2001

INHIBITION STUDIES OF PROTEIN PHOSPHATASE 2A BY KNOWN CATHARTIC PLANT DRUGS

Michael Ford
University of Rhode Island

INHIBITION STUDIES OF PROTEIN PHOSPHATASE 2A BY KNOWN CATHARTIC PLANT DRUGS

BY

MICHAEL FORD

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE

REQUIREMENTS FOR THE DEGREE OF

MASTER OF SCIENCE

IN

PHARMACOGNOSY

UNIVERSITY OF RHODE ISLAND

2001

MASTER OF SCIENCE THESIS

OF

MICHAEL FORD

APPROVED:

Thesis Committee

Major Professor

DEAN OF THE GRADUATE SCHOOL

UNIVERSITY OF RHODE ISLAND

2001

ABSTRACT

Protein phosphatases play significant roles in signal transduction pathways that regulate cellular processes in response to external/internal stimuli. They are crucial for the growth, division, and differentiation of all organisms. Examples of cell functions involving reversible phosphorylation include ion transport, metabolism, cell cycle progression, developmental control, and stress responses. Serine/threonine-specific protein phosphatases are of particular interest in this study. There are two types of serine/threonine-specific protein phosphatases: type 1 & type 2. Both protein phosphatase 1 (PP1) and protein phosphatase 2A (PP2A) are inhibited by a structurally diverse group of natural toxins produced by marine organisms that cause diarrhetic shellfish poisoning. Recent research into diarrhetic shellfish poisoning has contributed to the understanding of some of the mechanism of actions of cathartics. It was learned that hydroxy acid moieties are essential for receptor binding of diarrhetic shellfish toxins, such as okadaic acid, to the protein phosphatase enzymes in the intestines which regulate ion channels. The loss of ion channel regulation in the intestines leads to an efflux of electrolytes and water, causing diarrhea.

This study was undertaken in order to learn if four different crude cathartic plant drugs whose active components contain hydroxy-acid moieties, will act similarly to these diarrhetic shellfish toxins by inhibiting PP2A. First, two resin glycosides, jalapin and convolvulin, were isolated from *Ipomoea purga* and *Pharbitis nil*, of the Convolvulaceae family, respectively. After verifying their molecular structures by proton nuclear magnetic resonance (^{1}H NMR) spectroscopy, they were tested against PP2A. Pure ricinoleic acid from *Ricinis communis* and

podophyllotoxin from *Podophyllum peltatum* were purchased and also tested against PP2A. A fluorometric assay, recently developed in Dr. Shimizu's lab, was used to determine their activities toward protein phosphatase enzyme PP2A. The assay results indicated weak but significant inhibition of PP2A by these compounds. The doses of these crude drugs used to produce catharsis are six orders larger than the dose of okadaic acid which causes diarrhea in humans. Thus the weak activities of these compounds may be sufficient to account for the cathartic action of these drugs. The bioavailability of these compounds in the human body is unknown. Since the traditional standard dosages of these cathartics are too large to prepare as samples for a micro-titer assay due to their solubilities, they were not able to be assayed at higher concentrations at which more or total inhibition might take place. The possibility that these crude cathartic drugs inhibit PP2A can not be discounted based on the results of this study. It is also possible that these compounds may affect other enzymatic activities which were not tested. This matter remains open to question.

ACKNOWLEDGMENTS

I would like to express my sincerest gratitude to my major professor, Dr. Yuzuru Shimizu for his endless support, patience, mentorship and expertise in the field of pharmacognosy. My life has changed profoundly because of your presence.

Thanks are also extended to the other members of my defense committee, Drs. Bingfang Yan, Alison Roberts, and Zahir Shaikh for their time and consideration.

I am also thankful for the friendship and support of my colleagues in our lab: Katherine Drainville, Sudhakarao Thammana, Naiyu Zheng, Christopher Grant, Xinhua Qu, Zhongzhe Ma, Xuemin Yang, Hang Chen, Tomaharu Sano, Yuto Kamei, Y. Suzuki, David Rowley, Shireesha Alluri, Asakawa, Mitsunaga and M. Konishi.

I would also like to extend special thanks to Drs. Richard Koske and Ann Suter, for their understanding and support through my undergraduate studies at URI.

I would like to thank my parents, Virginia Vassett and Raymond Ford, my aunt and uncle, Francis and Carolyn Callahan, my sisters, Jane Gingerella and Susan Buffery for their encouragement, understanding, and support throughout my entire educational experience. I am also grateful to Evan Saia, Daniel Estes, Tom and Michel Buehler, Juliette de Bairicli Levy, Rosemary Gladstar, Jo-Anne Pacheco, Joanne O'Brien, Saika Messineo, Dr. James Whynot, Dale & Kathy Valentine, James Reynolds, Ellen Watt, Tom Berge, Sean Clarke, Amy Leinhos, James Verity, John Tarrats, Dylan Masson, Cheryl Sulyma-Masson, Philomena Curran, Darlene Spears, Norine LaFerriere, Priscilla Marshall, David Shafer, Susan Graham, Jonathon Deitch, Marie-Claude Moriarty, Robert Bankell, Evan Nickles, Jonelle Ference, Beverly Pino, and Janis, Black Kitty, and Blanche. I love you all.

PREFACE

This thesis was prepared with the standard thesis plan of the University of Rhode Island. The introduction will present an overview of the literature involved with the background of Convolvulaceae plants, phosphatase research, and diarrheic shellfish poisoning (DSP) including the 4-MUP assay for DSP detection. An Experimental chapter will be followed by a Results and Discussion chapter. Finally, a Conclusion and Bibliography will be presented.

TABLE OF CONTENTS

ABSTRACT	ii
ACKNOWLEDGMENT	iv
PREFACE	v
LIST OF TABLES	vii
LIST OF FIGURES	viii
INTRODUCTION	1
EXPERIMENTAL	18
RESULTS AND DISCUSSION	29
CONCLUSION	42
BIBLIOGRAPHY	61

LIST OF TABLES

Table 1. ^{1}H NMR Spectral Data for Jalapin Samples (400 MHz, pyridine-d5) 43

Table 2. ^{1}H NMR Spectral Data for Convolvulin (400 MHz, pyridine-d5) 45

Table 3. Effective Doses of Cathartic Drugs 47

\

LIST OF FIGURES

Figure 1. Anthracene Derivatives in Cathartic Plant Drugs 12

Figure 2. Structure of Phorbol Myristate Acetate (PMA) from Croton Oil, *Croton tiglium,* Euphorbiaceae 13

Figure 3. A Free Carboxyl at C1 and Hydroxyl Groups at C24 and C27 Are Essential for PP2A Receptor Binding of Okadaic Acid 14

Figure 4. 4-MUP Assay: PP1 & PP2A Dephosphorylate 4-MUP to 4-MU 15

Figure 5. Structure of Ricinoleic Acid (12-hydroxystearic acid) from the seeds of *Ricinus communis*, Euphorbiaceae 16

Figure 6. Structure of the lignan podophyllotoxin from the roots of *Podophyllum peltatum,* Berberidaceae 17

Figure 7. Outline for the Extraction of Jalapin from *Ipomoea purga* Roots 48

Figure 8. Outline for the Extraction of Convolvulin from *Pharbitis nil* Seeds 49

Figure 9. Thin Layer Chromatography of *Ipomoea purga* Isolates 50

Figure 10. ^{1}H NMR Spectra of *Ipomoea purga* Isolates 51

Figure 11. Proposed Structure of *Ipomoea purga* Isolates, Based on ^{1}H NMR Analysis 52

Figure 12. Thin Layer Chromatography of *Pharbitis nil* Isolates 53

Figure 13. ^{1}H NMR Spectra of *Pharbitis nil* Isolates 54

Figure 14. Proposed Structure of *Pharbitis nil* Isolates, Based on ^{1}H NMR Analysis 55

Figure 15. Standard Emission Curve of 4-Methylumbelliferone (4-MU) 56

Figure 16. The Inhibition Effect of Jalapin on PP2A 57

Figure 17. The Inhibition Effect of Convolvulin on PP2A 58

Figure 18. The Inhibition Effect of Ricinoleic Acid on PP2A 59

Figure 19. The Inhibition Effect of Podophyllotoxin on PP2A 60

INTRODUCTION

Cathartic and laxative plant drugs comprise a large class of important crude drugs. In the past, cathartics were used extensively and were thought to purge the body of a vast array of maladies. Among this class of crude plant drugs, some of the more famous and officially recognized cathartics included croton oil, castor oil, jalap roots, senna leaves, Cascara Sagrada bark, frangula bark, Aloe spp., and rhubarb roots (United States Pharmacopeia XX, 1979). Despite their widespread popularity and usage, only recently have a few mechanisms of action of such cathartic drugs been elucidated. Many of these mechanisms still remain unknown.

Senna leaves *(Cassia angustifolia),* Cascara Sagrada bark *(Rhamnus purshiana),* Frangula bark *(Rhamnus frangula),* the dried latex of Aloe leaves *(Aloe spp.)* and rhubarb roots *(Rheum palmatum)* all contain 1,8-oxygenated derivatives of anthracene in the form of anthraquinone, anthrone and anthranol (see Figure 1). They may occur as O- and C glycosides. Glycosides of anthranols and anthrones elicit a more drastic effect than do the anthraquinone glycosides causing a discomforting, griping action. (Stahl, E., 1973). These glycosides contribute significantly to the therapeutic activity of these crude plant drugs (Tyler, V. *et al.*, 1988). Taken orally, the free anthraquinone aglycones have little therapeutic activity, but in the glycosidic form the sugar moiety facilitates absorption and translocation of the aglycone to the site of action in the wall of the large intestine. Bacterial flora in the colon wall cleave the sugar residues and free the aglycones at the site of action. The anthraquinone

glycosides and their derivatives are stimulant cathartics and exert their action by increasing the tone of the smooth muscle in the wall of the colon. The exact mechanisms of these actions are not yet known (Fairbairn, J.W., 1977).

Relatively recent studies involving croton oil, cholera toxin, and diarrhetic shellfish poisoning have shed some light on the mechanisms by which cathartics can act. These mechanisms of action involve interference with signal transduction pathways responsible for regulating electrolyte balance.

Signal transduction regulates cellular processes in response to external/internal stimuli and is crucial for the growth, division, and differentiation of all organisms. Reversible protein phosphorylation is an essential component of almost all signaling pathways in living cells. Changes in the phosphorylation state of a protein are conducted by two types of enzyme activities: protein kinases and protein phosphatases. Protein kinases catalyze the covalent attachment of a phosphate group to an amino acid side chain, whereas protein phosphatases reverse this process (Luan, 2000). The attachment to or removal of a phosphate group from a protein often has profound effects on its structure and thereby modifies the functional property of the protein. Phosphorylation of key proteins with associated changes in their biological activity accounts for many physiological responses. The phosphate content of these proteins reflects a net balance of the protein kinases and protein phosphatases acting on them (Hanks *et al.*, 1988).

Phosphorylation of hydroxyl-bearing amino acid side chains (serine, threonine, and tyrosine) is catalyzed by protein kinases (PKs) using ATP as a

phosphoryl donor, whereas dephosphorylation is catalyzed by protein phosphatases, which induce changes in protein conformation, protein-protein or protein-ligand interactions, membrane permeability and solute gradients (Sheppeck, *et al.*, 1997). This simple cycle acts as an 'on-off' switch to selectively modulate the action of countless other proteins. Examples of enzyme regulation by phosphorylation include initiation of allosteric conformational changes which may directly block the access to an active site or regulation of the interaction among protein partners that must form complexes in order to function. Examples of cell functions involving reversible phosphorylation include ion transport, metabolism, cell cycle progression, developmental control, and stress responses.

Research conducted on croton oil points to the mechanisms by which cathartics interfere with signal transduction. Croton oil is a naturally occurring plant oil obtained from *Croton tiglium,* a shrub-like tree of the Euphorbiaceae family (Evans, F.J., 1986). Croton oil has been used traditionally in Western medicine as a powerful purgative (Trease, G.E. and Evans, W.C., 1972). Due to the highly potent tumor-promoting property of phorbol myristate acetate (PMA), the active ingredient of C*roton tiglium* oil, this drug was considered to be too toxic for human use and was eventually removed from modern pharmacopoeias (British Pharmaceutical Codex, 1934) (see Figure 2). This same compound, PMA, was later found to be a potent protein kinase C activator. This finding is consistent with the hypothesis that protein kinase C activation is part of the normal growth control process that becomes perturbed in tumorigenesis

(Matthews, C. K., Van Holde, K. E., 1997). Activation of protein kinase C causes hyperphosphorylation of proteins that control sodium secretion by intestinal cells. The increased phosphorylation of cytoskeletal or junctional moieties that regulate solute permeability result in the passive loss of fluids as diarrhea (Dho et al., 1990).

There are many causes of diarrhea, but the overall alterations in intestinal function are similar in that the intestine ceases to be an organ of net absorption of water and electrolytes. The fluid produced exceeds the absorptive capacity of the remaining intestine and water passes into the stool. The aim of diarrhea treatment is to enhance intestinal absorption of water by reducing the content of luminal electrolytes (by increasing active absorption of Na+ or decreasing secretion of anions). Absorption of fluid by the colon is secondary to active transport of Na^+ (Sellin, J. H., 1993).

The mechanism responsible for colonic absorption of Na^+ is primarily electrogenic transport, which relies on a Na^+/K^+/ATPase activity in the basolateral membrane of the colonic epithelium. Neutral absorption of NaCl may also be involved. The colon absorbs Cl^- by an electrically neutral mechanism that involves the exchange of Cl^- for HCO_3^- and by neutral uptake of NaCl. Agents that elevate intracellular cAMP in colonic enterocytes stimulate electrogenic secretion of Cl^- and may inhibit NaCl uptake. This causes net fluid secretion. The colon also secretes K^+, probably via an active mechanism that is stimulated by cAMP (Goodman & Gilman, 1996).

Most animal cells maintain large ionic gradients across their surface membranes such that intracellular fluid contains a higher concentration of K^+ ions and low concentration of Na^+ and Ca^{2+} ions relative to the extracellular fluid. These ionic gradients are maintained by the action of specific energy-dependent ion pumps. Ion channels mediating electrical signaling are intrinsic membrane proteins that form ion-selective pores through which ions can move down their electrochemical gradients into or out of cells. The responsiveness of voltage-gated ion channels to membrane potential is regulated by G-protein-coupled receptors (Fine, K. D. *et al.*, 1993). These regulatory processes are crucial in the control of hormone secretion, neurotransmitter release, muscle contraction, and gene transcription. Both direct binding of G proteins and phosphorylation of the ion-channel proteins are important effectors of this second order regulation of ion-channel function (Matthews, C. K., Van Holde, K. E., 1997).

Cholera toxin, a highly cathartic peptide produced by *Vibrio cholerae,* has been shown to stimulate Cl^- secretion in the small intestine and the colon by its ability to activate adenylate cyclase in the mucosa. The toxin consists of an A subunit surrounded by five B subunits. The B subunits attach the toxin to ganglioside G_{M1} on the cell surface. The A subunit catalyzes ADP-ribosylation of the α-subunit of G proteins, reducing GTPase activity. Activating the α-subunit of G proteins also catalyzes ADP-ribosylation of cell membrane adenylate cyclase (Chandana, S. *et al.*, 2001). This activation interferes with the role of the Gα-subunit in regulating the maintenance of epithelial cell tight junctions. Tight junctions serve two functions: regulation of the permeability barrier to

paracellular fluxes, and separation of the apical and basolateral membrane domains. Loss of regulation of epithelial cell tight junctions results in secretion of Cl- in the small intestine and colon, causing net fluid secretion (Wang, W. *et al.*, 2000).

Research into diarrhetic shellfish poisoning has also contributed to the understanding of the mechanism of action of cathartic drugs. Diarrhetic shellfish poisoning (DSP) is caused by consumption of shellfish which have been contaminated with natural toxins produced by dinoflagellates. The onset of the illness ranges from 30 minutes to several hours after consumption of the contaminated shellfish, but seldom exceeds 12 hours (Van Egmond *et al.*, 1993). Victims suffer from diarrhea, nausea, and stomach pain, but recover within three days without serious after-effects. DSP toxins accumulated in the shellfish inhibit the activity of serine/threonine specific protein phosphatases 1 and 2A in colonic endothelial cells, resulting in rapid accumulation of phosphorylated proteins. This rapid accumulation of phosphorylated proteins results in disruption of the maintenance of electrolyte balance, preservation of membrane potential, and control of cellular volume in tissues. Oral ingestion of one microgram of okadaic acid is sufficient to produce the diarrhetic effect in a human being.

Both protein phosphatase 1 (PP1) and protein phosphatase 2A (PP2A) are the intracellular targets for the toxins produced by marine organisms that cause diarrhetic shellfish poisoning. These DSP toxins include the polyketide inhibitors okadaic acid and dinophysistoxin-4 which are produced by dinoflagellates. Other natural protein phosphatase inhibitors include cyclic

peptide inhibitors such as microcystins and nodularins, as well as calyculin and tautomycin (Gupta *et al.*, 1997). The toxins, though structurally dissimilar from one another, all seem to bind at the active site of each phosphatase, where they contact multiple residues near the active site (Suganuma et al., 1989). A free carboxyl and hydroxyl group in the molecule is essential for receptor binding in the case of okadaic acid (see Figure 3). These moieties are important for such compounds to bind to a receptorial site on PP1 and PP2A, thus inhibiting their activity and interfering with signal transduction processes, including ion channel regulation (Sasaki, K. *et al.*, 1994).

These toxins have become important research tools for understanding the roles of PP1 and PP2A in signal transduction. There are two types of serine/threonine-specific protein phosphatases: type 1 & type 2 (Ingebritsen & Cohen). Type 1 protein phosphatases specifically dephosphorylate the β subunit of phosphorylase kinase and are inhibited by nM concentrations of inhibitor-1 (I-1) & inhibitor-2 (I-2), two small heat- and acid- stable proteins found in liver & muscle extracts, as well as okadaic acid, calyculin A, NaF, and orthovanadate.

A number of different forms of type-1 protein phosphatase have been characterized. They differ in their association with regulatory components, which determine their overall activity and subcellular location. Additionally, these forms differ in their substrate specificity measured *in vitro* using various phosphoproteins.

Type 2 protein phosphatases preferentially dephosphorylate the α subunit of phosphorylase kinase & are insensitive to Inhibitor-1 and Inhibitor-2. Type 2

protein phosphatases are subclassified into three distinct enzymes, PP2A, PP2B and PP2C, which are characterized by their cation dependence and use of various activators and inhibitors (Cohen, P., 1989).

PP1 holoenzymes are heterodimers composed of catalytic PP1c and a regulatory R subunit. The diverse functions of PP2A are attributed to the presence of at least 15 B regulatory subunits that individually assemble with each core heterodimer of PP2Ac and a 65-kDa A subunit. PP2A exists in either heterodimer or heterotrimer form (Catterall, W. A., 1997).

As the diarrheic effect of okadaic acid and related toxins has been attributed to the accumulation of phosphorylated proteins that control sodium secretion in intestinal cells, a detection method for such cathartic compounds based on the inhibition of protein phosphatases is of particular interest. Using the specific inhibition of both PP1 and PP2A catalytic subunits provides a sensitive method to detect diarrhetic shellfish poisoning . Recently, a highly sensitive fluorometric assay for DSP using PP1 and PP2A was developed in Dr. Y. Shimizu's lab (Shimizu *et al.*, 1997) and Dr. Vieytes's lab (Vieytes *et al.*, 1997), independently of each other. This assay is based on the dephosphorylation of 4-methylumbelliferone phosphate (4-MUP), a non-fluorescing compound, to fluorescent 7-hydroxy-4-methylcoumarin (4-MU), which fluoresces at approximately 446 nm, by PP1 or PP2A (see Figure 4). The substrate exhibits high substrate efficiencies for PP1 & PP2A.

After incubation of 4-MUP and purified PP1 enzyme in a 96-well microtiter plate, liberated 4-MU is measured with a fluorescent scanner.

Inhibition of PP1 or PP2A by a specific inhibitor, e.g., okadaic acid, is quantified using the above protocol. The fluorescent inhibition assay involves introducing a test sample to the enzyme PP1 or PP2A and then adding the substrate 4-MUP. An inhibitory sample will result in a fluorescence reading that is less than a control known to be free of such inhibitors.

This inhibition assay has advantages over other assays, such as a radioactive phosphoprotein assay using ^{32}P ATP or a para-nitrophenyl phosphate colorimetric assay (pNPP assay), due to the elimination of clean up and the increase in specificity, sensitivity, precision, rapidness, reproducibility, and percentage of recovery of the 4-MUP assay. A drawback to this assay is that the commercially available PP2A enzymes which are obtained from human erythrocytes are very expensive and no recombinant enzymes are available for PP2A (Baden *et al.*, 1995).

In this study, the 4-MUP assay was used to test the activity of jalapin and convolvulin, two hydroxy-acid containing resin glycosides from plants of the Convolvulaceae family, upon PP2A. Jalapin was isolated from roots of *Ipomoea purga* and convolvulin isolated from seeds of *Pharbitis nil.* At the same time other known cathartics whose active components contain hydroxy-acid moieties were also studied including ricinoleic acid (see Figure 5), isolated from the oil of *Ricinus communis* (castor oil), and podophyllotoxin (see Figure 6), isolated from podophyllum resin. The hydroxy acid moieties of these cathartic compounds, if released, may bind to a receptorial site on PP2A, as seen with the

DSP toxins, inhibiting its activity and interfering with signal transduction processes such as ion channel regulation (Sasaki, K. *et al.*, 1994).

Castor oil is cold-pressed from the seeds of *Ricinis communis* (Euphorbiaceae) and has traditionally been taken orally as a stimulant cathartic. It is composed of a mixture of triglycerides, of which about 75 % is triricinolein. Triricinolein is hydrolyzed by lipases in the duodenum to release ricinoleic acid, which exerts a cathartic effect (Tyler *et al.*, 1988). Podophyllotoxin is the active principle of podophyllum resin, also called podophyllin, which is isolated from the roots of *Podophyllum peltatum,* Berberidaceae (American Mandrake or Mayapple). The resin possesses drastic purgative properties and its active constituents consist of a mixture of lignans including podophyllotoxin (20%), α-peltatin (10%), and β-peltatin (5%) (Emmenegger, H. *et al.*, 1961).

It has long been known that certain plant species of the Convolvulaceae family produce drastic cathartic and purgative effects when consumed. Two species of this family, *Ipomoea purga* (Mexican Jalap), and *Pharbitis nil* (Morning Glory), were used in this study. Both of these plants produce resins, jalapin and convolvulin, respectively, which are both monomers of hydroxy-fatty acid oligoglycosides in which the sugar moiety is partially acylated by organic acids and can also combine with the carboxy group of the aglycone to form a macrocyclic ester in the case of jalapin (Noda, et al, 1987). Studies have identified these resins to be the active cathartic components of these crude drugs (Mannich, C. *et al.*, 1938; Shellard, E. J., 1961). Jalapin is characterized by the presence of an oligoglycoside of 6-deoxyhexoses (rhamnose, fucose), whose

aglycone is the hydroxylated fatty acid 11*S*-hydroxyhexadecanoic acid or jalapinolic acid. The aglycone found in convolvulin, also known as pharbitinis the hydroxylated fatty acid, 11*S*-hydroxytetradecanoic acid or convolvulinic acid (pharbitic acid) (Ono, 1990).

Jalapin is obtained from the dried tubercles of Convolvulaceae plants, including *Ipomoea purga,* which was chosen as the source of jalapin for this study, as well as *I. orizabensis, I. braziliensis, I. simulans,* and *Convolvulus scammonia.* Convolvulin is obtained from the seeds of Convolvulaceae plants, including *Pharbitis nil,* which was chosen as the source of convolvulin for this study. The plant materials were extracted and the chemical structures of the isolates were confirmed by proton nuclear magnetic resonance (NMR), prior to use in the 4-MUP PP2A inhibition assay.

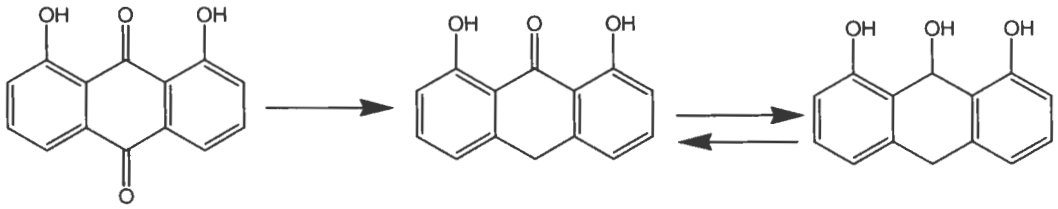

Figure 1. Anthracene Derivatives Found in Cathartic Plant Drugs

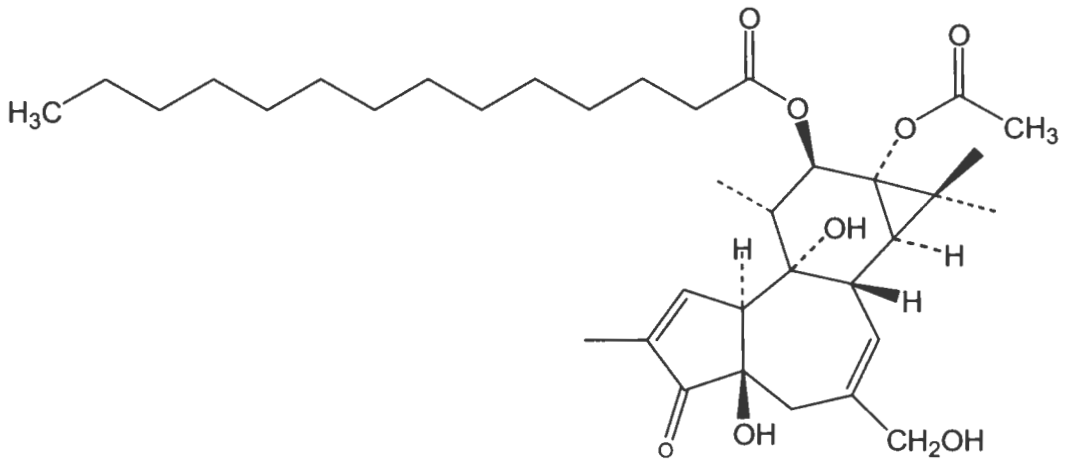

Figure 2. Structure of Phorbol Myristate Acetate (PMA) from Croton Oil, *Croton tiglium,* Euphorbiaceae.

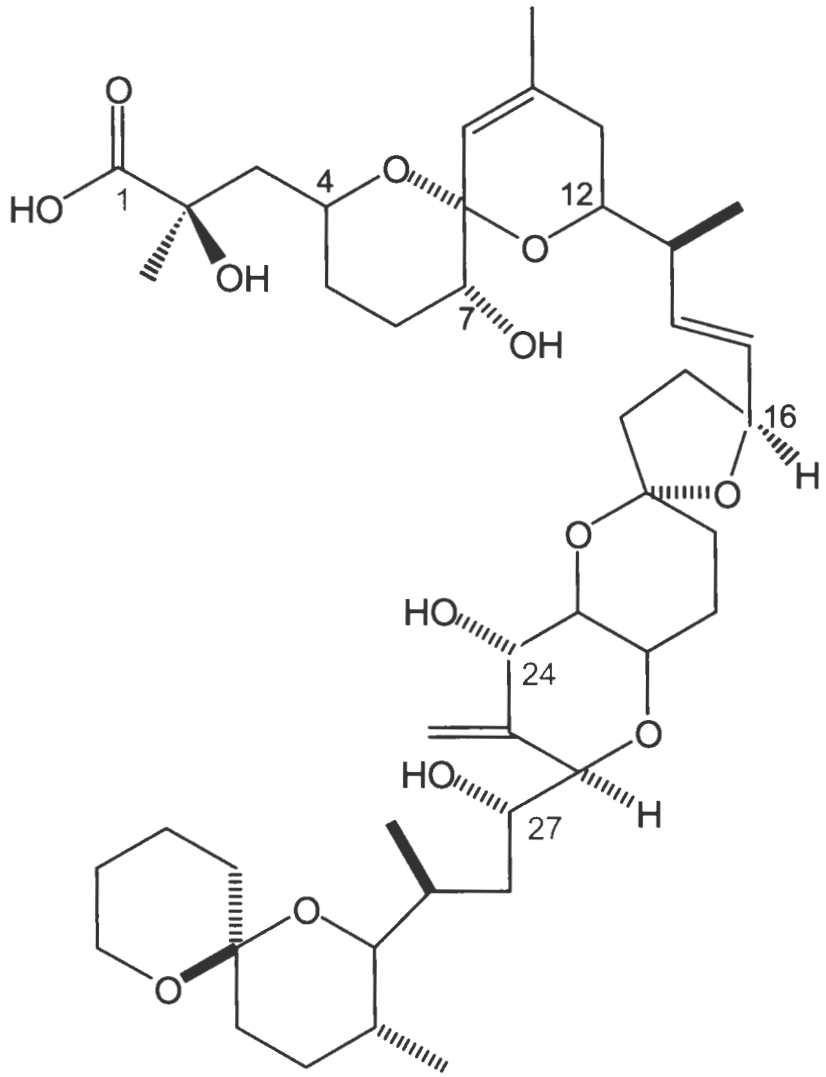

Figure 3. A Free Carboxyl at C1 and Hydroxyl Groups at C24 and C27 Are Essential for PP2A Receptor Binding of Okadaic Acid

Non-fluorescing
4-methylumbelliferone
phosphate (4-MUP)

Strongfluorescence
4-methylumbelliferone
(4-MU)
Ex. 315nm/Em. 446 nm

Figure 4. 4-MUP Assay
PP1 & PP2A Dephosphorylate 4-MUP to 4-MU

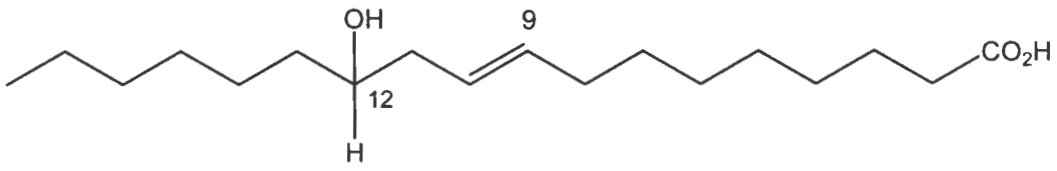

Figure 5. Structure of ricinoleic acid (12-hydroxystearic acid) from the seeds of *Ricinus communis*, Euphorbiaceae

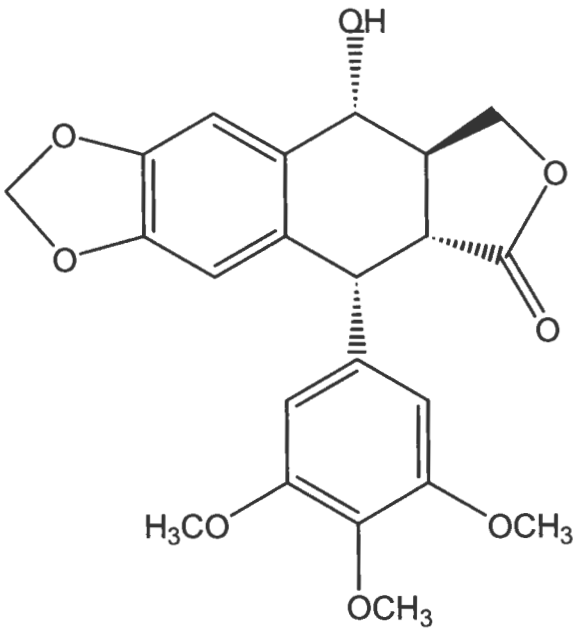

Figure 6. Structure of the lignan podophyllotoxin from the roots of *Podophyllum peltatum,* Berberidaceae

EXPERIMENTAL

Materials & Methods

Unless otherwise specified, all chemicals were reagent grade or better. Solvents for extraction, partitioning, and chromatography were HPLC grade. Distilled deionized water was used to make all solutions needed for the 4-MUP assay. Deionized water was used for all other procedures.

The following abbreviations have been used in this work: protein phosphatase 1 (PP1), protein phosphatase 2 (PP2A), protein phosphatase 2B (PP2B), protein phosphatase 2C (PP2C), protein phosphatase 2A catalytic subunit (PP2Ac), proton nuclear magnetic resonance (^{1}H-NMR), phorbol myristate acetate (PMA), 4-methylumbelliferyl phosphate (4-MUP), 4-methylumbelliferone (4-MU), high pressure liquid chromatography (HPLC), thin layer chromatography (TLC), protein kinase (PK), adenosine triphosphate (ATP), adenosine diphosphate (ADP), cyclic adenosine monophosphate (cAMP), inhibitor-1 (I-1), inhibitor-2 (I-2), guanosine triphosphate (GTP), diarrhetic shellfish poisoning (DSP), and hydroxymethyl aminomethane (Tris).

Dried tubercles of *Ipomoea purga* (Mexican jalap root) were purchased from Trinity Herb Co. (Graton, CA). Seeds of *Pharbitis nil* (morning glory seeds) were harvested at the URI Heber Youngken Medicinal Plant Garden in Kingston, RI. Ricinoleic acid and podophyllotoxin were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO). Okadaic acid was purchased from LC Laboratories (Woburn, MA). 4-Methylumbelliferyl phosphate (4-MUP) and 4-methylumbelliferone (4-MU) were purchased from Molecular Probes, Inc.

(Eugene, OR). Tris (hydroxymethyl) aminomethane (electrophoresis purity) and 2-mercaptoethanol were obtained from Bio-Rad Laboratories (Richmond, CA). Protein phosphatase 2A (PP2A) from human red blood cells (purified enzyme) was purchased from Upstate Biotechnology Inc. (Lake Placid, NY). NMR solvent pyridine-d^5 was purchased from Cambridge Isotopes, Inc. (Andover, MA). Assorted laboratory glassware, magnesium chloride-$6H_20$, hydrochloric acid, 1-butanol, chloroform, methanol, and methylene chloride were purchased from Fisher Scientific Inc. (Fairlawn, NJ). Ethanol was purchased from Aaper Alcohol, Inc. (Shelbyville, KY). For column chromatography, Baker silica gel (J. T. Baker, Inc. Phillipsburg, NJ) (40 μm average particle diameter) and Bio-gel LH-20 (Amersham Pharmacia Biotech, Piscataway, NJ) were used. Thin layer chromatography plates were purchased from Whatman, Ltd. (Maidstone, Kent, England).

The following equipment and instruments were employed in this study: MTX Labsystems, Inc. Titertek Fluoroskan II version 4.0 of Bio-Tek FL500 Fluorescence Reader, Yamato Rotary Evaporator model RE-46 with water bath model BM-41, Amsco Autoclave, Eppendorf micropipettors, Dell Latitude C600 Laptop computer, Fuji Digital Camera, HP Scanjet 5200C Color Scanner, Fisher Vortex Genie 2, Sonicor Ultrasonic Cavitator, Precision Scientific Co. Vaccuum Pump model S-35 and Vacuum Dessicator, Fisher Scientific Accumet 15 pH meter, and VMR I 550 Incubator from VMR Scientific. Microsoft Excel t-test was used to determine P values.

Nuclear Magnetic Resonance (NMR) Spectroscopy

Nuclear Magnetic Resonance (NMR) spectrum of ^{1}H-NMR was recorded on the Bruker Model DPX-400 Avance NMR spectrometer, College of Pharmacy, University of Rhode Island. Data processing was performed with the standard software by Silicon Graphics Indy Station NMR Analysis and Dell 450MHz NT Workstation NMR Analysis Computer. Five milligrams of each sample was dissolved respectively in HPLC grade methanol, filtered through non-adsorbant cotton, and dried under the flush of nitrogen gas and further subjected to vacuum desiccation. The dried samples were each dissolved in 0.6 ml pyridine-d^5 (Cambridge Isotopes, Inc.) to take ^{1}H-NMR spectrum.

Thin Layer Chromatography (TLC)

TLC was performed on silica-gel coated aluminum sheets and silica-gel coated glass plates using developing solvent $CHCl_3$:MeOH:H_2O (6:4:1). Resin glycosides such as jalapins and convolvulins lack UV absorbance, so in order to visualize the compounds on TLC plates. The vanillin/sulfuric acid spray reagent (3.0 g vanillin, 40 ml glacial acetic acid, 30 ml concentrated sulfuric acid, and 450 ml 95% ethanol) was used, followed by heating on a hot stage.

Extraction of *Ipomoea purga* Roots

Extraction and fractionation were generally based on the methods of Ono *et al.* (1990). The outline of the extraction is shown in Figure 12. Fifty grams of

root tubercles of *Ipomoea purga* were pounded into a powder and macerated in methanol, sonicated, and extracted three times at room temperature, each time 200 ml methanol was used. Each methanol extraction was suctioned-filtered through a Buchner funnel lined with filter paper into a filtration flask, giving an orange-colored solution. The three filtered methanol extracts were combined and evaporated to dryness with a rotary evaporator, yielding a golden-orange resin (6.15 grams). Two grams of this methanol extractive was suspended in 25 ml of water in a separatory funnel and extracted three times with 1-butanol, each time 25 ml 1-butanol was used. The aqueous layer was a yellow color and was evaporated with a rotary evaporator to give a yellowish resin (330 milligrams). The three 1-butanol extracts were orange in color and were combined together, then evaporated in a rotary evaporator to give a golden-orange resin (1.65 grams). This 1-butanol extractive was defatted with methylene chloride three times, each time using 25 ml of methylene chloride. The three methylene chloride extracts were combined and evaporated with a rotary evaporator to yield a yellow oil (100 milligrams). The defatted 1- butanol-soluble extractive (1.55 grams) was further purified by liquid chromatography.

Purification of *I. purga* Resin Glycosides by Liquid Chromatography

a. Silica Gel Column Chromatography of Defatted 1-Butanol-soluble Extractive

Twenty-five grams of silica gel was suspended in chloroform:methanol solution (10:1) and then immediately and continuously poured into a 2 cm diameter glass chromatography column. After the silica gel settled down and

there was barely any chloroform:methanol solution left above the surface of the silica gel, the defatted 1-butanol soluble extract (630 mg of 1.55 grams) was dissolved in the chloroform:methanol solution (10:1) and loaded. The column was eluted with chloroform and methanol in 60 ml quantities in which the percentage of methanol was increased stepwise with the following ratios of chloroform to methanol: 10:1, 5:1, 5:2, 5:3, and 5:4. Fractions were collected in 4 ml aliquots and each fraction was checked by TLC. Fractions containing the same components were combined based on TLC results.

Extraction of *Pharbitis nil* Seeds

Extraction and fractionation were generally based on the methods of Ono *et al.* (1990) as previously described. The outline of the extraction is shown in Figure 13. One hundred grams of seeds of *Pharbitis nil* were ground into a powder and used as starting materials for the extraction. The methanol extraction produced a yellow resin (4.17 grams). After partitioning 2.00 g of this methanol extractive between water and 1-butanol, 0.73 grams of water-soluble extract and 1.26 grams of 1-butanol soluble material were obtained. This 1-butanol extract was defatted with methylene chloride, as described previously, to yield a yellow oil (1.21 grams). The remaining defatted 1-butanol extract was evaporated with rotary evaporator and weighed 50 milligrams. Both the water-soluble extract and the defatted 1-butanol extract were further purified by liquid chromatography.

Isolation of *P. nil* Resin Glycosides by Liquid Chromatography

a. Silica Gel Column Chromatography of Water-soluble Extract

Twenty-five grams of Baker silica gel was suspended in chloroform:methanol:water solution (6:4:1) and then immediately and continuously poured into a 2.25 cm diameter glass chromatography column to a height of 20 cm. The water- soluble extract (700 mg of 730mg) was dissolved in chloroform:methanol:water solution (6:4:1) and loaded onto the silica gel column, using just enough solution to dissolve the sample. The column was eluted with the chloroform:methanol:water solution (6:4:1). Fractions were collected in 10 ml aliquots and each fraction was checked by TLC. Fractions containing the same components were combined based on TLC results.

b. LH-20 Biogel Column Chromatography of Defatted 1-Butanol-soluble Extract

A 1.5 cm diameter glass chromatography column was packed to a height of 14 cm with Bio-gel LH-20 previously suspended in methanol. After the LH-20 settled down and there was barely any methanol left above the top of the gel, the defatted 1-butanol extract (50 mg) dissolved in methanol was loaded. The column was eluted with methanol. Fractions were collected in 3 ml aliquots and each fraction was checked by TLC. Fractions containing the same components were combined based on TLC results.

Standard Emission Curve of 4-Methylumbelliferone (4-MU)

One 10 µM 4-MU stock solution and one 5 µM 4-MU stock solution were made by dissolving 0.00018 grams and 0.00009 grams of 4-MU, respectively, in 100 ml of Tris buffer which was made of 50 mM, 20 mM $MgCl_2$, and 1 mM 2-mercaptoethanol, pH 8.5 adjusted with 1N HCl. Serial dilutions were made by 50 mM Tris buffer, pH 8.50 to make concentrations of 10, 5, 1, 0.5, 0.1, 0.05, 0.01, 0.005, 0.001, 0.0005, and 0 µM. Fifty microliters of each solution was pipetted into each well used, on a 96-well microtiter plate. Each row consisted of six replicates of the given concentration for that column. In total, sixty-six wells were filled on the plate, which was read on the Fluoroskan II, version 4.0 of Biotek FL500 Fluorescence Reader at time zero and time 30 minutes.

Preparation of Tris Buffer, pH 8.50 [50 mM]

Tris (3.04 grams) and $MgCl_2.6H_20$ (2.04 grams), were weighed and combined in a 500 ml glass beaker which was filled to the 400 ml mark with distilled, deionized water, and 2-mercaptoethanol (36 µl) was added. The pH of the solution was adjusted to 8.50 with 1 N hydrochloric acid. The contents of the beaker were transferred to a 500 ml volumetric flask. The beaker was rinsed with 100 ml of distilled, deionized water, which was added to the 500 ml volumetric flask. The flask was capped and inverted several times. The concentration of the Tris buffer was 50 mM.

Preparation of 4-MUP Solution, pH 8.50 [50 mM]

4-MUP (6.36 milligrams), was added to 250 ml 50 mM Tris buffer, pH 8.50.

Preparation of Test Solutions of Plant Extracts for 4-MUP Assay

Each of the plant samples isolated in this experiment, jalapin and convolvulin, were dissolved in 500 µl of methanol and combined with 9.5 ml of 50 mM Tris buffer, pH 8.50. From these 5% methanolic solutions, serial dilutions were prepared using the Tris buffer. The ricinoleic acid and podophyllotoxin samples were also prepared in serial dilutions, but starting with a 1% methanolic solution. The PP2A 4-MUP inhibition assay is sensitive to alcohol in concentrations of 5% or greater in the total well concentration. One percent methanol was the highest concentration of methanol in any of the 50 µl wells of the microtiter plates used in this experiment.

Jalapin (15.9 milligrams) was added to a small vial containing 500 µl of methanol and sonicated until completely dissolved. Into this vial, 9.5 ml of 50 mM Tris buffer, pH 8.50, was pipetted to give the jalapin solution a concentration of 1.59 mg/ml. Three serial dilutions were made by taking one milliliter of this 1.59 mg/ml solution and pipetting it into nine milliliters of 50 mM Tris buffer, pH 8.50. Each newly prepared dilution was used in place of the stock solution used before it. The concentrations of the jalapin samples made were 1.59 mg/ml, 0.159 mg/ml, 0.0159 mg/ml, and 0.00159 mg/ml, respectively.

Convolvulin (14.81 milligrams) was added to a small vial containing 500 µl of methanol and sonicated until completely dissolved. Into this vial, 9.5 ml of

50 mM Tris buffer, pH 8.50, was pipetted to give the convolvulin solution a concentration of 1.48 mg/ml. Three serial dilutions were made starting with the 1.48 mg/ml convolvulin solution. The concentrations of the convolvulin solutions made were 1.48 mg/ml, 0.148 mg/ml, 0.0148 mg/ml, and 0.00148 mg/ml.

Ricinoleic acid (100 mg) was pipetted into a small vial containing one milliliter of methanol to give a 100 mM concentration of ricinoleic acid. Into a small vial containing 900 µl of methanol, 100 µl of 100 mM ricinoleic acid solution was pipetted, to make a 10 mM concentration of ricinoleic acid. One hundred microliters of 10 mM ricinoleic acid solution was pipetted into a small vial containing 900 µl of 50 mM Tris buffer, pH 8.50, to make a 1 mM concentration of ricinoleic acid, containing ten percent methanol. Three more serial dilutions were made, starting with the 1 mM ricinoleic acid solution. These three serial dilutions were diluted with 900 µl of 50 mM Tris buffer, pH 8.50, instead of 900 µl of methanol. The concentrations of these ricinoleic acid solutions were 100 µM, 10 µM, and 1 µM, respectively.

Podophyllotoxin (4.14 milligrams) was pipetted into a small vial containing 1 ml of methanol to make a 10 mM solution. One hundred microliters of 10 mM podophyllotoxin solution was pipetted into 900 µl of 50 mM Tris buffer, pH 8.50, to make a podophyllotoxin solution with a 1 mM concentration that contained ten percent methanol. Three more serial dilutions were made starting with the 1 mM podophyllotoxin solution. These three serial dilutions were diluted with 900 µl of 50 mM Tris buffer, pH 8.50. The concentrations of these podophyllotoxin solutions were 100 µM, 10 µM, and 1 µM, respectively.

4-Methylumbelliferone Phosphate Fluorometric Assay (4-MUP Assay)

In a 96-well microtiter plate, placed on ice, 1 µl of purified PP2A enzyme from Upstate Biotechnology was added to 10 µl of the compound being tested for potential inhibitory activity. The concentration of purified PP2A enzyme was 0.03 units per well. The mixture of the potential inhibitory compound with the enzyme was incubated at room temperature on a 3-D rotator (Lab-Line Instruments, Inc., Melrose Park, IL) for five minutes before adding the substrate. Thirty-nine microliters of the substrate, 50 mM 4-MUP in 50 mM Tris buffer (20 mM $MgCl_2$, and 1 mM 2-mercaptoethanol, pH 8.5 adjusted with 1N HCl), was added to each well to make a total volume of 50 µl per well. Each row of wells on the plate consisted of six replicates of the given reaction mixture for that row. Fluorescence intensity measurements were performed using ICN Titertek model Fluoroskan II fluorescence reader using ex. 355 nm / em. 460 nm filters. Readings were taken at time zero, thirty, and sixty minutes, after incubating the reaction mixtures at 37 °C. Inhibition of PP2A by a specific inhibitor, i.e. okadaic acid, was quantified using the above protocol. Four 96-well microtiter plates were used in this experiment.

Okadaic acid was used as a reference inhibitor on each of the four microtiter plates used. The okadaic acid sample was previously prepared in Dr. Shimizu's lab by Dr. X. Qu, in a 60 nM concentration. A 60 nM concentration of okadaic acid is known to completely inhibit the activity of PP2A (X. Qu, 1998). Each plate contained a row of wells with 60 nM okadaic acid in the presence of

the PP2A enzyme and 4-MUP substrate. Each plate also contained a row of control wells with 60 nM okadaic acid in the presence of the substrate with no enzyme. In the row of control wells with no enzyme, the wells were filled with 1 µl of 50 mM Tris buffer, pH 8.50, in place of the 1 µl of PP2A enzyme.

Each microtiter plate was used to test the potential inhibitory activity of each of the following compounds on PP2A, respectively: jalapin (plate 1), convolvulin (plate 2), ricinoleic acid (plate 3) and podophyllotoxin (plate 4). Each row of wells to be filled on the plate, consisted of six replicates of the given concentration of compounds to be tested. Each microtiter plate also had a row of control wells which contained 39 µl of 4-MUP substrate in the presence of 1 µl of PP2A enzyme, without any inhibitory compound being present. These control wells were filled with 10 µl of 50 mM Tris buffer, pH 8.50, in place of any potential inhibitory compounds.

Plates 1 and 2 tested four concentrations of jalapin and convolvulin, respectively, for their inhibitory effects on PP2A. Plates 3 and 4 tested three concentrations of ricinoleic acid and podophyllotoxin, respectively, for their inhibitory effects on PP2A. For each concentration of compound tested on each plate, there was a row of control wells filled on the same plate containing the same concentraton of compound being tested, but in the presence of the 4-MUP substrate without any PP2A enzyme. In the rows of wells containing no enzyme, the wells were filled with 1 µl of 50 mM Tris buffer, pH 8.50, in place of the 1 µl of PP2A enzyme.

RESULTS AND DISCUSSION

A schematic representation of the extraction and isolation of jalapin from *Ipomoea purga* roots is shown in Figure 7. Several purple spots were observed on silica-gel TLC of the 1-butanol-soluble portion of the methanol extract of the *I. purga* roots (1.65 grams). This 1-butanol extract was separated by silica-gel chromatography to afford a crude glycoside mixture. The fractions did not have color, had no UV absorbance, and displayed a purple color after being sprayed with the vanillin/sulfuric acid reagent. One of the TLC plates is shown in Figure 14. These fractions were further identified by ^{1}H NMR. The total weight of the two samples identified by ^{1}H NMR was 279 milligrams (14% of crude methanol extract) and 186 milligrams (9% of crude methanol extract). These fractions were combined prior to the assay. These two fractions, each appearing as a single bluish-purple spot, had Rf values of 0.71 and 0.75. These normal phase Rf values are reasonable values for high molecular weight amphoteric resin glycosides containing lipophilic fatty acids and hydrophilic sugar moieties.

Based on their ^{1}H NMR spectra, these samples were determined to contain a mixture of homologues or very closely related compounds. Variations in the structures of these resin glycosides primarily come from the differing organic acid moieties such as tiglic acid, isobutyric acid, etc. which can occur. Variations also occur due to the different types and numbers of sugars within the glycosides. However, these resin glycosides do share a basic structure which includes the hydroxy-acid moieties. The percentages of these resin glycosides present in the total crude extract are exceptionally high, reaching up to 26.9% of the crude

methanol extract in some Convolvulaceae species (Noda, N., 1987). The predominance of these resin glycosides in the crude extract may account for the ^{1}H NMR data fitting the molecular structure so well, despite the fact that the separation procedure was not so thorough.

The ^{1}H NMR spectra of these two fractions were identical (Figure 10) and gave similar ^{1}H NMR spectra as those compared to in the literature (H. Kogetsu *et al.,* 1991), based on the peak assignments, integration, and ratios of hydrocarbon protons to protons of sugar and organic acids. This suggests that the resin glycosides are similar to what Mayer called the ether-soluble resin glycosides (Shellard, 1961). The ^{1}H NMR showed three acetoxy methyl signals [δ 1.96 (8H), 2.34 (2H), 2.69 (2H)], the signals characteristic of 1 mol of isobutyric acid [δ 2.92, (1 H, sept, H-2), 1.21 (3H, d, H_3-3), 1.07 (3H, d, H_3-3')] and 2 mols of tiglic acid groups [δ 7.02 (1H, dq, H-3), 1.59 (3H, dd, H_3-5), 1.82 (3H, s, H_3-4)] and [δ 7.17 (1H,dq, H-3),), 1.36 (3H, dd, H_3-5), 1.65 (3H, s, H_3-4)]. The ^{1}H NMR spectrum showed five anomeric protons (δ 5.51, 5.13, 4.82, 5.67 and 4.81) and four secondary methyls due to 6-deoxyhexose (δ 1.56, 1.26, 1.45 and 1.65) as well as a 2-methylene (δ 2.69) and a primary methyl (δ 0.96) attributable to a jalapinolic acid moiety (Table 1). Based on this data, it is reasonable to presume that the *Ipomoea* resin glycoside isolated in this experiment is similar to the proposed chemical structure depicted in Figure 11.

A schematic representation of the extraction and isolation of convolvulin from *Pharbitis nil* seeds is shown in Figure 8. The crude methanol extract

obtained from 100 grams of seeds weighed 4.17 grams. After partitioning 2.0 grams of this crude extract between 1-butanol and water, the amount of water-soluble extract was 730 milligrams (36.5 % of crude methanol extract). The amount of 1-butanol-soluble extract was 50 milligrams (2.5 % of crude methanol extract). These fractions had a slight yellowish-white color and had no UV absorbance. The water-soluble portion of the methanol extract from *Pharbitis nil* seeds exhibited several brownish-gold spots on silica-gel TLC. The LH-20 biogel column chromatography was employed to separate them. However these further purified fractions obtained from the chromatography were found to be pure sugars of di- and oligo-saccharides based on their ^{1}H NMR spectra, indicating that the convolvulinic acid aglycone had been hydrolyzed from the sugars and remained in the column. For this reason, the crude extracts obtained after the partitioning were identified by ^{1}H NMR and employed to run the PP2A 4-MUP inhibition assay. Two fractions, one being water-soluble and the other 1-butanol-soluble, each appeared as a group of three spots and had identical Rf values of 0.08, 0.14, and 0.32 (see Figure 12). These normal phase Rf values are reasonable values for high molecular weight amphoteric water-soluble resin glycosides containing lipophilic fatty acid and hydrophilic sugar moieties. Since the carboxy group of the aglycone (convolvulinic acid) is free and does not combine with a hydroxy group of the sugar moiety to form an intramolecular macrocyclic ester structure, as seen in *Ipomoea purga* resin glycosides, there are more free hydroxyl groups present, contributing to the water-soluble property of these resin glycosides (Ono, M. et al., 1990). Due to not being chromatographed, the purities of these samples

were crude compared with the *Ipomoea purga* samples. Based on the data from the ^{1}H NMR spectra, it was determined that they contained the resin glycosides as a mixture of homologues. It is possible that other contaminants may have been present, since many glycolipids were isolated in the extraction process.

Although the *Pharbitis nil* samples were rather crude preparations, their ^{1}H NMR spectra (Figure 13) gave similar ^{1}H NMR spectra as those compared to in the literature, based on the peak assignments, integration, and ratios of hydrocarbon protons to protons of sugar and organic acids. Again this suggests that the resin glycoside was an overwhelmingly major component in these samples. They are similar to what Mayer called ether-insoluble resin glycosides with a free carboxy group of the convolulinic acid aglycone (Shellard, 1961). These suggestions were supported by the presence in the ^{1}H NMR of the signals characteristic of 1 mol of isobutyric acid [δ 2.55, (1 H, sept, H-2), 1.27 (3H, d, H_3-3), 1.27 (3H, d, H_3-3')], 2 mols of (+)-2-methylbutyric acid [δ 2.53 (1H,tq, H-2),), 1.69 (2H, m, H_3-3), 0.99 (3H, s, H_3-4), 1.27 (3H, d, H_3-5) and [δ 2.43 (1H,tq, H-2),), 1.60 (2H, m, H_3-3), 0.94 (3H, s, H_3-4), 1.27 (3H, d, H_3-5)] and 1 mol of tiglic acid groups [δ 7.01 (1H, dq, H-3), 1.27 (3H, dd, H_3-5), 1.93 (3H, s, H_3-4)]. The ^{1}H NMR spectrum exhibited the signals of five anomeric protons (δ 6.32, 5.85, 5.48, 5.30 and 4.89) and three secondary methyls due to 6-deoxyhexose (δ 1.93, 1.67 and 1.63) as well as a 2-methylene (δ 2.55) and a primary methyl (δ 0.87) attributable to a convolvulinic acid moiety (see Table 2). Based on this data, it is reasonable to presume that the *Pharbitis* resin glycoside

isolated in this experiment is similar to the proposed chemical structure depicted in Figure 14.

In the assay experiments, the standard emission curve of 4-MU was established (Figure 15). The fluorescence intensity of 4-MU was in a linear function with its concentration in the experimental range (0 to 10 μM). The enzyme activity could be analyzed by using the standard emission curve to compare the amount of 4-MU from the dephosphorylation of 4-MUP by PP2A.

The okadaic acid at 60 nM was used as a reference inhibitor to verify that the enzyme inhibition assay was working correctly. The results showed at least 99% significant inhibition of PP2A by 60 nM okadaic acid on all four plates, with the largest p value of the four sets of okadaic acid data being less than 0.01. These results were expected since 60 nM of okadaic acid is a known concentration to completely inhibit PP2A activity. For each row of compounds tested on PP2A on each microtiter plate, a duplicate row of controls wells was prepared on the same plate that contained 1 μl of Tris buffer, pH 8.50 in place of the 1 μl of PP2A enzyme. These controls indicated that none of the compounds tested in this assay fluoresced on their own and therefore quenching could not occur that might produce false results. The row of control wells on each plate that contained 1 μl of PP2A enzyme, 39 μl of 4-MUP and 10 μl of Tris buffer, pH 8.50 was used to determine the normal activity that would be expected from the enzyme with no other compounds present. All of the wells on each plate containing potential inhibitory compounds were compared against these respective control wells to observe any change in activity of the enzyme.

The jalapin samples tested against PP2A were prepared in the following concentrations: 1.59 mg/ml, 0.159 mg/ml, 0.0159 mg/ml, and 0.00159 mg/ml. These concentrations would correspond to 1 mM, 100 μM, 10 μM, and 1 μM if the samples were pure, according to the molecular weight of the proposed structure of the glycoside. Similarly, the convolvulin samples were prepared in the following concentrations: 1.48 mg/ml, 0.148 mg/ml, 0.0148 mg/ml, and 0.00148 mg/ml, which, likewise, would correspond to 1 mM, 100 μM, 10 μM, and 1 μM of pure glycoside. The molecular weight of the proposed jalapin structure is 1,590 and the molecular weight of the proposed convolvulin structure is 1, 480.

The results of the inhibition assays are shown in Figures 21 – 24. A 4% increase in activation occurred at the 1.59 mg/ml concentration of jalapin, however the p value was equal to 0.3, so this data should be considered to be insignificant. Whereas, 12 % inhibition was observed at the concentrations of 0.159 mg/ml and 0.0159 mg/ml, with the p values both less than 0.01. The jalapin samples showed a maximum inhibition of 20% at the 0.00159 mg/ml concentration with a p value, 0.04. (see Figure 16). According to this data, the activity of PP2A did not change when the concentration of jalapin was decreased ten-fold from 0.159 mg/ml to 0.0159 mg/ml. The PP2A inhibition increased when the concentration of jalapin was decreased ten-fold from 0.0159 mg/ml to 0.00159 mg/ml. However, the data from the 1.59 mg/ml concentration of jalapin was not significant, and there was no observable trend for the inhibition to increase as the concentration of jalapin decreased. The effective dose of jalapin

(as a powdered resin) consumed orally which will produce catharsis is 2 grams (see Table 3). Because of the six order difference between the effective dose and the 0.00159 mg/ml concentration of jalapin which caused 20 % inhibition of PP2A in vitro, the possibility can not be excluded that jalapin inhibits PP2A .

The convolvulin showed a maximum inhibition of 50.9 % at the 1.48 mg/ml concentration with a p value less than 0.01, 5.7 % inhibition at the 0.148 mg/ml concentration with a p value of 0.045, 15.5 % inhibition at the 0.0148 mg/ml concentration with a p value of 0.12, and 8% increase in enzyme activity occurred at the 0.00148 mg/ml convolvulin concentration with a p value less than 0.01 (see Figure 17). The inhibition of PP2A activity was significantly increased by 45.2% when the concentration of convolvulin was increased ten-fold from 0.148 to 1.48 mg/ml, increasing the inhibition from 5.7 % to 50.9 %. This was the largest significant change observed among two concentrations of the same compound with a ten-fold difference in concentration, out of the four cathartic plant drugs tested with this assay. The 15.5 % inhibition of PP2A activity reported for the 0.0148 mg/ml concentration of convolvulin should not be considered significant based on the 0.12 p-value. An 8% increase in activation occurred at the 0.00148 mg/ml concentration of convolvulin. This does not seem to be consistent with the rest of the convolvulin data, despite the data's significance due to the low p-value below 0.01. The effective dose of convolvulin (as a powdered resin) consumed orally to produce catharsis is 2 grams (see Table 3). Because of the three order difference between the effective dose and the 1.48

mg/ml concentration of convolvulin which caused 50.9 % inhibition of PP2A in vitro, the possibility can not be excluded that convolvulin may inhibit PP2A .

The ricinoleic acid samples showed a maximum of 9.7 % inhibition of enzyme activity at the 100 μM ricinoleic acid concentration with a p value of 0.02, a 6.7 % inhibition at 10 μM with a p value of 0.01, and a 0.04 % inhibition of enzyme activity occurred at the 1 μM ricinoleic acid concentration with a p value less than 0.01 (see Figure 18). This data is significant, therefore ricinoleic acid may have a weak inhibitory effect on PP2A. According to this data, there was a slight trend for the inhibition of PP2A to increase as the concentration of ricinoleic acid increased. The effective dose of ricinoleic acid (as castor oil) consumed orally which will produce catharsis is 30 milliliters (see Table 3). Because of the substantial difference between the effective dose and the concentration of ricinoleic acid which caused 9.7 % inhibition of PP2A in vitro, the possibility can not be excluded that ricinoleic acid may inhibit PP2A .

The podophyllotoxin samples showed a maximum inhibition of 7.2% at the 100 μM concentration with a p value of 0.03. There is 97% confidence that this data is significant, so podophyllotoxin may have a weak inhibitory effect on PP2A. A 3.4 % increase in activation occurred at the 10 μM concentration of podophyllotoxin, whereas the 1 μM concentrations showed an inhibition of 5.6 % PP2A activity (see Figure 19). However, these values should not be considered significant since there is only 15% and 25% confidence in this data based on the p values. Therefore it is unclear whether the inhibition is increasing or decreasing as the concentration of podophyllotoxin increases. The effective dose of

podophyllotoxin consumed orally which will produce catharsis is 1.25 grams (see Table 3). Since there is a four-order difference between the effective dose and the concentration of podophyllotoxin which caused 7.2 % inhibition of PP2A in vitro, the possibility can not be excluded that podophyllotoxin may inhibit PP2A.

The cathartic compounds studied were chosen because of their partial structural resemblance to the DSP toxin okadaic acid and its derivatives. Their structural resemblance may cause them to act in the same manner as okadaic acid. These plant-derived compounds all contain unique hydroxy acid moieties which can be easily cleaved in the intestines to produce catharsis. Although okadaic acid and its derivatives have complicated structures, the functional groups essential for binding to protein phosphatase 1 and 2A have been determined to be a carboxyl group and hydroxyl groups. They are located on the straight-chain carbon-carbon backbone. With the folding of the molecule, a free carboxyl group at carbon one and free hydroxyl groups at carbons 24 and 27 become available and are essential for receptor binding in the case of okadaic acid (see Figure 3). This was determined by x-ray crystallography of okadaic acid bound to the protein phosphatase 1 and 2A enzymes (Sasaki, K. *et al.*, 1994). These moieties are important for such compounds to bind to a receptorial site on protein phosphatases 1 (PP1) and 2A (PP2A), thus inhibiting their activity and interfering with signal transduction processes, such as ion channel regulation. The loss of ion channel regulation results in the efflux of electrolytes and water as diarrhea.

Resin glycosides are well known as the purgative ingredients of some crude drugs such as Pharbitidis Semen and Jalapae Tuber which originate from Convolvulaceae plants. Chemical investigations on these resin glycosides were conducted as early as 1840 by J. F. W. Johnston. When these resin glycosides are subjected to alkaline hydrolysis, a hydroxyfatty acid oligoglycoside (glycosidic acid) and some organic acids (isobutyric, 2-methylbutyric, tiglic acids, etc.) are provided. The glycosidic acid is cleaved by acid hydrolysis to yield a hydroxyfatty acid and several kinds of monosaccharides such as glucose rhamnose, quinovose, etc. (Noda, N. *et al.*,1987).

Jalapae Tuber, the dried sliced root of *Ipomoea purga,* a Convolvulaceae species indigenous to Mexico, is well known as a purgative crude drug. The resin obtained from the root is called *Ipomoea* resin. Its resin glycoside is typically known as Mayer's "jalapin", an ether-soluble resin glycoside (Mayer, W., 1852). The hydroxyfatty acid (jalapinolic acid) obtained by alkaline and subsequent acid hydrolyses of *Ipomoea* resin was determined to be 11-hydroxyhexadecanoic acid (Asahina, Y. *et al.*, 1922). In 1961, Shellard reexamined the components of this resin and identified seven organic acids (acetic, propionic, isobutyric, tiglic, 2-methylbutyric, n-valeric and isovaleric acids), three sugars (glucose, fucose, and rhamnose) together with the jalapinolic acid from the ether-soluble portion. The same organic acids and sugars were isolated from the ether-insoluble portion along with ipurolic and convolvulinic acids. The parent glycosides were not isolated.

Pharbitidis Semen, the seeds of *Pharbitis nil,* a species of morning glory, is a cathartic crude drug. Its resin glycoside is typically known to be a Mayer's "convolvulin," an ether-insoluble resin glycoside (Mayer, W., 1852). Early chemical investigations on the resin glycoside of this plant revealed the presence of a hydroxytetradecanoic acid (convolvulinic acid) and tiglic acid (named for *Croton tiglium*, the source from which it was initially isolated), along with a glycosidic acid by alkaline hydrolysis and two crystalline fatty acids and D-glucose by acid hydrolysis of the glycosidic acid. (Kromer, N., 1896). More detailed investigations were later reported that alkaline hydrolysis of the crude glycoside named pharbitin (convolvulin) gave an organic acid named nilic acid (2-methyl-3-hydroxybutyric acid), together with tiglic acid and (+)-2-methylbutyric acid (Asahina Y. *et al.*, 1922). Mannich and Schumann in 1938 presumed Mayer's "convolvulin" to be a complex glycoside composed of a number of the repeating unit which is a glycosidic acid partially acylated by some organic acids at the sugar moiety. However, any pure resin glycoside had not yet been isolated and the chemical studies had been limited only to characterization of the component glycosidic acids and organic acids afforded by alkaline hydrolysis of a crude resin glycoside (Wagner, H., 1974).

In 1970, Okabe *et al.* reinvestigated the components of *Pharbitis* resin and isolated two glycosidic acids, pharbitic acids C and D, along with valeric, tiglic, nilic, isobutyric acid, and (+)-2-methylbutyric acid from its alkaline hydrolysis. A reexamination of the chemical components of pharbitin was carried out again in

1990 by Ono, M. *et al.*, who characterized the glycosidic acids as ipurolic acid 11-O-penta- and 11-O-hexaglycoside.

The effects of the hydroxy acid-containing cathartics on PP2A were determined using the PP2A 4-MUP inhibition assay. An assay that perhaps uses a crude PP2A preparation would be much more economical for further studies to be conducted. Testing these compounds on other enzymes may help to further explain their cathartic activities. Also, the development of an assay that is not performed on microscale might be more appropriate for future studies due to the solubilities of the test samples. Having a maximum volume of 50 µl per well is a limitation to this assay. This limitation prevents the drugs from being tested at a scale that is relevant to their actual therapeutic dosage and prevents them from being assayed at higher concentrations at which more or total inhibition might take place.

Another limitation of this experiment was the uncertainty of the purity of the jalapin and convolvulin samples. It may be possible that the slight activation observed in each data set from the jalapin and convolvulin samples could have been due to contamination of the enzyme assay by highly hydrophobic compounds which may have been present in the crude extracts. If this experiment were to be improved upon, separation of the crude extracts by HPLC would improve the ability to attain better purity of the isolated samples. ^{13}C NMR studies in addition to the ^{1}H NMR studies would enhance the ability to confirm the molecular structure of the isolated compounds. Performing replicates

of each microtiter plate would provide more data, which would allow for better statistical analyses and more certainty in interpreting the results of the experiment.

CONCLUSIONS

The assay results indicated weak but significant inhibition of PP2A by these compounds. Okadaic acid almost completely inhibits PP2A *in vitro* at a 60 nM concentration and can produce diarrhea in humans by consumption of 1 μg of the toxin. The doses of these crude drugs used to produce catharsis are six orders larger than the dose of okadaic acid which causes diarrhea in humans. Thus the weak activities of these compounds may be sufficient to account for the cathartic action of these drugs. However, in the human body, the bioavailability of these compounds is unknown. Since the traditional standard dosages of these cathartics are too large to prepare as samples for a micro-titer assay due to their solubilities, they were not able to be assayed at higher concentrations at which more or total inhibition might take place. The possibility that these crude cathartic drugs inhibit PP2A can not be discounted based on the results of this study. It is also possible that these drugs may cause catharsis by affecting other enzymatic activities which were not tested. This matter is still open to question.

Table 1. ^{1}H NMR Spectral Data for Samples N76-24-5 & N76-24-6 (YSN2246 & YSN2247) (400 MHz, pyridine-d5)

Moiety	Carbon #	Chemical Shift δ (ppm)	# of H's	Peak splitting
Rhamnopyranosal	1	5.51	1 H	d
	2	5.67	1 H	dd
	3	5.78	1 H	dd
	4	4.18	1 H	dd
	5	4.26	1 H	dq
	6	1.56	3 H	d
Glucopyranosal	1	5.13	1 H	d
	2	4.09	1 H	dd
	3	5.78	1 H	dd
	4	5.39	1 H	dd
	5	4.17	1 H	ddd
	6	4.42	1 H	dd
		4.68	1 H	dd
Quinovopyranosal	1	4.82	1 H	d
	2	4.09	1 H	dd
	3	5.39	1 H	dd
	4	5.04	1 H	dd
	5	3.68	1 H	dq
	6	1.26	3 H	d
Rhamnopyranosal'	1	5.67	1 H	d
	2	5.92	1 H	dd
	3	5.99	1 H	dd
	4	4.53	1 H	dd
	5	4.61	1 H	dq
	6	1.45	3 H	d
Quinovopyranosal'	1	4.81	1 H	d
	2	4.01	1 H	dd
	3	3.55	1 H	dd
	4	3.62	1 H	dd
	5	3.91	1 H	dq
	6	1.65	3 H	d
Jalapinoyl	2	2.69	2 H	m
	11	3.77	1 H	m
	16	0.96	3 H	t
	3-10, 12-15	1.65	24 H	m

Moiety	Carbon #	Chemical Shift δ (ppm)	# of H's	Peak splitting
Tiglyl	3	7.02	1 H	dq
	4	1.59	3 H	dd
	5	1.82	3 H	s
Tiglyl'	3	7.17	1 H	dq
	4	1.36	3 H	dd
	5	1.65	3 H	s
Acetyl		1.96	8 H	
		2.34	2 H	
		2.69	2 H	
Isobutyryl	2	2.92	1 H	sept
	3	1.21	3 H	d
	4	1.07	3 H	d

Table 2. ^{1}H NMR Spectral Data for Samples N57-174-8 & N57-175-3 (YSN2170 & YSN2171) (400 MHz, pyridine-d5)

Moiety	Carbon #	Chemical Shift d (ppm)	# of H's	Peak splitting
Glucopyranosal	1	4.89	1 H	d
	2	4.36	1 H	dd
	3	4.58	1 H	dd
	4	3.85	1 H	dd
	5	3.91	1 H	ddd
	6	4.28	1 H	dd
		4.46	1 H	dd
Glucopyranosal'	1	5.85	1 H	d
	2	4.25	1 H	dd
	3	4.23	1 H	dd
	4	4.21	1 H	dd
	5	3.85	1 H	ddd
	6	4.3	1 H	dd
		4.47	1 H	dd
Rhamnopyranosal	1	5.48	1 H	d
	2	4.54	1 H	dd
	3	4.53	1 H	dd
	4	4.25	1 H	dd
	5	4.38	1 H	dq
	6	1.67	3 H	d
Rhamnopyranosal'	1	6.32	1 H	d
	2	4.71	1 H	dd
	3	4.76	1 H	dd
	4	4.5	1 H	dd
	5	5.04	1 H	dq
	6	1.93	3 H	d
Quinovopyranosal	1	5.30	1 H	d
	2	4.21	1 H	dd
	3	4.29	1 H	dd
	4	3.49	1 H	dd
	5	3.85	1 H	dq
	6	1.6	3 H	d
Convolvulinyl	2	2.55	2 H	m
	11	3.91	1 H	m
	14	0.87	3 H	t
	3-10, 12-13	1.32	20 H	m

Moiety	Carbon #	Chemical Shift d (ppm)	# of H's	Peak splitting
Tiglyl	3	7.01	1 H	dq
	4	1.27	3 H	dd
	5	1.93	3 H	s
Methylbutyryl	2	2.43	1 H	tq
	3	1.6	1 H	m
		1.93	1 H	m
	4	0.94	3 H	t
	5	1.27	3H	d
Methylbutyryl'	2	2.53	1 H	tq
	3	1.69	1 H	m
			1 H	m
	4	0.99	3 H	tq
	5		3 H	d
Isobutyryl	2	2.55	1 H	sept
	3	1.27	3 H	d
	4	1.27	3 H	d

Table 3. Effective Doses of Cathartic Drugs

Cathartic Drug	Drug Source	Chemical Classification	Effective Dose
Okadaic Acid	Dinoflagellates, marine sponge	Polyketide	1 μg
Croton Oil	Seeds of *Croton tiglia*	Phorbol esters	10-50 mg*
Castor Oil	Seeds of *Ricinus communis*	Hydroxy-fatty acid (Ricinoleic Acid)	30 ml*
Podophyllotoxin	Roots of *Podophyllum peltatum*	Lignan	1.25 g*
Jalapin	Roots of *Ipomoea purga*	Resin glycoside	2 g*
Convolvulin	Seeds of *Pharbitis nil*	Resin glycoside	2 g*

*Effective doses listed in the United States Pharmacopeia XX

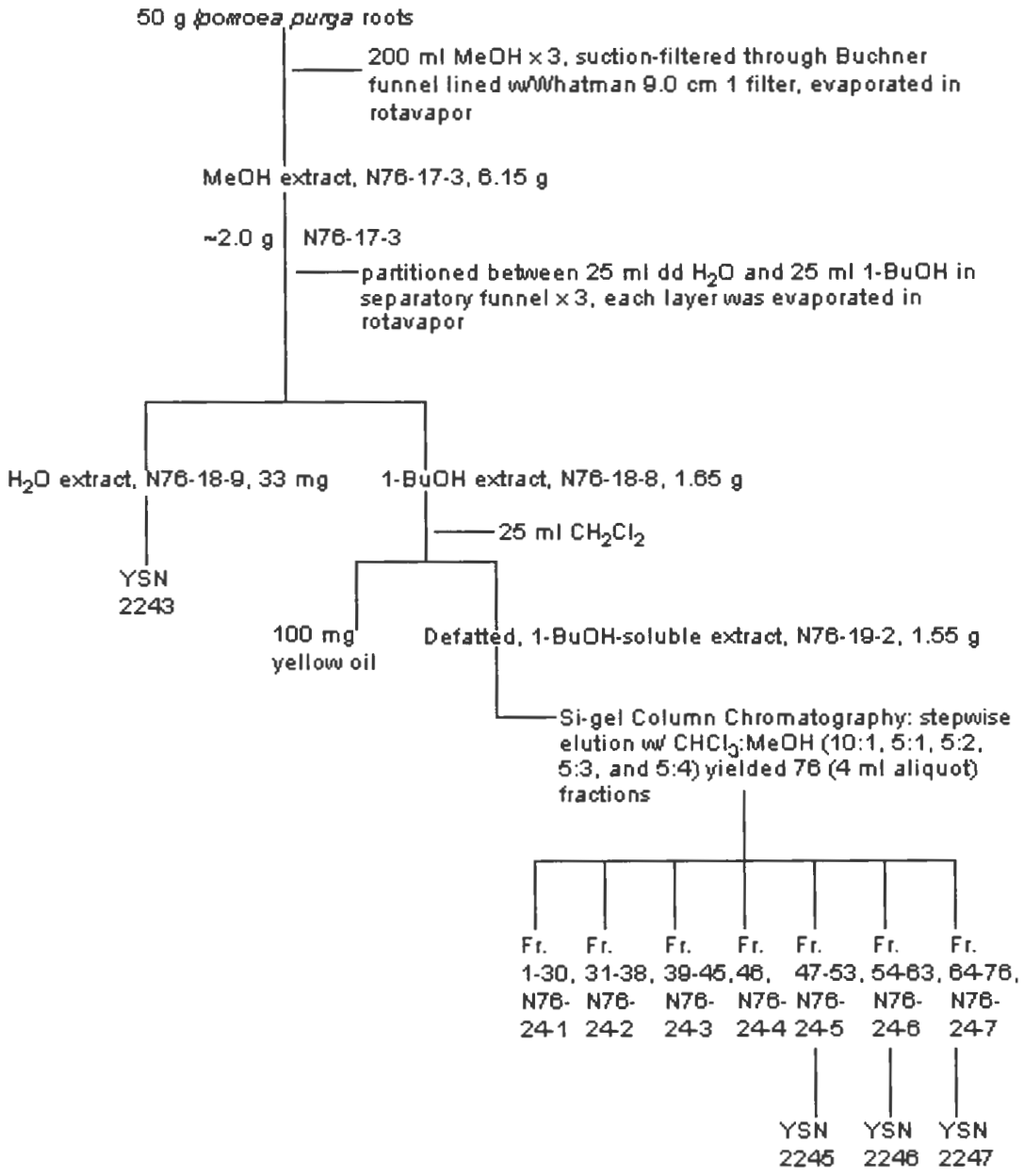

Figure 7. Outline for the Extraction of Jalapin from Roots of *Ipomoea purga*

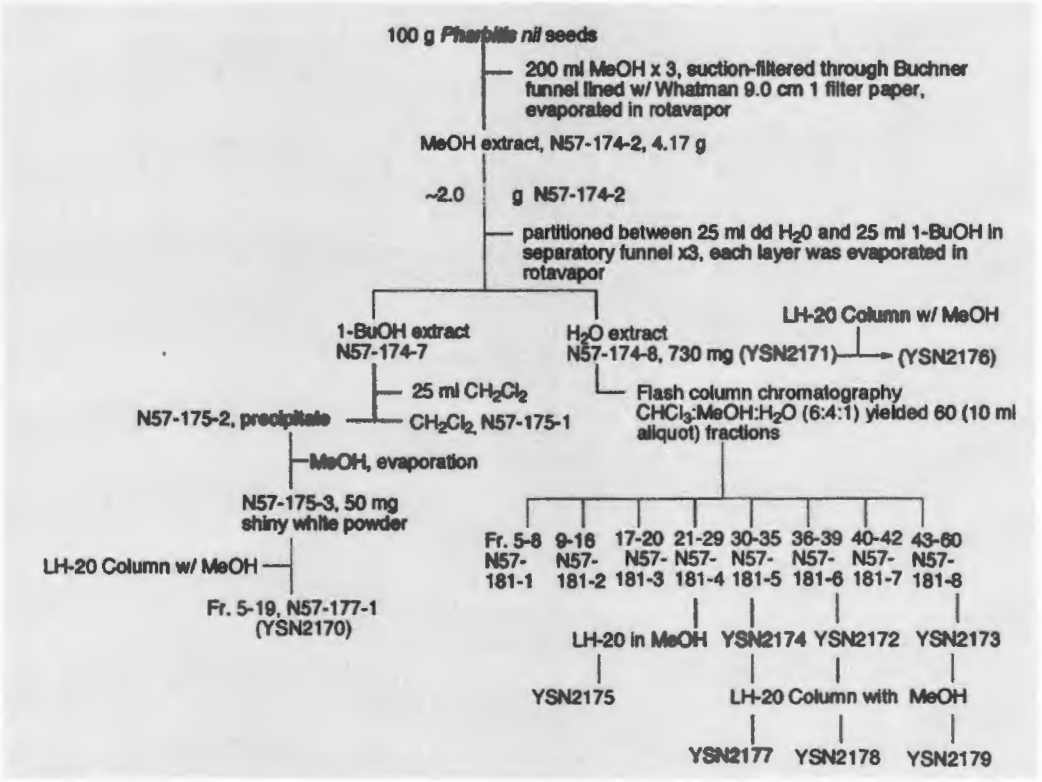

Figure 8. Outline for the Extraction of Convolvulin from Seeds of *Pharbitis nil*

Sample #	cm sample traveled/ cm solvent front	Rf Value
N76-24-5	5.7 cm / 7.6 cm	0.75
N76-24-6	5.4 cm / 7.6 cm	0.71
N76-24-7	4.6 cm / 7.6 cm	0.61
	2.3 cm / 7.6 cm	0.30
	1.4 cm / 7.6 cm	0.18

Solvent system: $CHCl_3$:MeOH:H_2O (6:4:1)

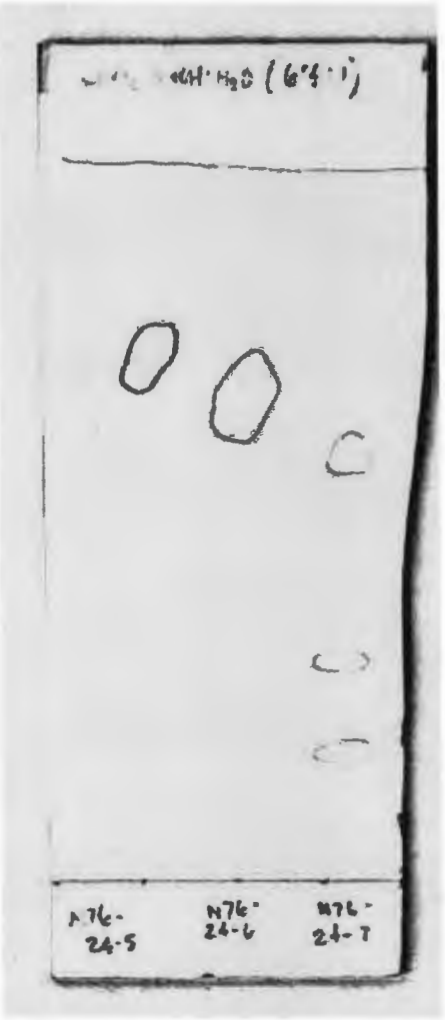

Figure 9. Thin Layer Chromatography of *Ipomoea purga* Isolates

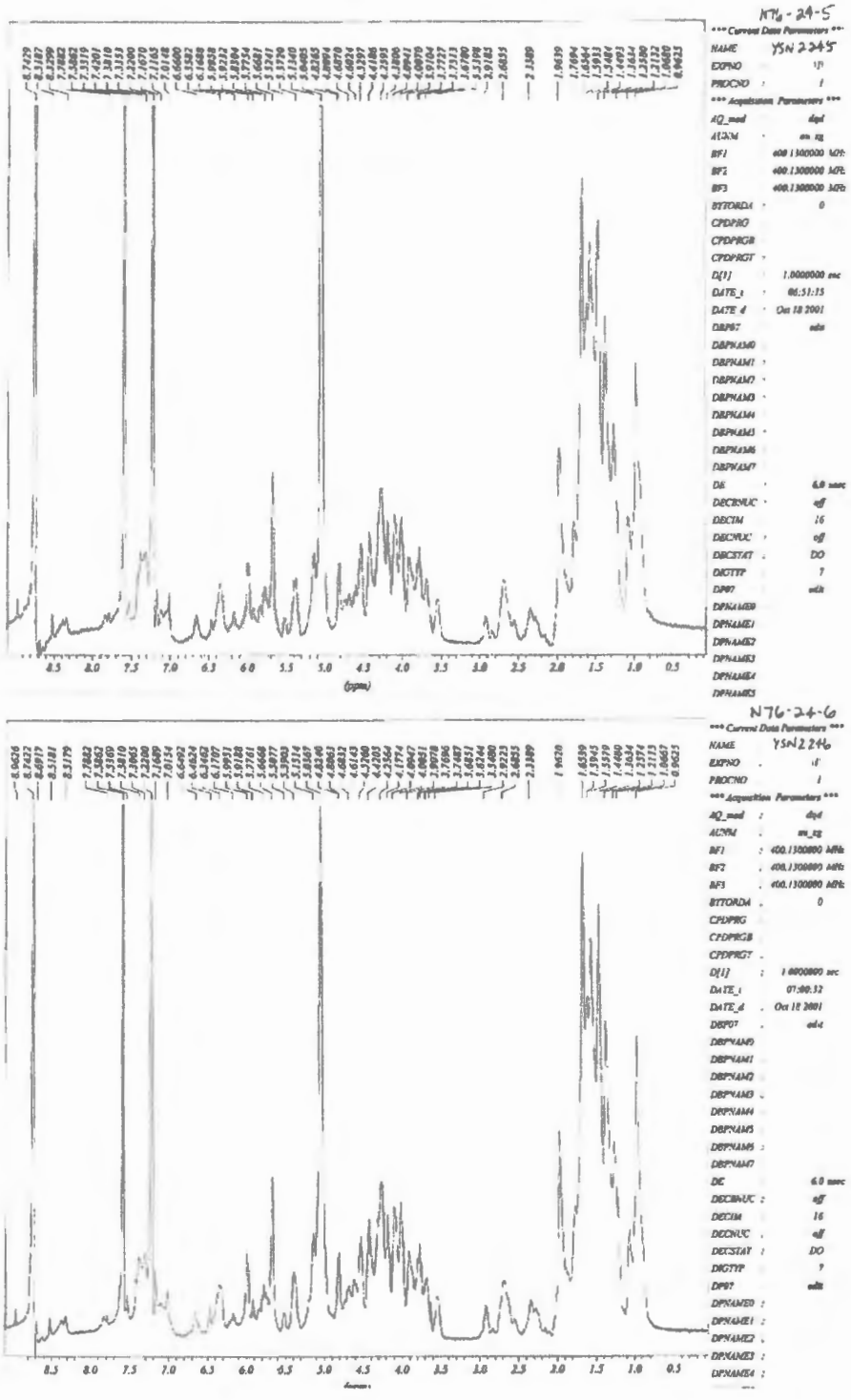

Figure 10. ^{1}H NMR Spectra of *Ipomoea purga* Isolates

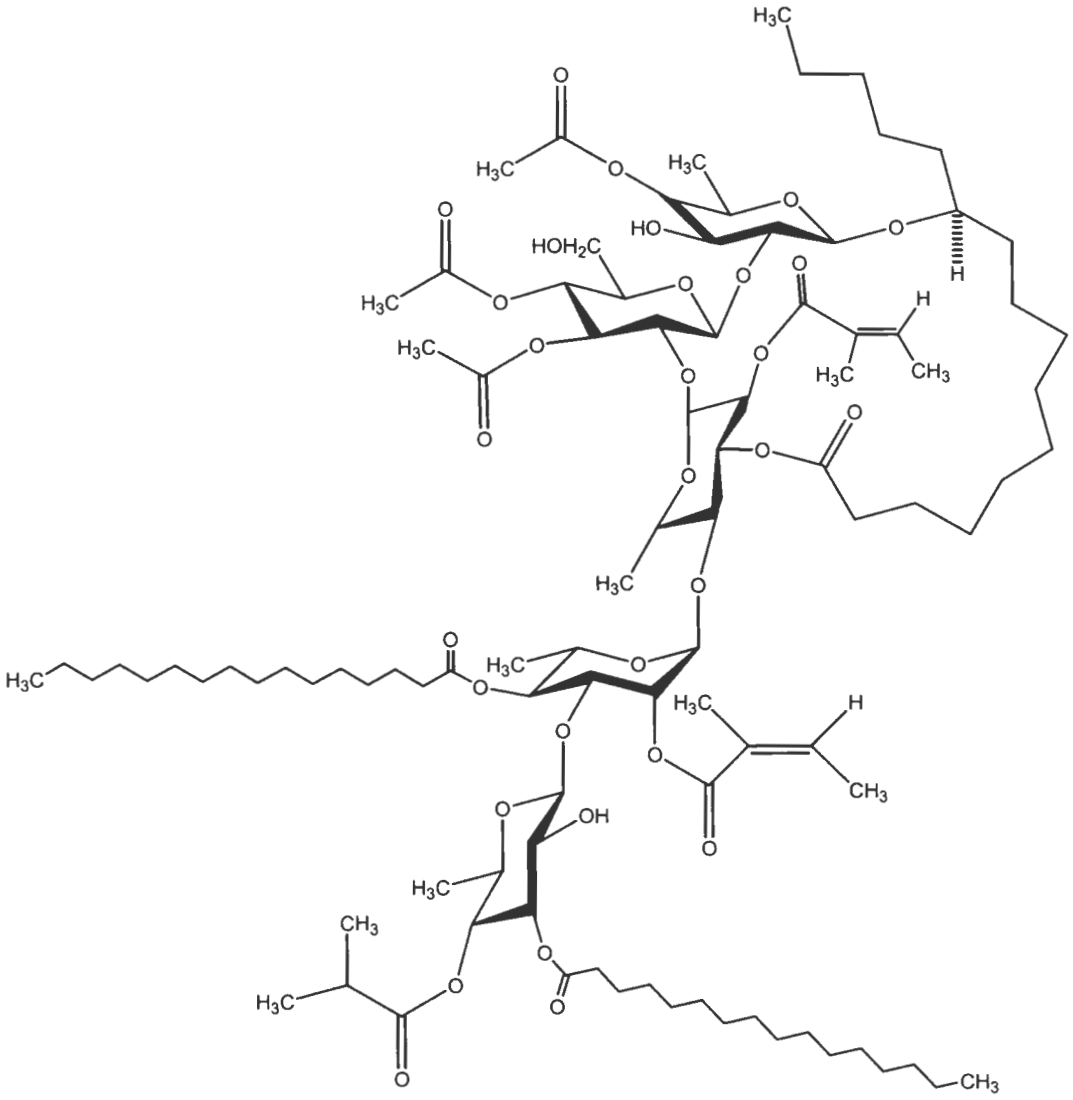

Figure 11. Proposed Structure of *Ipomoea purga* Isolates, Based on ^{1}H NMR Analysis

2.5 cm / 7.7 cm	Rf = 0.32
1.1 cm / 7.7 cm	Rf = 0.14
0.6 cm / 7.7 cm	Rf = 0.08

Solvent system: $CHCl_3$:MeOH:H_2O (6:4:1)

Figure 12. Thin Layer Chromatography of *Pharbitis nil* Isolates

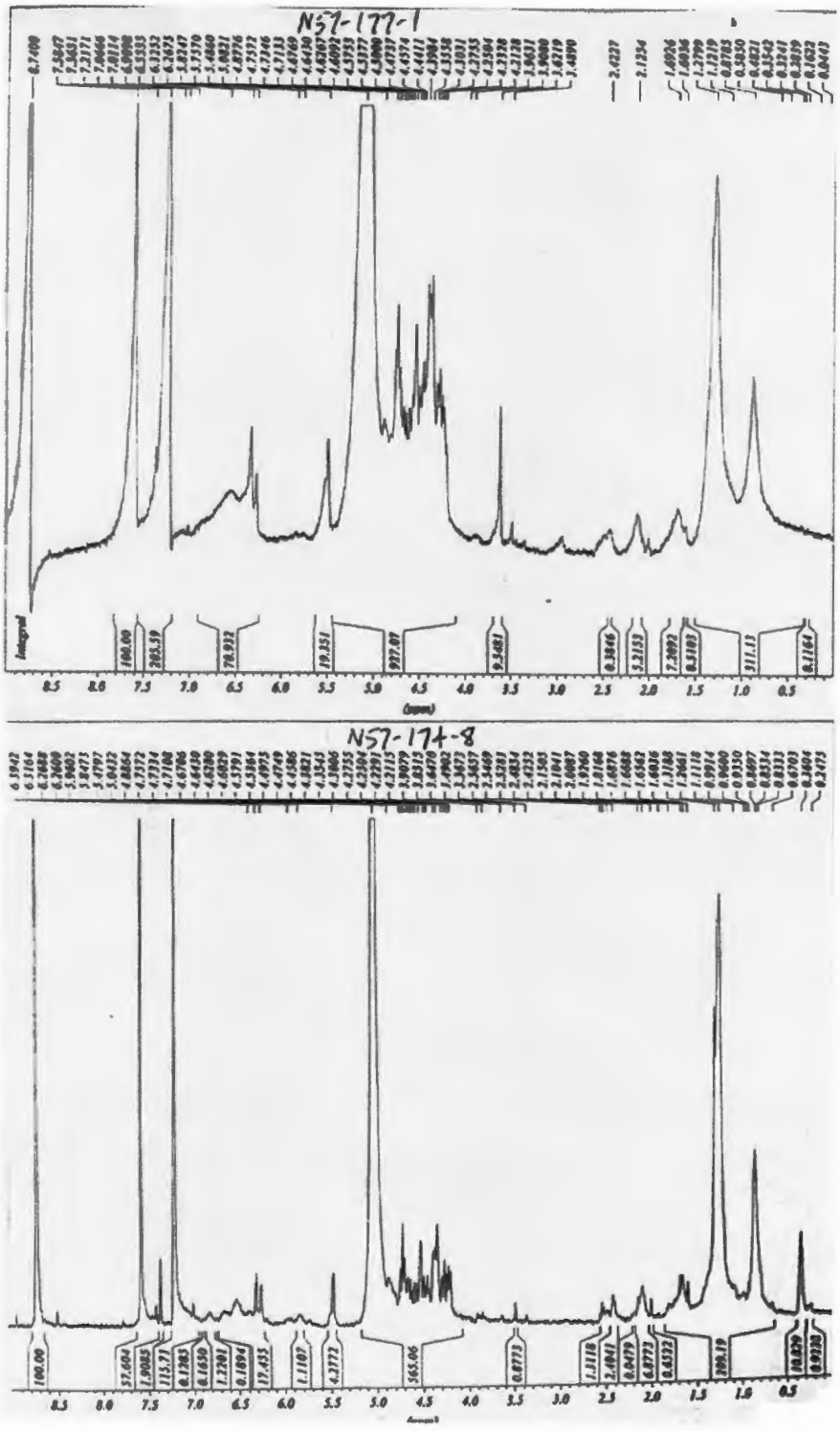

Figure 13. ^{1}H NMR Spectra of *Pharbitis nil* Isolates

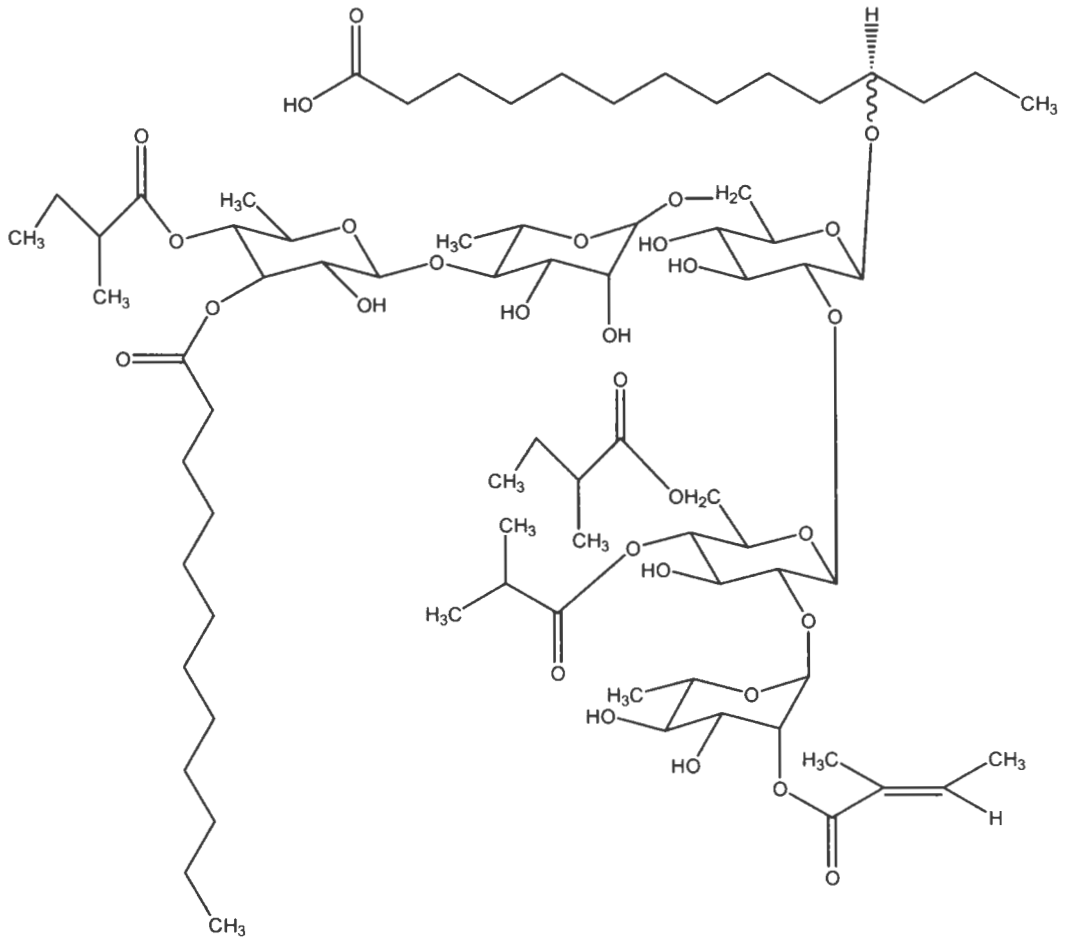

Figure 14. Proposed Structure of *Pharbitis nil* Isolates, Based on ^{1}H NMR Analysis

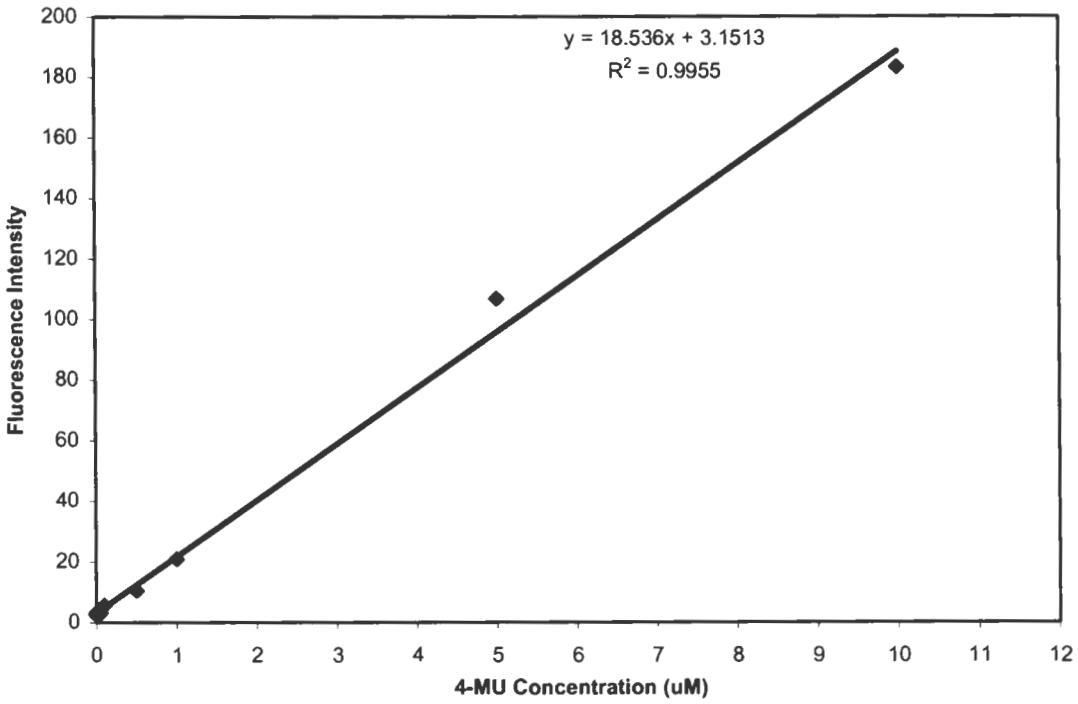

Figure 15. Standard Emission Curve of 4-Methylumbelliferone (4-MU)

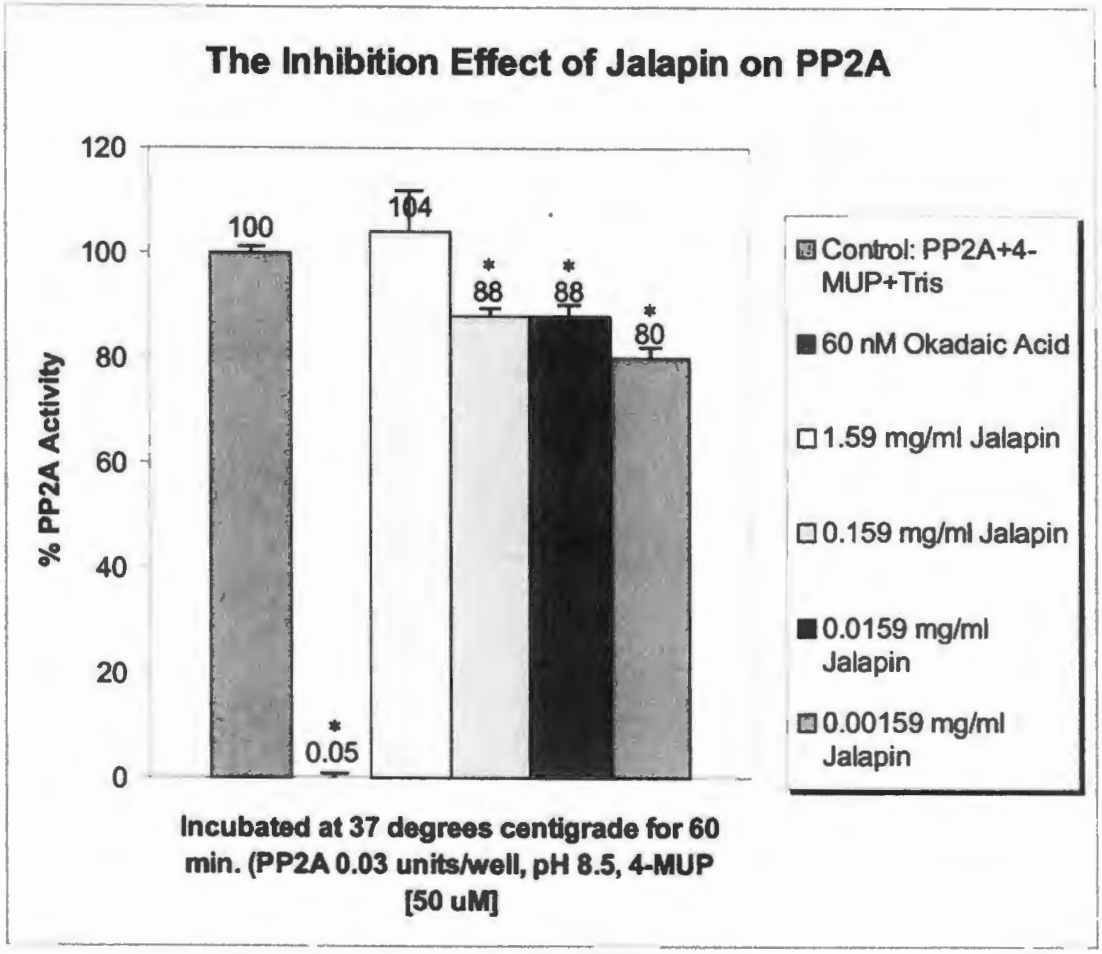

Figure 16. The Inhibition Effect of Jalapin on PP2A

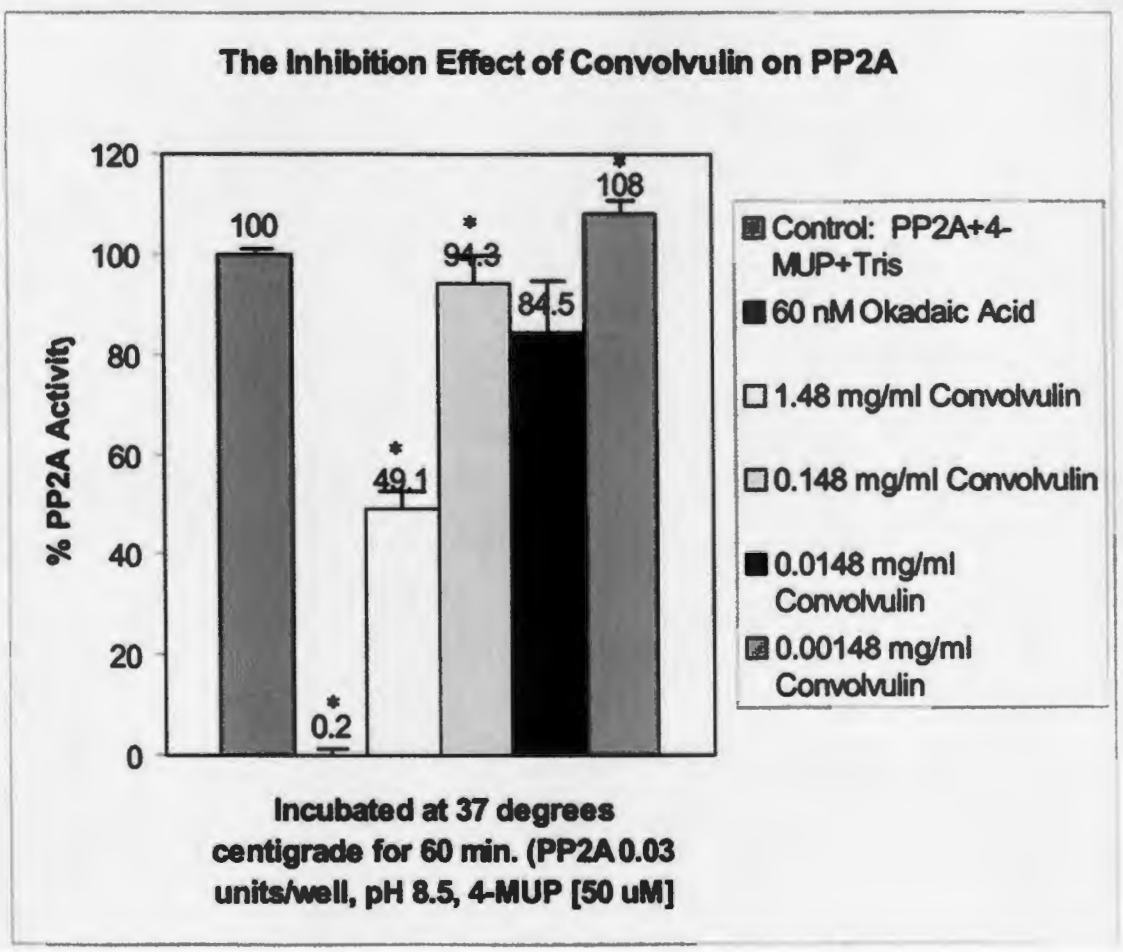

Figure 17. The Inhibition Effect of Convolvulin on PP2A

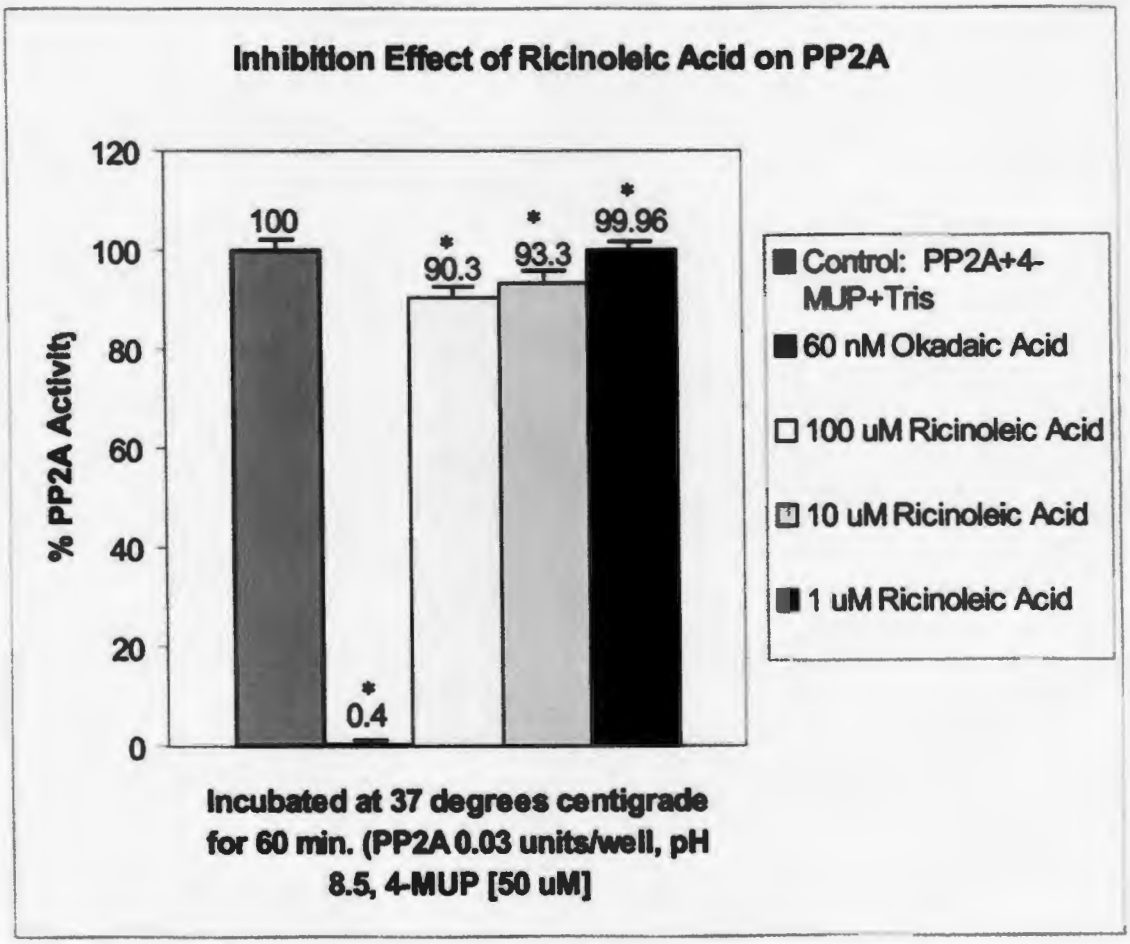

Figure 18. The Inhibition Effect of Ricinoleic Acid on PP2A

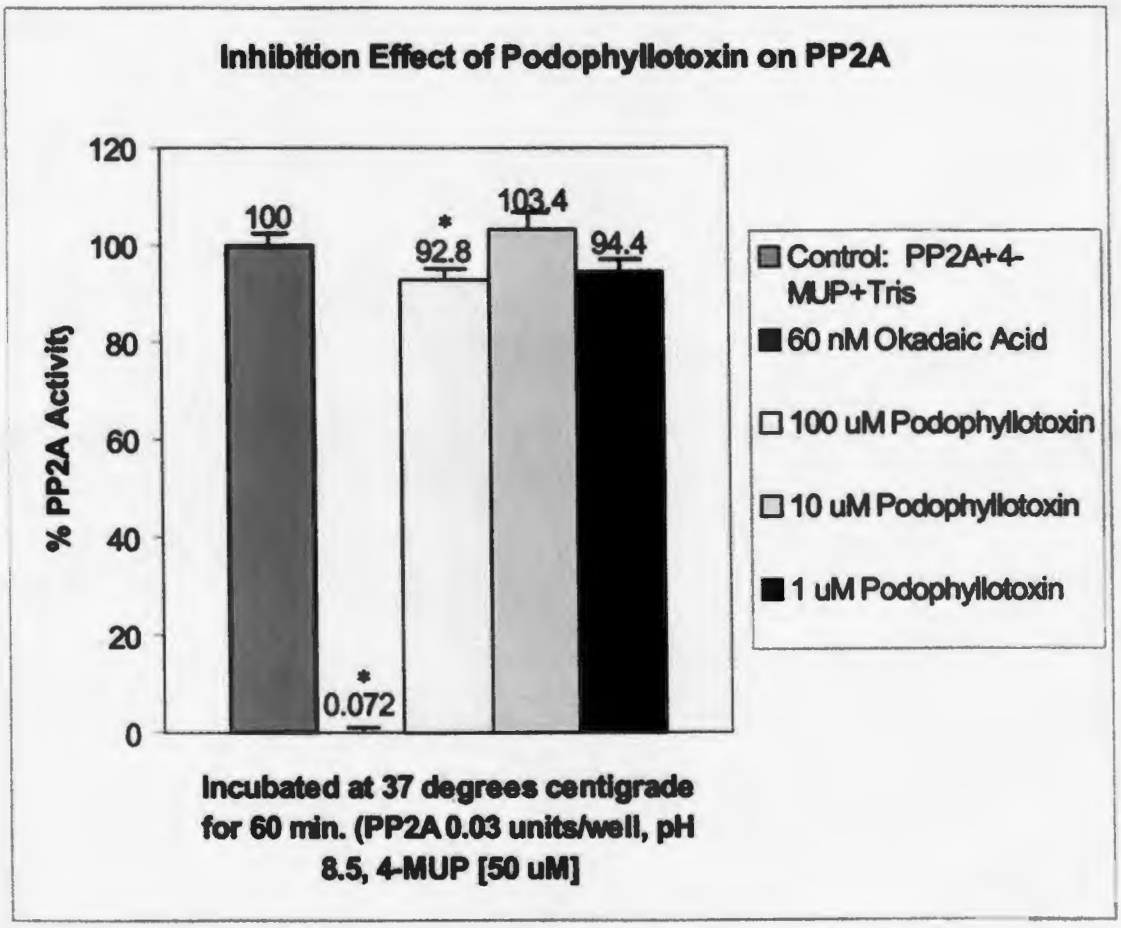

Figure 19. The Inhibition Effect of Podophyllotoxin on PP2A

BIBLIOGRAPHY

Asahina, Y. and Akasu, T., Journal of Pharmacological Society of Japan, 1925, Vol. 45, p.779

Barford, D. Structural Studies of Reversible Protein Phosphorylation and Protein Phosphatases, Biochemical Society, 1999, Vol. 27, part 6, pp. 751-766.

Bialojan, C., and Takai, A., Inhibitory Effect of a Marine-Sponge Toxin, Okadaic Acid, on Protein Phosphatases. Biochemistry Journal, 1988, Vol. 256, pp. 283-290

Brautigan, D.L. Phosphatases as Partners in Signaling Networks, Signal Transduction in Health and Disease, Advances in Second Messenger and Phosphoprotein Research, 1997, Vol. 31, pp. 113-124.

British Pharmaceutical Codex, Pharmaceutical Society of Great Britain, London, 1934, p. 429.

Catterall, W.A. Modulation of Sodium and Calcium Channels by Protein Phosphorylation and G Proteins, Signal Transduction in Health and Disease, Advances in Second Messenger and Phosphoprotein Research, 1997; Vol. 31, pp. 159-181.

Chandana, S., Sanjay, K. N., and Denker, B. M. Expanding role of G proteins in tight junction regulation: Gas stimulates TJ Assembly. Biochemical and Biophysical Research Communications, 2001, Vol. 285, pp. 250-256.

Cohen, P., The Structure and Regulation of Protein Phosphatases. Annal Review of Biochemistry, 1989, Vol. 58, pp.453-508.

Cohen, P., Holmes, C. F. B., and Tsukitani, Y. Okadaic Acid: A New Probe for the Study of Regulation. Trends in Biochemical Sciences, 1990, Vol.15, pp.98-121.

Dho, S., Stewart, K., Lu, D., Grinstein, S., Buckwald, M., Auerbach, W., Foskett, J. K. Phosphatase Inhibition Increases Tight Junctional Permeability in Cultured Human Intestinal Epithelial Cells, Journal of Cell Biology, 1990, Vol.111, p.410A.

Emmenegger, H., Stahelin, H., Rutschmann, J., Renz, J., and von Wartburg, A.: Zur Chemie und Pharmakologie der Podophyllum-Glucoside und ihr Derivate, Arzneim Forsch, 1961, Vol. 11, p.327, p.459.

Evans, F. J., Naturally Occurring Phorbol Esters, 1986, CRC Press Boca Raton, Florida, pp. 2-5.

Fairbairn, J. W.: Anthraquinone Laxatives, 1977, New York, John Wiley & Sons, Inc., pp. 117-119.

Fine, K. D., Krejs, G.J., and Fordtran, J. S. Diarrhea. Gastrointestinal Disease, 1993, 5th ed. (Sleisenger, M.H., and Fordtran, J. S., eds.) W.B. Saunders Co., Philadelphia, pp. 1043-1072.

Goodman and Gilman's The Pharmacological Basis of Therapeutics, 9th Ed., 1996, pp. 918-919.

Gupta, G., Ogawa, A., Du X., Houk, K. N., Armstrong, R. A Model of Structurally Diverse Natural Product Inhibitors of Protein Phosphatases PP1 and PP2A,; Journal of Medicinal Chemistry, 1997, Vol. 40, pp. 3199-3206.

Hanks, S. K., Quinn, A. M., and Hunter, T. The protein kinase family: conserved features and deduced phylogeny of the catalytic domains. Science, 1988, Vol.241, pp. 42-52.

Kitagawa, I., Baek, N. I., Ohashi, K., Sakagami, M., Yoshikawa, M., Shibuya, H. Mammosides B and H1, New Ionophoric Resin Glycosides from the Tuber of Merremia mammosa, An Indonesian Folk Medicine. Chemical & Pharmaceutical Bulletin, 1989, Vol. 37, No. 4, pp.1131-1133.

Kogetsu, H., Noda, N. Kawasaki, T., Kazumoto, M. Scammonin III-VI, Resin Glycosides of *Convolvulus scammonia,* Phytochemistry, 1991; Vol. 30, No. 3, pp. 957-963

Kromer, N., Journal of Practical Chemistry, 1896, Vol. 57, pp. 448

Linajes, A., Rico-Gray, V., Carrion, G. Traditional Production System of Jalapa, Ipomoea purga (Convolvulaceae), in Central Veracruz Mexico, Economic Botany, 1994, Vol. 48 No.1, pp. 84-89.

Luan, S. Protein Phosphatases: Structure, Regulation, and Function, Plant Protein Kinases: Advances in Botanical Research, 2000;Vol. 32, pp. 67-95.

Mannich, C., and Schumann, P., Arch Pharm. Berlin Deutsch, Pharm. Ges., 1938, Vol. 276, p. 221.

Mayer, W., Justus Liebigs Annals Chemie, 1852, Vol. 51, p. 81.

Noda, N., Ono, M., Miyahara, K., and Kawasaki, T. Resin Glycosides I. Isolationand Structure Elucidation of Orizabin-I, II, III, and IV, Genuine Resin Glycosides from the Root of *Ipomoea orizabensis,* Tetrahedron, 1987, Vol. 43, No. 17, pp. 3889-3902.

Noda, N., Kobayashi, H., Miyahara, K., Kawasaki, T. Resin Glycosides II. Identification and Characterization of the Component Organic and Glycosidic Acids of the Crude Resin Glycosides from the Seeds of *Ipomoea muricata*, Chemical & Pharmaceutical Bulletin ,1988, Vol. 36 No.2, pp. 627-633.

Noda, N., Kobayashi, H., Miyahara, K., Kawasaki, T. Resin Glycosides III. Isolation and Structural Study of the Genuine Resin Glycosides, Muricatins I-VI, from the Seeds of *Ipomoea muricata,* Chemical & Pharmaceutical Bulletin , 1988, Vol. 36, No. 3, pp. 920-929.

Noda, N., Nishi, M., Miyahara, K., Kawasaki, T. Resin Glycosides IV. Two New Resin Glycosides, Muricatins VII and VIII, from the Seeds of *Ipomoea muricata*, Chemical & Pharmaceutical Bulletin, 1988, Vol. 36, No. 5, pp. 1707-1713.

Okabe, H., Kawasaki, T., Tetrahedron Letters, 1970, p.3121.

Ono, M. Kawasaki, T. and Miyahara, K. Resin Glycosides V. Identification and Characteriazation of the Component Organic and Glycosidic Acis of the Ether-soluble Crude Resin Glycosides ("Jalapin") from Rhizoma Jalapae Braziliensis (Roots of Ipomoea operculata), Chemical & Pharmaceutical Bulletin, 1989, Vol. 37, No.12, pp. 3209-3213.

Ono, M., Kubo, K., Miyahara, K., Kawasaki, T. Operculin I and II, New Ether-soluble Resin Glycosides ("Jalapin") with Fatty Acid Ester Groups from Rhizoma Jalapae Braziliensis (Roots of *Ipomoea operculata*), Chemical & Pharmaceutical Bulletin, 1989, Vol. 37, No. 1, pp. 241-244.

Ono, M., Noda, N., Kawasaki, T., Miyahara, K. Resin Glycosides VII. Reinvestigation of the Component Organic and Glycosidic Acids of Pharbitin, the Crude Ether-Insoluble Resin Glycoside ("Convolvulin") of Pharbitidis Semen (Seeds of *Pharbitis nil*), Chemical & Pharmaceutical Bulletin, 1990; Vol. 38, No. 7, pp. 1892-1897.

Ono, M., Fukunaga, T., Kawasaki, T., and Miyahara, K. Resin Glycosides. VIII. Four New Glycosidic Acids, Operculinic Acids D, E, F, and G, of the Ether – soluble Crude Resin Glycosides ("Jalapin") from Rhizoma Jalapae Braziliensis (Roots of *Ipomoea operculata*), Chemical & Pharmaceutical Bulletin, 1990, Vol. 38 No.10, pp. 2650-2655.

Ono, M., Nishi, M., Kawasaki, T., Miyahara, K. Resin Glycosides. IX. Operculins I, II, V, VII and VIII, New Ether-soluble Resin Glycosides of Rhizoma Jalapae Braziliensis (the Roots of *Ipomoea operculata*), Chemical & Pharmaceutical Bulletin, 1990, Vol. 38 No. 11, pp. 2986-2991.

Ono, M. Kawasaki, T. and Miyahara, K. Resin Glycosides XI. Operculins II, IV, IX, X, XVI, XVII, and XVIII, New Ether-soluble Reswin Glycosides of Rhizoma Jalapae Braziliensis (Root of *Ipomoea operculata*), Chemical & Pharmaceutical Bulletin, 1991, Vol. 39, No. 10, pp. 2534-2539.

Qu, X., Efficient Assay for Okadaic Acid Class Diarrhea Shellfish Poison Using Protein Phosphatase 2A from Bean Sprouts, 1998, Biomedical Sciences Dept., University of RI.

Sellin, J. H., Intestinal electrolyte absorption and secretion. Gastrointestinal Disease, 5th ed., 1993, (Sleisenger, M.H., and Fordtran, J. S., eds.) W.B. Saunders Co., Philadelphia, pp. 1844-1857.

Shellard, E. J., The Chemistry of some Convolvulaceous Resins, Planta Medica, 1961, Vol. 9, pp. 102-116.

Shenolikar, S., Nairn, A. Protein Phosphatases: Recent Progress, Advances in Second Messenger and Phosphoprotein Research, 1991, Vol. 23, pp. 3-95.

Sheppeck, J.E., Gauss, C.M., Chamberlin, A.R. Inhibition of the Ser-Thr Phosphatases PP1 and PP2A by Naturally Occurring Toxins, Bioorganic & Medicinal Chemistry, 1997; Vol. 5, No. 9, pp. 1739-1750.

Shimizu, Y., Kinoshita, M., Ooi, F. Highly Sensitive, Non-radioactive Assays for Protein Phosphatase 1 and Protein Phosphatase 2A, In: Proceedings of the VII International Conference on Harmful Algae, Vigo, Spain, 1997, pp. 537-540.

Stahl, Egon. Drug Analysis by Chromatography & Microscopy, 1973, Ann Arbor Science Publishers, Inc., pp. 69-82

Suganama, Y., Fujiki, T., Manual On Harmful Marine Microalgae, 1988, Intergovernmental Oceanographic Commission (of UNESCO), Methods for Diarrhetic Shellfish Poisons, M. A. Quillian and J.L.C. Wright, pp. 95-111.

Trease, G. E. and Evans, W. C., Pharmacognosy, 10th ed., 1972, Bailliere Tindall, London, p. 473.

Tubaro, A., Florio, C., Luxich, E. Sosa, S., Loggia, R. D., and Yasumoto, T., A Protein Phosphatase 2A Inhibition Asay for a Fast and Sensitive Assessment of Okadaic Acid Contamination in Mussels, Toxicon, 1996, Vol. 34, No.7, pp. 743-752.

Tyler, V. E., Brady, L. R., Robbers, J. E. Pharmacognosy, 9th edition, 1988, Lea & Febiger, Philadelphia, PA, pp. 59-61.

United States Pharmacopeia XX, National Formulary XV, 1979, Mack Printing Co., Easton, PA 18042, pp. 121-123, 635-636, 722-723.

Vieytes, M. R., Fontal, O. J., Leira, F., Baptist de Sousa, J. M. V. and Botana, L. M., A Fluorescent Microplate Assay for Diarrheic Shellfish Toxins, Analytical Biochemistry, 1997; Vol. 248, pp.258-264.

Wang,W., Uzzau, S., Goldblum, S. E., and Fasano, A. Human zonulin, a potential modulator of intestinal tight junctions, Journal of Cell Science, 2000, Vol. 113, pp. 4435-4440.