sieb. Et Zucc. (Cornaceae), *Schisandra chinensis* (Turcz.) Baill. (Schisandraceae), and *Alisma orientale* (Sam.) Juz. (Alismataceae) located in the main herbarium.

With careful handling under your supervision, Jordan Gunn is permitted to remove small samples of ***selected*** specimens for microscopic authentication, without causing damage to the entire specimen. In fact, he should examine pieces of fallen parts of a mounted specimen placed in the envelope attached to that specimen, before removing any part of the plant. Be sure to place a small slip on the specimen from which the part has been removed, providing details of the name of the researcher, the date of removal, and the title of the project for which the parts have been removed.

With best wishes for a successful proposal.

Sincerely,

Thorsten Lumbsch, Ph.D.

Chairman, Botany Department

Roosevelt Road at Lake Shore Drive Chicago, Illinois 60605-2496 (312) 922-9410

APPENDIX B. INSTRUMENTATION

- Virtis 25EL Feezemobile Lyophilizer
- Gowe Dry Heated Nitrogen Evaporator
- Vacuum Desiccating Cabinet w/Thermo SPCPOF110 vacuum source
- Shimadzu HPLC Prominence UFLC XR series
- AmScope 5.0 Megapixel Microscope Color Digital Camera
- 400 MHz Bruker AVIII HD NMR Spectrometer
- 400 MHz Bruker DPX-400 NMR Spectrometer
- Micromass Quattro II Triple Quadrupole Mass Spectrometer
- Beckman Coulter LS6500 Multi Purpose Scintillation Counter
- Synergy H1 Hybrid Multi-Mode Reader Spectrophotometer
- Biotek Synergy H4 Hybrid Multi-Mode Microplate Reader

VITA

Jordan Gunn

EDUCATION

Ph.D., Pharmacognosy, University of Illinois at Chicago, Chicago, Illinois, 2018

B.A., Philosophy, Coe College, Cedar Rapids, IA, 2009

HONORS

- Ruth L. Kirschstein National Research Service Award Individual Predoctoral Fellowship (F31) 2012-2015
- Edward Benes Scholarship 2013
- Chancellor's Student Service Award (CSSA) 2011
- Presidential scholarship, Coe College 2005-2009
- Philosopher of the year award, Coe College 2006
- Roy J. Carver Scholarship, 2005
- Honor roll 2000-2005
- AP Chemistry Washington HS 2004
- Top ten letter 2001 & 2002
- President's student achievement award, 2001
- Eagle scout award, 2001
- Order of the Arrow

PROFESSIONAL MEMBERSHIP

- Web Committee 2011
- Graduate Committee 2009-2011
- ICBG Lao Project Staff 2009-2011
- Graduate Studies Committee 2009-2011
- Graduate Student Council Representative 2010-2011
- Faculty Senate Student Representative 2010-2011
- Research Committee 2010-2011
- CampusCare Committee Chair 2011

PUBLICATIONS

Gunn, J., Zhao, M., Zhang, X., Che, C. α-Glucosidase Inhibitors from the Rhizome of *Alisma plantago-aquatica* subsp. *orientale*. (Submitted)

Cao, L., Zhu, K., Jiang, C., Gunn, J., Che, C., Zhang, J., Yin, Z. Intervention effects of the Extract of *Alismatis Rhizoma* on Streptozotocin-Induced Type 2 Diabetic Rats. Journal of China Pharmaceutical University, 2017, 48, 601-608.

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Gunn, J., Sydara, K., Southavong, B., Soejarto, D., Farnsworth, N. Literature-based Evaluation on Potential Toxicity of Lao Medicinal Plants (In review)

Gunn, J., Sombat, S., Damrong, S. Thai Medicinal Herbs used in Public Healthcare (To be published)

Whitlatch, A., Monahan, K., Stegeman, A., Kandel, K., Gunn, J., Brandt, C., Dean, M. and Tauer, T.J. Phragmatopoma lapidosa hypothetical protein mRNA, complete cds. Accepted August 2005. GenBank Accession Number: DQ167588