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PHYTOCHEMICAL INVESTIGATION OF THE TRADITIONAL BOTANICAL MEDICINE *IRESINE CELOSIA*

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PHYTOCHEMICAL INVESTIGATION OF THE TRADITIONAL
BOTANICAL MEDICINE *IRESINE CELOSIA*

BY

CAROLINE GRACE KILLIAN

A DISSERTATION SUBMITTED IN PARTIAL FULFILLMENT OF THE
REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

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2014

ABSTRACT

Iresine celosia L. is a traditional medicine used by the indigenous Mayan people for a variety of ailments. It is also the sole active ingredient in the Odyliresin™ formulation currently marketed to humans worldwide as an antioxidant and for the promotion of prostate health. However, Odyliresin™ has not been characterized phytochemically, or evaluated biologically for its intended use. This work represents the first comprehensive phytochemical investigation of the Odyliresin™ formulation botanical extract. To better understand its constitutive phytochemistry, a tailored isolation scheme was developed, using various chromatography resins (silica gel, LH-20), separation and purification techniques. In all, eleven compounds were isolated from the extract. Relevant marker compounds were isolated and characterized using HR-MS, NMR, HPLC-UV, FT-IR and CD spectroscopy. These marker compounds were then quantified, analytical methods developed, and analytical fingerprint profiles generated to standardize formulation extracts. Among the compounds isolated, a novel pair of cyclic guanidine alkaloids is reported. To our knowledge, this is the second reporting of 2-substituted imidazoline alkaloids isolated from a plant source. These compounds were screened *in silico* for their ability to bind to the human androgen receptor (AR), a target for anti-androgen prostate cancer therapies, and further tested for their biological activity in AR-positive (LNCaP) and AR-negative (PC3) prostate cancer cell lines. These compounds show activity against AR-positive LNCaP cells in the 12.5-50 μ M range, while AR-negative PC3 cells were unaffected. In addition, compounds isolated from *Iresine celosia* L. were screened using drug metabolism and toxicology simulation studies *in silico*, to predict the formation of reactive metabolites and their possible toxicological

endpoints using Simulation's Plus ADMET Predictor proprietary software. The isolation and structure elucidation of eleven compounds from the formulation, two of which are new, the development of analytical methods to quantify their presence within the extract, and initial *in silico* toxicology screening offer information that can be used to support a level of quality production and a reasonable expectation of safety when using this dietary supplement as directed for the promotion of prostate health.

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PREFACE

This dissertation is written in manuscript format.

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1. Natural Products: History and What it Has Become

The timeless use of plants as medicine by man was formally christened a Western science by the German C.A. Seydler in his 1815 thesis and book, Analecta Pharmacognistica. Combining the Greek words for “drug” (φάρμακον, pharmakon), and the verb “to know, to discover,” (γινώσκω, *gignosko*), he coined the phrase pharmacognosy to describe the scientific field that deals with the chemistry, biological activity, and biosynthesis of natural products, or secondary metabolites.

The use of these specific Greek words remains to this day a fitting choice, as pharmacognosy continues to be primarily a discipline of drug discovery [1]. The discovery of pharmacological agents from natural sources or based on natural pharmacophores was made possible by advancements in separation science. Seydler’s treatise was published at a time when new methods resulting from the rise of industrial expansion in Britain, western Europe and the United States in the 19th century allowed for the manufacture of fine chemicals, including the isolation of medicinal substances from plants, such as quinine, camphor and ether, ingredients employed in the pharmacy of the day [2]. This included advancements in the large-scale extraction of drugs from plant material by means of solvents, usually water or alcohol, which was still a function of the pharmaceutical manufacturer at the end of the 19th century [3].

By the early years of the 20th century, researchers had indicated that the action of “vegetable drugs” was due to the presence of definite chemical compounds, many of which had been isolated already. Among them were certain alkaloids, notably quinine and morphine. The isolation of these active compounds

in a pure state had far-reaching consequences in medicine and in the industry of fine chemicals. For one, more accurate dosing for the drugs of that time became possible; it is important to appreciate that this had previously been impossible, as patients were treated with crude drugs of unknown or variable composition. Secondly, harmful effects due to the presence of impurities in the crude drugs could be mitigated or avoided and a level of quality control of the formulated dose could be achieved [4]. In the case of quinine, its isolation from *Cinchona* bark in 1820 by Pelletier and Caventou revolutionized the treatment of malaria, as it was possible to give a measured dose of the pure alkaloid compound instead of the nauseous bitter brews that had been the prescription since the 1630s. Thirdly, investigation of the chemical composition of the active principles was possible. Among other advancements, this third point led to the development of synthetic organic chemistry, which aimed to synthesize active principles and related substances. The advent of synthetic products, however, did not diminish the large-scale extraction of drugs from plant material by means of solvents, usually water or alcohol. At the beginning of the 20th century, preparation of plant materials comprised about half of medicines in the United States Pharmacopeia [5].

To this day, natural products and structures derived from natural products continue to play a vital role in the therapeutic armament [6]. Centuries of co-evolution have taken place between organisms that produce these compounds (thought by some to be produced by the organism for the purposes of protection, to ward off predators, harmful effects of UV rays, or as a strategy to be more widely cultivated, by pollinators or cultivators like humans, for example), and those that use them for medicines. Therefore, it is not surprising that natural product structures offer leads of higher quality than mass efforts to synthesize

bioactive compounds. Natural product secondary metabolites have been honed for biological function.

For the most part, formal research in the area of natural products chemistry has continued to take the approach to isolate and extract single active ingredients, to search for “silver bullet”-type molecules that may go on to be used as pharmaceutical-like medicines. Some have made attempts to understand the synergistic function of active ingredients [7, 8]. These “active principles” from natural sources can be of great value in the search for bioactive molecules because they often possess intricate architecture, with more stereogenic centers and chiral complexity than can be synthesized in a lab. For these reasons, as well as their coevolution among living systems, natural products have been the single most productive source of leads for the development of drugs, and the source of most of the active ingredients of medicines.

Emphasis on isolation of single active principles has resulted in natural products being evaluated, both for efficacy and safety, under a pharmaceutical and food additive-type paradigm [9]. That scientific paradigm for the evaluation of natural products in commercial use, in the form of dietary supplements, is outlined in the 2011 Draft New Dietary Ingredient Guidance document, which is discussed in detail elsewhere in this work (see Chapter 4). The historical and ethnobotanical uses of herbs have given way to evaluation and testing of dietary supplements, mostly through the isolation of single active principles. As mentioned above, this has allowed for desirable outcomes, like more accurate dosing, quantification, and specifications for formulation and its testing. However, it is important to keep in mind that the reduction of complex botanical mixtures down to single active principles is a model in itself that simplifies at the outset. Nevertheless, the

reduction of the action of an herbal medicine to individual active principles makes possible their pharmacological and toxicological evaluation. Toxicology and pharmacology, sciences that have been built on the evaluation of single active ingredient pharmaceutical compounds and environmental toxins, is now being applied to the evaluation of herbal medicines.

However, even when reduced to single active principles, natural products differ structurally, in their activity, and in their modes of action from these types of chemicals. In general, these compounds tend to differ from synthesized pharmaceutical compounds in the structural features that they employ. Natural products possess exceptional chemical functionality that keep them compatible with the aqueous milieu of biological microenvironments [10]. Nature often exploits reactive functional groups in biologically active natural products. Natural products typically have more stereogenic centers, and are more architecturally complex than synthetic molecules. Natural products contain relatively more carbon, hydrogen, and oxygen, and less nitrogenated or other elements than synthetic agents. Synthetic medicinal chemistry prefers a high proportion of aromatic and heteroaromatic rings, fewer stereogenic centers, lower molecular weights, lipid-soluble molecules, with a lack of chemical reactivity. Natural products tend to be more polar, water soluble, and adaptable to fluctuations in pH.

Furthermore, single active principles isolated from botanicals that are not necessarily developed and consumed primarily by humans as drug molecules, but that are consumed primarily as one of many phytochemical constituents within dietary supplement formulations in relatively minute quantity as compared with pharmaceutical prescription dosing, present a different pharmacological profile altogether. Standardization of extracts can reveal that what are thought to be the

active ingredients are present in very small quantities, on the microgram, nanogram and picogram scale – concentrations that may be well below a threshold needed to see a toxic effect. While most of these single active principles within the context of their use in a botanical dietary supplements can be considered safe, there exist a few compounds that can be considered toxic.

When considered as a whole, these compounds tend to exert toxic effects through oxidative metabolism into biologically reactive intermediates (BRIs) [11-14]. When dosed properly, these compounds can also be found to exert chemopreventive effects. The overall risk/benefit of botanical dietary supplements can be thought of as the result of BRI formation and interaction with resulting biological targets, which likely depends on the level of reactivity and selectivity of the BRI, as well as the dose and time of exposure.

Biotransformation of a compound into reactive metabolites is largely a function of its chemical properties. The presence of certain chemical functional groups has been associated with toxicity due to the formation of reactive metabolites. Phytochemicals containing structural features like conjugated systems, simple Michael acceptors, epoxides, carbocations, quinoids, terminal alkenes, acetylenes, and benzodioxoles, among others, or the ability to form them, have been commonly associated with reactive metabolite-mediated adverse effects in botanical dietary supplements [12-15]. For decades, structural alerts have also been used to mitigate toxic liabilities for pharmaceuticals, environmental chemicals, and food additives, though the structural features present in these classes of compounds tend to differ from those associated with botanical natural products [11, 16-20]. Mitigating metabolism-related liabilities for pharmaceuticals and environmental toxins is often accomplished through *in silico* screening of

compounds for these structural alerts. This strategy has been used successfully both by manufacturers and regulators of these products, who find these computational SAR toxicology models to be both reasonably sensitive and specific [17, 18, 21-25]. A similar methodology could be applied to mitigate risk from dietary supplements, but such an approach should be specific to the risks presented by the particular chemistry of natural products.

The benefit of this approach is a substantial reduction, replacement, and refinement in the need for biological and particularly animal toxicological testing required to establish the safety of chemical substances. Within the food additive regulatory paradigm, screening for structural alerts also allows for the development of thresholds of concern, which then trigger requirements for further testing before the product can be introduced to the marketplace. If similar structural alert screening could be conducted for dietary supplements, and threshold of concern regarding a dietary supplement's risk profile could be determined, the extensive, resource intensive, and costly testing outlined within the New Dietary Ingredient Draft Guidance of 2011 may not be entirely necessary for every phytochemical ingredient present in every natural product brought to market.

When combined with phytochemical characterization and standardization, such an approach may help to mitigate the health risks posed by botanical dietary supplements. The work that follows attempts to apply such an approach to the evaluation on one of the nearly one thousand new dietary supplements brought to market each year.

2. Justification for and Significance of the Study

The use of natural product medicine in the form of dietary supplements in the U.S. has increased significantly over the past two decades [26]. Since the passage of the Dietary Supplement Health and Education Act (DSHEA) in 1994, the botanical dietary supplement market in the US has grown from \$2.9 billion in 1995 to \$4.8 billion in 2008 [27, 28]. It is estimated that over one thousand new dietary supplement products enter the market each year [29]. For the natural products chemist, this trend translates into new challenges to evaluate the quality of botanical ingredients, which is linked directly to the safety and efficacy of the final dietary supplement product.

For all their popularity, however, many commercial botanical products are poorly defined scientifically. In the United States, consumers take it on faith that the supplement they are ingesting is the same as is listed on the label, and that it contains the reportedly “active” constituents they seek [30]. While a number of commonly used herbs are generally regarded as safe (GRAS), many herbs that have been in use for centuries as traditional medicines are not necessarily guaranteed to be either safe for consumption or efficacious, in part due to a lack of scientific investigation. In the words of the Institute of Medicine, “the same principles and standards of evidence of treatment effectiveness apply to all treatments, whether currently labeled as conventional medicine or [as] complementary and alternative medicine (CAM)” [31, 32]. Whether botanical or synthetically derived, medicines should be made in a safe and reproducible manner, with an understanding of the degree of efficacy related to the medicine’s intended use. The study goes on to explain that in the case of CAM, “safety trumps efficacy”, and “the absence of evidence of effectiveness does not imply

absence of effectiveness” [29]. Whether or not clear evidence of effectiveness for a given condition is found, these therapies can and should be scientifically investigated, for their safety as well as for the presence of known and/or novel phytochemicals present that may possess biological activity.

Odyliresin™ is a botanical supplement formulation currently marketed to humans for the promotion of prostate health [33]. The tincture is comprised of a 30% ethanol extract of the botanical *Iresine celosia*. The plant is a member of the Amaranthaceae family and goes by several botanical synonyms and common names, as listed in Table 1 [34-38].

Table 1: Botanical Synonyms and Common Names for *Iresine*

celosia^[34-38]

<u>Taxonomical synonym for <i>Iresine celosia</i> L.</u>	<u>Common names</u>
<i>Iresine diffusa</i> Humb. & Bonpl. ex Willd.	Juba bush Bloodleaf, Camarón, shrimp plant, <i>hierba de la plata</i> (silver herb, Central America), <i>plumaria</i> (little feather, Columbia), <i>velo de princessa</i> (princess's veil, Guatemala), nerve wist
<i>Iresine canescens</i> Humb. & Bonpl. ex Willd	
<i>Iresine celosioides</i> (L.)	tlatlancuaya
<i>Iresine chalk</i>	tlatlancuaya
<i>Iresine elongata</i> Humb. & Bonpl. ex Willd	
<i>Iresine paniculata</i> (L.) Kuntze, non Poir	
<i>Iresine flavescens</i> Humb. & Bonpl. ex Willd	
<i>Iresine gracilis</i> Humb. & Bonpl. ex Willd	
<i>Alternanthera flavescens</i> Humb. & Bonpl. ex Willd	
<i>Iresine calea</i> (Bftenz)	
<i>Iresine latifolia</i>	
<i>Gomphrena latifolia</i>	
<i>Iresine laxa</i>	Amargosilio, tepozan, erba del tabardluo herba de la calenture; mosquintero; clacancuayo (derived from the Nahuatl tlatiancua-ye "which has knees," referring to jointed stems)
<i>Alternanthera paniculata</i>	

The plant is native to the Southeastern United States, Mexico, Central and South America, and the West Indies. It has a history of use by indigenous peoples, including the Highland Mayans of Chiapas, Mexico, who referred to the plant as *tlatlancuaya* [39]. A variety of anecdotal indications accompany the plant, including its ancient use for the treatment of skin conditions such as dropsy, reproductive health, gonorrhea and malaria [40]. The plant is also one of several ingredients included in Jamaican root tonics for male virility and overall health, among other varied indications [35, 36, 41-43]. The Odyliresin™ formulation of *Iresine celosia* has been marketed as a treatment of tumorigenic conditions at large and for epilepsy as far back as 1965, and more recently as an antioxidant [44].

Previous phytochemical analysis may have been conducted on the plant under one of the plant's many botanical synonyms. Using classic techniques for the isolation and elucidation of phytochemicals, Djerassi and his colleague Pierre Crabbé reported two main chemical constituents of the plant. The first was the then unknown drimane iresin, reported in 1953, which took six years to elucidate its structure [39, 45-49]. Its spectral assignment was not reported until 2005 in a paper that also identified the presence of the previously reported compounds triacontanol, beta-sitosterol, stigmasterol, alpha-amyrin-3-*O*-beta-D-glucopyranoside, beta-amyrin-3-*O*-beta-D-glucopyranoside, beta-sitosteryl-beta-*O*-D-glucopyranoside and iresin [50]. The 2005 paper by Rios and colleagues also identified three new drimanes from the plant: 3,14-dihydroxy-17,8-drimen-11,12-acetonide; 3,7,14-trihydroxy-18,9-drimen-11,12-olide; and 3,7,14-trihydroxy-18,9-drimen-11,12-olide. The 2005 Rios paper did not report a second compound isolated by Djerassi and Crabbé, *tlatlancuayin*, or 2',5-dimethoxy-6,7-

[methylenebis(oxy)]isoflavone;2',5-Dimethoxy-6,7 methylenedioxyisoflavone [48]. The drimane structures, along with iresin and isoflavone tlatlancuayin, are listed in Table 2 and Figure 1. However, it is not clear that these plants were definitively identified as *Iresine celosia*.

This work represents the first phytochemical evaluation of the *Iresine celosia* herbal supplement formulation.

Table 2: Compounds Isolated from *Iresine diffusa*

Compounds Isolated From <i>Iresine diffusa</i>
iresin
tlatancuayin
3-beta,7-alpha,14-trihydroxy-delta-8,9-drimen-11,12-olide
3-beta,7-beta,14-trihidroxy-delta-8,9-drimen-11,12-olide
3-beta,14-dihydroxy-delta-7,8-drimen-11,12-acetonide
dimethoxy-6,7-[methylenebis(oxy)]isoflavone;2',5-Dimethoxy-6,7 methylenedioxyisoflavone
triacontanol
beta-sitosterol
stigmasterol
alpha-amyrin-3- <i>O</i> -beta-D-glucopyranoside
beta-amyrin-3- <i>O</i> -beta-D-glucopyranoside
beta-sitosteryl-beta- <i>O</i> -D-glucopyranoside

Figure 1: Structures of Chemical Constituents Identified in *Iresine diffusa*

3. Aims of This Work

The work set out to evaluate the quality, safety and efficacy of the dietary supplement Odyliresin™ formulation of the botanical *Iresine celosia* through the following aims:

- Aim 1: Ensure quality through biological classification of plant starting material, the isolation and characterization of known and/or novel chemical constituents of the *Iresine celosia* extract, and the development of methodologies for preparation where needed.
- Aim 2: Evaluate the safety of isolated phytochemicals using *in silico* drug metabolism and toxicology simulation studies, to predict possible metabolites generated *in vivo* and their possible toxicological endpoints using ADMET predictor proprietary software.
- Aim 3: Biological investigation using in-house bioassays and *in silico* molecular docking experiments with targets involved in prostate cancer, with a focus on the human androgen receptor.

4. Outline of Chapters

Chapter 1 set a backdrop for the history and current conditions under which the present study is being conducted. We set out our aim of investigating phytochemically a traditional herbal medicine made dietary supplement, currently used in humans to promote prostate health.

Chapter 2 goes into detail describing the isolation and structural elucidation of 11 compounds from the *Iresine celosia* Odyliresin™ formulation extract, with the reporting of two new and structurally rare (see Table 3) guanidine alkaloids that show activity as possible androgen receptor (AR)-binders *in silico*, and inhibit

growth of AR-dependent prostate cancer cells. Until now, guanidine alkaloids have not been found in *Iresine celosia*.

Table 3: Plant Species Known to Contain Guanidine Alkaloids

Order	Family	Genus, species
Asterales	Asteraceae	<i>Verbesina peraffinis</i>
Caryophyllales	Plumbaginaceae	<i>Plumbago zeylanica</i>
Fabales	Fabaceae	<i>Canavalia rosea</i>
Fabales	Fabaceae	<i>Milletia laurentii</i>
Fabales	Fabaceae	<i>Pterogyne nitens</i>
Malpighiales	Euphorbiaceae	<i>Alchornea cordifolia</i>
Ranunculales	Ranunculaceae	<i>Cimicifuga racemosa</i>
Solanales	Solanaceae	<i>Solanum cernuum</i>

Chapter 3 outlines a method for the HPLC-analysis and the calibration of these two new, species-specific compounds as analytical standards to identify the extract chemically, as well as an analytical fingerprint profile to which a crude chromatogram can be compared. Chapter 3 also allows for understanding of concentrations of these compounds in dosing of the supplement in its current formulation.

Chapter 4 discusses current regulatory environment calling for more stringent testing of botanicals for the evaluation of their safety and risk, a perspective on the proper evaluation of risks presented by phytochemicals used as medicine, and how *in silico* ADME/Tox screening can be applied to the safety evaluation by regulators and manufacturers of botanical dietary supplements, as it has been for food additives, environmental toxins, and pharmaceuticals. This

strategy offers an opportunity for cost-savings, and reduced and conscientious use of animal testing,

Chapter 5 evaluates the ability of these compounds to form reactive metabolites, the ability of parent compounds and metabolites to cause CYP enzyme inhibition, and for other structural features that may be related to toxic liabilities. This is accomplished through the use of Simulation's Plus ADMET Predictor software designed for ADME/TOX (PK) screening.

At the end of this work, it is hoped that perspective on the chemistry, biological activity, and potential risk profile of this particular botanical formulation is gained, through the understanding of pharmacognosy.

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Rare Cyclic Guanidine Alkaloids from *Iresine celosia*

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Abstract: Among eleven compounds isolated from the plant, two new, structurally rare cyclic guanidine alkaloids have been isolated and characterized from the aerial portion of *Iresine celosia*. The structures of **1** and **2**, celosiadine A and B, were confirmed by 1D and 2D NMR spectroscopy as well as HRESIMS. Since a commercial formulation of the botanical is currently consumed by humans for the purpose of promoting prostate health, compounds isolated from the plant were screened *in silico* for their ability to bind to the human androgen receptor (AR). Compounds **1** and **2** showed favorable binding affinity (-6.6 and -6.8 kcal/mol respectively) in wild type AR and mutant AR T877A and H874Y crystal structures that were then evaluated for their activity *in vitro* against androgen-independent prostate cancer cell line PC3, and the androgen-sensitive prostate adenocarcinoma cell line LNCaP. Preliminary *in vitro* work confirmed findings from the *in silico* docking experiments. Statistically significant changes were observed in LNCaP cells following 24 hour exposure at concentrations ranging from 12.5 - 50 μ M were observed, while PC3 cells remained almost entirely unaffected by the compounds, suggesting that the compounds isolated from this formulation could have therapeutic implications in androgen-dependent prostate cancers.

Introduction:

For centuries in Asia, Africa and India, phytotherapy has been considered a first line treatment for conditions of the prostate, and in the United States and Europe, the number of people using medicinal plants for prostate related conditions, including prostate cancer, has been on the rise [1]. *Iresine celosia*, a member of the family Amaranthaceae, is native to the Southeastern United States, Mexico, Central and South America, and the West Indies. The plant has 14 or more botanical synonyms [2-5], and has been previously investigated for its phytochemistry under the name *Iresine celosiodies* [6-11], and more recently under the name *Iresine diffusa* [12]. It has a history of use as a remedy by indigenous peoples, including the Highland Mayans of Chiapas Mexico, who referred to the plant as tlatlancuaya [9]. A variety of anecdotal indications accompany the traditional use of the plant, including those for skin conditions like dropsy, gonorrhea and malaria. A formulation of the aerial portions of the plant was developed in the 1960s and has since been promoted for a variety of applications, including epilepsy, as an antioxidant, and for promoting health in tumorigenic conditions at large, especially of the prostate [33]. This formulation, comprised of a 30% ethanol extract of the aerial portions of the plant, is currently consumed by humans. However, the chemical constituents of this formulation are not known. Herein, the isolation and structure elucidation of two new guanidine alkaloids, celosiadine A and B (**1**, **2**), along with the purification of hydroxygalegine (**3**), and eight other compounds from the formulation and aerial portions of the plant are reported (Fig. 2).

As a class, guanidine compounds are relatively rare as secondary metabolites, and are found more commonly in marine sponges than in higher

plants. They possess a wide range of biological activity and impressive chemical structures [13, 14]. To our knowledge, this is the first reporting of guanidine alkaloids in the Amaranthaceae family, the second reporting within the Caryophyllales order, and the second ever reporting of cyclic guanidine alkaloids isolated from a plant source [15]. Cyclic guanidine alkaloids have been found as the products of secondary metabolism in sponges. Sponge-derived guanidine alkaloids, and imidazoles in particular, are well known for their anti-cancer properties [16]. Thus, as the formulation is used for promoting health in tumorigenic conditions, especially that of the prostate, further biological experiments were conducted to evaluate the therapeutic potential of the compounds.

In silico docking experiments have previously helped to elucidate the anti-cancer activity of plant natural product compounds against prostate cancer cell lines expressing the T877A mutation [17, 18]. Small molecule plant natural products like epigallocatechin gallate from green tea (*Camellia sinensis*) and atraric acid from *Pygeum africanum* have been found to act as anti-androgens, both active at an *in vitro* concentration of 10 μ M. In particular, atraric acid has demonstrated the ability to inhibit transport of the AR to the nucleus, and has been shown to efficiently repress the growth of androgen-dependent LNCaP prostate cancer cells, but not androgen-independent cell lines, like PC3 [19]. Therefore, to test whether compounds isolated from *Iresine celosia* could act in a similar fashion, Compounds **1** and **2** were screened *in silico* for their ability to bind the androgen receptor crystal structures for both wild type and prostate cancer-related mutants.

Compounds **1** and **2** were screened *in silico* for their ability to bind to the crystal structures of AR wild type (PDB ID: 2YHD), and two mutant varieties relevant to the progression of prostate cancer with crystal structures available in the Protein Databank, T887A (PDB ID: 1I37) and H874Y (PDB ID: 2Q7K) [20, 21]. Binding affinities resulting from docking experiments were compared with the AR's natural ligands, testosterone and dihydrotestosterone (DHT), known non-AR binders beta-sitosterol and stigmaterol, known AR-binding small molecule natural products EGCG and atraric acid, and compounds implicated in BPH-related prostate health: apigenin, emodin, baicalein, genistein, icaritin, xanthohumol (see Tables 7-9) [22]. The lab constituted *Iresine celosia* formulation extract, and pure Compounds **1** and **2**, were then evaluated for their ability to inhibit prostate cancer cell growth as compared to control, using LNCaP prostate cancer cells, that express the AR mutant T877A, and PC3 AR-independent cell lines.

Figure 2: Structures of Compounds Isolated from *Iresine celosia*

Results and Discussion:

In total, eleven compounds were isolated from *Iresine celosia* (Fig. 2). Alongside two novel cyclic guanidine alkaloids, celosiadine A and B, compounds (1) (25 mg) and (2) (5.7 mg) respectively, a third known guanidine containing alkaloid, hydroxygalegine, was isolated, compound (3) (2.3 mg). The 30% ethanol extract of the plant also possesses a number of nucleosides, among them, 2'-deoxy-uridine (7) (3.5 mg), 2'-deoxy-thymidine (8) (13 mg), uracil (4) (0.7 mg) and adenine (5) (2.8 mg), along with some additional small molecule structures, 3-indole-carboxylic acid (6) (2.8 mg), anisic acid (10) (1.8 mg), and phenylacetamide (11) (3 mg). The identity of these structures was assigned based on analysis of the ^1H spectra and the ^{13}C NMR spectra and in accord with assigned values from the literature.

Biosynthesis

The biosynthesis of the two new compounds, celosiadine A and B, could be explained by the isolation of a third, known compound whose biosynthesis has been investigated through the use of radiolabeled feeding experiments. Alongside celosiadine A and B, or compounds (1) and (2) respectively, 4-hydroxygalegine (3) was also isolated. Its parent compound galegine (1-guanidino-3-methyl-2-butene) is a guanidine derivative found together with hydroxygalegine in the seeds of Goat's Rue, *Galega officinalis*. Galegine is synthesized in the shoot of the plant and is found to accumulate in the seeds. Surprisingly, its isoprenoid hydrocarbon chain does not arise from the mevalonic acid-isopentenyl pyrophosphate sequence of terpenoid biosynthesis. Rather, a series of radiolabeling experiments conducted between 1965-1968 by Reuter *et al.* showed that the guanidino group is added by

a transamidation of an amidino group of arginine [23-26]. More specifically, ^{14}C -radiolabeled arginine and its biosynthetic precursor, ornithine, were both applied to young seedlings of *Galega officinalis*. Pyruvate was found to be a precursor of the galegine compound, but mevalonic acid was poorly incorporated. Later, when ^{14}C -amidino labeled guanidino-acetic acid hydrochloride was added to seedlings of *Galega officinalis*, 90% of radioactivity was found in the amidino group of galegine, suggesting that guanidine-acetic acid is the biosynthetic precursor of galegine. Thus, it is likely that related compounds **1** and **2** are biosynthesized in a similar manner (see Figure 4).

A simple rearrangement of the hydroxygalegine molecule could allow for a cyclization of the compound between the double bond and the free amine. Double bond isomerization could account for the imidazoline ring. The primary alcohol at the end of the alkyl chain off of the now cyclized imidazoline ring could be oxidized to form a carboxylic acid. Condensation of the cyclized compound with the alkyl moiety of an additional guanidinoacetic acid molecule could be responsible for the additional side chain seen in Celosiadine B. Nucleophilic attack of an alcohol group from water results in the alkene isomerization of the double bond into the terminal alkene seen in Celosiadine A.

Figure 4. Proposed biosynthesis of Celosiadine A and B from Hydroxygalegine

Spectral Analysis

Compound (**1**), 2-[2-Amino-1-(2-hydroxy-3-methyl-3-butenyl)-4-imidazoliny]propionic acid, a light yellow amorphous solid, was assigned the name Celosiadine A, and assigned the formula $C_{11}H_{19}N_3O_3$ based on HRESIMS data at m/z 242.1500 $[M+H]^+$ (calcd $C_{11}H_{19}N_3O_3$, for 242.1499). The IR absorptions revealed the presence of peaks at 3380 cm^{-1} and 1700 cm^{-1} , indicating the carboxylic acid functionality; a broad band ($3400\text{-}2000\text{ cm}^{-1}$) indicated N-H, and an additional band at 1600 cm^{-1} supported the presence of the guanido group.

Detailed analysis of the 1D and 2D NMR (^1H - ^1H COSY, HSQC, HMBC) data allowed for the construction of the structure of Compound (**1**). The ^1H and the ^{13}C NMR spectra (Table 4) characteristic of a guanidine moiety (δ_C 158.75), a carboxylic acid group (δ_C 175.60), a quaternary carbon (δ_C 145.93) of a terminal vinyl group (δ_C 112.16), also indicated by the presence of two singlets at 5.07 and 4.93 (H-1) that integrated for one proton each. In addition, two methyls, two other methylenes, and three methines were detected.

Table 4. Spectral Assignments for Compound (1)

<u>Position</u>	<u>δ C (mult.)</u>	<u>δ H (mult., J in Hz)</u>	<u>COSY</u>	<u>HMBC</u>
1	112.16, 2H	5.07, 4.93, s	H-3, H-5	C-5, C-3
2	145.93			H-5, H-4, H3, H1
3	71.29, 1H	4.22, dd (7.8, 3.9)	H-1, H-4	C-5, C-4, C-2, C-1
4	48.70, 2H	3.42, dd (15.1, 3.9)	H-3	C-3, C-2, C-4', C-3'', C-2''
		3.38, dd (15.1, 7.8)	H-3	C-3, C-2, C-4', C-3'', C-2''
5	18.67, 3H	1.77, s	H-1, H-3	C-3, C-1
6		8.42, s		
3'		7.87, s		
4'	158.75			H-4, H-4'', H-3''
1''	12.92, 3H	1.18, d (7.8)	H-2''	C-5'', C-3'', C-2''
2''	43.79, 1H	2.59, m	H-3'', H-1''	C-5'', C-3'', C-1''
3''	54.54, 1H	4.11, m (10.1, 6.6)	H-4'', H-2''	C-5'', C-4'', C-2'', C-1''
4''	52.60, 2H	3.92, dd (10.1)	H-3''	C-4', C-3'', C-2''
		3.55, dd (10.1, 6.6)	H-3''	C-4', C-3'', C-2''
5''	175.60			
6''		12.51, s

There were ^1H - ^1H COSY correlations between H-5/H-1/H-3/H-4, and between H-4"/H-3"/H-2"/H-1". In the HMBC spectrum, the guanidine carbon signal at δ_{C} 158.75 showed correlations with protons δ_{H} 3.92 and 3.55 (H-4), and δ_{H} 3.42 and 3.38 (H-4"), and δ_{H} 4.11 (H-3"). The carboxylic acid group at δ_{C} 175.60 had correlations with protons δ_{H} 4.11 (H-3") 2.59 (H-2") and 1.18 (H-1"). The quaternary carbon at δ_{C} 145.93 correlated with protons δ_{H} 5.07 and 4.93 (H-1), δ_{H} 4.22 (H-3), 3.42 and 3.38 (H-4), and δ_{H} 1.77 (H-5). The terminal alkene at δ_{C} 112.16 was shown to correlate with protons δ_{H} 4.22 (H-3) and 1.77 (H-5). Also, the carbon at the secondary alcohol, δ_{C} 71.29, revealed correlations with protons δ_{H} 5.07 and 4.93 (H1), and 3.42 and 3.38 (H-4). For key HMBC (H $\rightarrow$ C) and ^1H - ^1H COSY correlations, see Figure 3.

The relative configuration of stereocenters at C-3" and C-2" was determined by 1D and 2D NOESY experiments, with emphasis on couplings at protons C-4" to protons at these stereocenters (see Figure 14-15). The proton H-4" at δ_{H} 3.92 showed the effect of being coupled to H-3" at δ_{H} 4.11, while proton H-4" at δ_{H} 3.57 showed the effect of being coupled to the H-2" at δ_{H} 1.18. This information provided information about the relative configuration of these protons with respect to each other, but could not be extrapolated to provide information on the absolute stereochemistry of these stereocenters. In addition, the proton H-1 at δ_{H} 4.93 was found to be coupled to H-5 at δ_{H} 1.77, while H-1 at δ_{H} 4.93 was coupled to H-3 at δ_{H} 4.22, providing further confirmation of the assignment of the terminal alkene to its position on the alkyl chain substituent branching off from the imidazoline ring.

Chiral derivatization of the stereocenter at the secondary alcohol present at C-3 using Mosher's esterification failed, perhaps due to the conformational flexibility and rotatability of both the imidazoline ring's side chains. With three stereocenters, eight stereoisomers are possible, and it is possible that all eight may

be present when isolated from the crude botanical mixture. Attempts to crystalize the compound were also unsuccessful. To resolve the absolute configuration of stereocenters present within Compound (**1**), theoretical calculation of electronic circular dichroism (ECD) by time-dependent density functional theory (TDDFT) will be used. This method has previously been used to determine the absolute configuration of highly flexible compounds, including alkaloids, as well as other conformationally rigid natural product molecules [51].

**Figure 3. Key $^1\text{H} \rightarrow ^1\text{H}$ COSY (bold lines) and HMBC ($\text{H} \rightarrow \text{C}$)
(arrows) Correlations for Compound (1)**

Table 5. Spectral Assignments for Compound (2)

<u>Position</u>	<u>δ C (mult.)</u>	<u>δ H (mult., J in Hz)</u>	<u>COSY</u>	<u>HMBC</u>
1	18.17, 3H	1.73, s	H-4	C-5, C-3, C-2
2	138.45			H-5, H-4, H-1
3	117.42			H-5, H-4, H-1
4	41.87, 2H	3.95, dd (7.1)	H-6, H-1	C-3, C-2, C-4', C-4''
5	25.86, 3H	1.79, s	H-4	C-3, C-2, C-1
6		5.18, t (7.1)	H-4	C-5, C-1
3'		8.05, s		
4'	159.14			H-4, H-4''
1''	12.87, 3H	1.14, d (7.1)	H-2''	C-5'', C-3'', C-2''
2''	43.73, 1H	2.56, m	H-3'', H-1''	C-5'', C-3'', C-1''
3''	54.53, 1H	4.11, m (10.1, 6.3)	H-4'', H-2''	C-5'', C-4'', C-1''
4''	50.88, 2H	3.72, dd (10.1)	H-4'', H-3''	C-3'', C-2''
		3.36, dd (10.1, 6.3)	H-4'', H-3''	C-3'', C-2''
5''	175.59			H-2'', H-1''
6''		12.69, s

Compound (**2**), 2-[2-Amino-1-(3-methyl-2-butenyl)-4-imidazoliny]propionic acid, a light yellow powder, is chemically related to compound **1**, with a molecular formula of $C_{11}H_{19}N_3O_2$ as determined by HRESIMS at m/z 226.1547, $[M+H]^+$ (calcd for $C_{11}H_{19}N_3O_2$, 226.155). The NMR chemical shifts of compound (**2**), presented in Table 5, were similar to that of Compound (**1**), except for differences in the carbon chemical shifts of C-1 (δ_C 18.17) and C-3 (δ_C 117.42), reflecting a change in the placement of the double bond between the two compounds. The IR spectrum of Compound (**2**) was similar to Compound (**1**), in that it revealed the presence of peaks at (3500-3350) and 1700, indicating the carboxylic acid functionality; 3400-2000 broad indicated N-H, and an additional band at 1600 supported the presence of the guanido group, with slight variation with the peak at 1000 cm^{-1} more pronounced in compound (**2**) than in the fingerprint region from its related compound (**1**).

The structure of compound (**3**), hydroxygalegine, was determined through analysis of the 1D and 2D NMR experiments (see Table 6) and comparison with the literature [27]. The isolation of this compound helped to support rationale for the proposed biosynthesis of compounds (**1**) and (**2**) as deriving from arginine.

In addition, eight other compounds were isolated from the butanol fraction of the 30% ethanol extract of the plant. Among them were nucleosides 2'-deoxyuridine (**7**), 2'-deoxy-thymidine (**8**), uracil (**4**), and adenine (**5**) [52]. Three other small molecule structures, 3-indole-carboxylic acid (**6**), anisic acid (**10**), and phenylacetamide (**11**) were also isolated and confirmed by comparison with NMR spectra from the literature [53-55].

**Table 6. Spectral Assignments for Compound (3),
Hydroxygalegine**

Position	δ C (mult.)	δ H (mult., J in Hz)	COSY	HMBC
2	156.34			H-5
5	45.5, 2H	4.09, d (7.1)	H-10, H-6	
6	119.52, 1H	5.31, t (7.1)	H-10, H-5	H-8, H-5. H-10
7	140.28			H-8, H-5. H-10
8	60.53, 2H	4.12, s		H-6, H-10
10	20.97, 3H	1.81, s	H-6, H-5	H-6, H-8

Compounds (1) and (2) were screened *in silico* for their ability to bind to the crystal structures of AR wild type (PDB ID: 2YHD), and two mutant varieties relevant to the progression of prostate cancer with crystal structures available in the Protein Databank, T887A (PDB ID: 1I37) and H874Y (PDB ID: 2Q7K) [20, 21]. Binding affinities resulting from docking experiments were compared with the AR's natural ligands, testosterone and dihydrotestosterone (DHT), known non-AR binders beta-sitosterol and stigmaterol, known AR-binding small molecule natural products EGCG and atraric acid, and compounds implicated in BPH-related prostate health: apigenin, emodin, baicalein, genistein, icaritin, xanthohumol (see Tables 7-9) [22]. Compound (1) and (2) were found to have binding affinities comparable to atraric acid (-6.1, -6.3, -6.8 kcal/mol in 2YHD, 1I37 and 2Q7K respectively), and EGCG (-6.8, -4.2, -6.0 kcal/mol in 2YHD, 1I37 and 2Q7K respectively) but did not bind as well as natural ligand testosterone (-11.2, -11.2, -11.8 kcal/mol in 2YHD, 1I37 and 2Q7K respectively) or DHT (-10.9, -10.2, -11.3 8 kcal/mol in 2YHD, 1I37 and 2Q7K respectively). The top three modes of each compound docked are listed in Tables 7-9.

Because of promising *in silico* results, we sought to evaluate the ability of celosiadine A and B to inhibit the growth of prostate cancer cell lines *in vitro*. Testing of compounds (1) and (2) in *in vitro* prostate cancer cell assays supported *in silico* screening results by inhibiting the growth of AR-sensitive prostate cancer cells. Compound (1) and (2) were tested at 12.5, 25, and 50 μ M concentrations. The butanol extract was tested at the 50 and 250 ppm concentration. Exposure time was for 6 hours and 24 hours. Statistically significant changes in the LNCaP, AR-sensitive cell line, were observed following 24 hours of exposure from the compounds as well as the crude extract from which they were derived. This effect

was not observed in the PC3, androgen-independent prostate cancer cell lines (Fig. 34, 35, 36).

These results suggests that celosiadine A and B may be the health relevant principles responsible for the use of *Iresine celosia* in promoting prostate health, and that their mechanism of action may be androgen-receptor specific. Further testing is warranted to determine the ability of these compounds to bind to the AR *in vitro*, and their ability to act in an anti-androgenic mechanism, as is seen with other plant-derived compounds like EGCG and atraric acid. These non-steroidal compounds work by preventing translocation of the AR to the nucleus, and thus preventing the transcriptionally active conformation of the AR from turning on genes relevant to the proliferation and differentiation of prostate cancer. Because antiandrogen therapies are often later converted by the body into androgen receptor antagonists, resistance to antihormone therapy can be a major problem for long-term treatment of prostate cancer. Should celosiadine A and B prove effective as antiandrogens, they could add to the current armament of therapies a structurally novel and natural aid to help promote prostate health.

EXPERIMENTAL SECTION

General Experimental Procedures: Silica gel (230–400 mesh, Sorbent Technologies) and Sephadex LH-20 gel (Amersham Biosciences) were used for column chromatography, and pre-coated silica gel GF254 plates (Whatman Ltd., Maidstone, England) were used for TLC analysis. Semi-preparative HPLC separations were performed on a Hitachi Elite LaChrom system consisting of an L2130 pump, an L36 2200 auto-sampler, an L-2455 diode array detector and a Phenomenex Luna C18 column (250 ~ 10 mm, S-5 μ m), all operated by EZChrom

Elite software. The UV spectra were measured on a Shimadzu UV-2550 UV–visible spectrophotometer. All solvents were of ACS- or HPLC-grade and were obtained from Sigma-Aldrich (St. Louis, MO, USA) through Wilkem Scientific (Pawtucket, RI, USA). 1D and 2D NMR data were recorded on a Varian 500 MHz instrument with dimethyl sulfoxide- d_6 as solvent and TMS as internal standard. NOESY experiments were conducted using a three-pulse sequence with a mixing time of 400 milliseconds and 700 milliseconds. 1D NOE experiments were conducted with pulse width of 90 degrees, a relax delay of 1, 512 scans with a mix time of 500 mms and a block size of 16. HRESIMS data were acquired using an LTQ Orbitrap XL mass spectrometer (Thermo Scientific). Optical rotations were measured on an Auto Pol III automatic polarimeter (Rudolph Research, Flanders, NJ, USA) at room temperature. The IR spectra were recorded on a Bruker Tensor 27 FT-IR and analyzed using OPUS Data Collection and Analysis software.

Plant Material: Three kilos of the aerial portions of the *Iresine celosia* plant were collected in Masaya, Nicaragua in September of 2008 and kindly provided to our laboratory by Iresine International, Inc. (Miami, FL, USA). The plant was authenticated by Edda Contreras (Iresine International, Inc., Miami, FL) and Mr. Peter Morgan, Master Gardener (University of Rhode Island College of Pharmacy, Kingston, RI), and a voucher specimen (16CK37-IRE21712H) has been deposited at the University of Rhode Island, College of Pharmacy, Medicinal Plant Greenhouse.

Extraction and Isolation: In order to reproduce the commercial formulation, comprised of 30% ethanol, in the laboratory, air-dried aerial portions (2.0 kg) of *Iresine celosia* were extracted by maceration with 30% ethanol (16 L, 3 times for

7 days per time period of 21 days) at room temp. 9 L of the aqueous solution was recovered and dried to 1.2 mL through rotary evaporation, and then extracted successively with *n*-Hexanes (1.2 L, 3 times), ethyl acetate (1.2 L, 3 times), and *n*-butanol (1.2 L, 3 times) successively. The *n*-butanol fraction (8.93g) was purified through gravity column chromatography using silica gel (CHCl₃-MeOH, 19:1 v/v to 1:1 v/v) to yield four major fractions (A-F). Fraction A was further purified using Sephadex LH-20 eluted with MeOH affording four fractions (20B-G). Fraction 20E was separated by semi-preparative HPLC, eluted with MeOH-H₂O with 0.1% TFA (10:90 v/v to 95:5 v/v in 25 min, 2 mL/min) to yield compounds **7**, **8**, **9**, and **11**. Fraction 20F was similarly separated by semi-preparative HPLC, eluted with MeOH-H₂O with 0.1% TFA (10:90 v/v to 100:0 v/v in 30 min, 2.5 mL/min) to yield compounds **4** and **10**. Fraction 20G was also separated by semi-preparative HPLC, eluted with MeOH-H₂O with 0.1% TFA (10:90 v/v to 95:5 v/v in 25 min, 2 mL/min) to yield compounds **5** and **6**. Fraction E was further separated using Sephadex LH-20 eluted with MeOH to afford two main fractions (24A and 24B). Fraction 24B was separated by semi-preparative HPLC, eluted with MeOH-H₂O (10:90 v/v to 100:0 v/v in 27 min, 3 mL/min) to yield compounds **1**, **2**, and **3**.

Compound (**1**): light yellow amorphous solid; $[\alpha]_D^{20} = +3.4^\circ$ (MeOH); IR $\nu_{\max}$ 3380, 1700, 1600 cm⁻¹; ¹H and ¹³C NMR data, see Table 1; HRESIMS m/z 242.1500 [M+H]⁺ (calcd C₁₁H₁₉N₃O₃, for 242.1499).

Compound (**2**): a light yellow powder; $[\alpha]_D^{20} = +10^\circ$ (MeOH); IR $\nu_{\max}$ 3380, 1700, 1600 cm⁻¹; ¹H and ¹³C NMR data, see Table 5; C₁₁H₁₉N₃O₂ as determined by HRESIMS at m/z 226.1547, [M+H]⁺ (calcd C₁₁H₁₉N₃O₂, for 226.155).

Compound (**3**): ^1H and ^{13}C NMR data, see Table 6 and were compared with previously reported values in the literature [27].

***In silico* Molecular Modeling Study:** Crystal structures of the wild type human Androgen Receptors (PDB ID: 2YHD), the T877A mutant human AR (PDB ID: 1I37) and the H874Y mutant human AR (PDB ID: 2Q7L) were obtained from the Protein Data Bank (PDB) as a template for docking of compounds isolated from *Iresine celosia* [28]. Receptor macromolecules were checked for completeness and accuracy by overlapping with other known, reported androgen receptor crystal structures using Chimera [29]. Structures were cleaned and split from ligands with which they were crystalized, and residual water removed using the Accelrys Discovery Studio 3.5. Autodock Tools (version 1.5.4) was used to add all hydrogens, including non-polar, Kollman charges, and solvation parameters [30]. After adding charges, all non-polar hydrogens were merged. Autogrid, a program included within Autodock Tools, was used to generate grid map size parameters, which were then incorporated into configuration files used for docking in Autodock Vina, with grid coordinates centered on the known ligand binding domain. Grid box dimensions were set to 24.051 x 1.363 x 5.150, adjusting the spacing between the grid points to be 1 Å.

ChemDraw3D was used to create protein databank (.pdb) files for all compounds used in docking experiments. Ligands were further prepared, with Gasteiger charges assigned, all non-polar hydrogens merged, and the number of torsions set using Autodock Tools. All bond rotations for ligands were automatically set in ADT using the Lamarckian generic algorithm (LGA). The exhaustiveness number was set to 16 and the solutions for each docked ligand were evaluated through analysis of the text files containing binding affinity data

(kcal/mol) and root mean square deviations (RMSDs), and inspection of .pdbqt files to assess physical configuration of docked molecules and reproducibility among docking modes. Batch files used to script automated docking procedures were prepared using the text editing program TextWrangler. Docking was conducted using Autodock Vina.

Known non-androgen receptor binders stigmasterol and beta-sitosterol were used as negative controls, while natural androgen receptor ligands testosterone and 5 α -dihydrotestosterone (5 α -DHT) were used as positive controls for docking studies [31]. In addition, a set of natural product structures associated with prostate health, including apigenin, emodin, baicalein, genistein, icaritin, xanthohumol, and known AR-binders atraric acid and EGCG were used as a set for comparison [22].

Prostate Cancer Assay:

The cellular cytotoxicity of the *Iresine celosia* extract and isolated compounds celosiadine A and B was evaluated using the Alamar blue assay (Invitrogen, Burlington, ON) as per manufacturer's instructions. The Alamar blue assay measures cellular metabolic activity (and thus cellular viability) via the reduction of a nonfluorescent redox indicator to a fluorescent product by viable cells. The reduction of Alamar blue reagent by *Iresine celosia* extract-treated, compound (1)-treated, and compound (2)-treated cells were compared to that of vehicle control-treated cells.

Human LNCaP and PC3 prostate adenocarcinoma cells (ATCC, Manassas, VA) were cultured on 100 mm plastic tissue culture dishes (Falcon, Mississauga, ON) in alpha-MEM supplemented with 1% (v/v) antibiotic/antimycotic

(Invitrogen, Burlington, ON) and 10% (v/v) fetal clone III bovine serum (Hyclone/VWR Canlab, Mississauga, ON) and were grown at 37°C in 5% CO₂.

Compounds (1) and (2) were tested at 12.5, 25, and 50 µM concentrations. The butanol extract was tested at the 50 and 250 ppm concentration. Exposure time was for 6 hours and 24 hours.

The cellular cytotoxicity of *Iresine celosia* extract was evaluated using the Alamar blue assay (Invitrogen, Burlington, ON) as per manufacturer's instructions. The Alamar blue assay measures cellular metabolic activity (and thus cellular viability) via the reduction of a non-fluorescent redox indicator to a fluorescent product by viable cells. The reduction of Alamar blue reagent by the individual compounds and extract-treated and fraction-treated cells was compared to that of vehicle control-treated cells.

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ASSOCIATED CONTENT

Supporting information. The 1D and 2D NMR spectra and mass spectrometry data for compounds (1) and (2) are available free of charge via the Internet at <http://pubs.acs.org>.

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High-Performance Liquid Chromatography Characterization of New, Species-Specific Guanidine Alkaloid Marker Compounds in the Botanical Dietary Supplement *Iresine celosia*

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This chapter is written in the style of the Pharmaceutical Biology journal.

Abstract

Iresine celosia is the sole active ingredient of the Odyliresin™ dietary supplement formulation currently marketed to humans worldwide as an antioxidant for the promotion of prostate health. Like many botanical dietary supplements, the ingredient lacks authentication methods to characterize its phytochemistry. This work presents an analytical method for high-performance liquid chromatography (HPLC) and development of an analytical finger print profile that can be used to standardize the *Iresine celosia* botanical extract. Of the eleven compounds isolated from this plant and identified on the fingerprint chromatogram, the appearance of two new cyclic guanidine alkaloid compounds unique to this botanical specimen are among the most abundant and serve as important marker compounds for the extract. In this study, a method for the quantitative analysis of the two new guanidine alkaloids (GAs) present in the *Iresine celosia* botanical extract formulation was developed, celosiadine A and B, or compounds (1) and (2) respectively. A lab-produced formulation of the aerial plant material was extracted with 30% ethanol and subject to sequential liquid-liquid partitioning with n-hexanes, ethyl acetate, and n-butanol. Samples of the manufacturer's Odyliresin™ formulation were also subject to the same sequential partitioning. Butanol fractions, Lab-bu and Odyli-bu, were analyzed and the GAs quantified by HPLC. Calibration curves were tested in triplicate and showed good linearity ($r^2 > 0.99$) within the tested ranges and the concentrations of these compounds in the extracts were quantified based on the standard curves. The Lab-bu fraction appeared to have almost ten times as much of compounds (1) and (2) as the Odyli-bu fraction, with compound (1) comprising 0.032% of the Lab-bu formulation, and 0.0032% of the Odyli-bu formulation, and compound (2) comprising 0.4% of the Lab-bu formulation, and 0.04% of the Odyli-bu formulation respectively. Identification of relevant marker compounds, analytical method development, and analytical finger print profiling can help to address the deficiency of knowledge

needed for botanical dietary supplement formulations like Odyliresin™ to achieve a level of quality production and safe use.

Introduction

Analytical method development for botanicals used by humans in the form of dietary supplements has been identified as an important area of research by a variety of regulatory bodies, including the U.S. Congress, National Institutes of Health (NIH), Food and Drug Administration (FDA), National Institute of Standards and Technology (NIST), clinical researchers, including those seeking funding from the National Center for Complementary and Alternative Medicines (NCCAM), product manufacturers, and other industry stakeholders who seek to ensure that consumers have access to quality dietary supplement products [56-58]. Current good manufacturing practice (cGMP) regulations for dietary supplements require manufacturers to demonstrate that “specifications are met for the identity, purity, strength, and composition of the dietary supplements” [59]. However, regulations do not instruct manufacturers about particular analytical methods required to meet these stipulations. Rather, each botanical component requires a specific, scientifically valid authentication method in order to provide the necessary proof to comply with regulations.

In particular, analytical separation techniques like high performance liquid chromatography (HPLC) currently provide the most reliable and applicable authentication methods for botanicals. To accomplish this, however, it is necessary to isolate and identify selected “marker” compounds that make up an analytical fingerprint that is distinct for the selected species. Especially for less widely known botanicals, this work requires extensive phytochemical investigation of the plant, and usually includes the development of tailored isolation schemes, compound purification, and structure elucidation experiments, alongside the development of analytical methodology fitting to the botanical specimen to standardize extracts.

Iresine celosia is a member of the Amaranthaceae family. The plant is native to Central America, Mexico and southern portions of the United States like Texas and Florida [60]. It has a history of use as a traditional medicine by the Mayan people of Chiapas Mexico, who used this plant for a variety of conditions, among them malaria, gonorrhea, and dropsy [35, 36, 38-40, 42, 43, 61]. To them, it was known as *tlatlancuaya*, although this name has been used to describe other botanical medicines in the region. In addition, the plant also has over 27 botanical synonyms [34-36, 38, 60]. Because of the many names ascribed to the plant, there is added utility to the development of chemical reference standards as this information can also be used in the taxonomical identification of the plant and products made therefrom.

Today, *Iresine celosia* is best known for its use as the sole active ingredient in the Odyliresin™ Antioxidant botanical formulation, used for the promotion of prostate health in humans [33]. The product is produced in a 30% ethanolic aqueous extract of the aerial portions of the plant. Although produced on small (1L or less) scale in single batches, cGMP regulations apply also to small scale manufacturers of dietary supplements like the makers of the Odyliresin™ Antioxidant botanical formulation. This work represents the first phytochemical investigation of this formulation resulting in the identification of marker compounds and their use in analytical profiling of the formulation.

Materials and Methods

Plant Material

Aerial portions of the *Iresine celosia* plant were collected in Masaya, Nicaragua in September of 2008 and kindly provided to our laboratory by Iresine International, Inc. (Miami, FL, USA). The plant was authenticated by Edda Contreras (Iresine International, Inc., Miami, FL) and Mr. Peter Morgan, Master Gardener (University of Rhode Island College of Pharmacy, Kingston, RI), and a voucher specimen (16CK37-IRE21712H) has been deposited at the University of Rhode Island, College of Pharmacy, Medicinal Plant Greenhouse. In addition, three Odyliresin™ Antioxidant, 50 ml dispenser bottles were provided by the manufacturers from one batch of product, the only available at the time.

HPLC Conditions

Semi-preparative HPLC separations were performed on a Hitachi Elite LaChrom system consisting of an L2130 pump, an L36 2200 auto-sampler, an L-2455 diode array detector and a Phenomenex Luna C18 column (250 ~ 10 mm, S-5 µm), all operated by EZChrom Elite software. The UV spectra were measured on a Shimadzu UV-2550 UV–visible spectrophotometer. All solvents were of ACS- or HPLC-grade and were obtained from Sigma-Aldrich (St. Louis, MO, USA) through Wilkem Scientific (Pawtucket, RI, USA). A linear gradient chromatographic technique was used at room temperature with the following solvent system: solvent A = 0.1% trifluoroacetic acid with filtered, deionized water; solvent B = methanol; starting at 10% A:90% B and rose via a gradient to 40% A:60% B at 30 minutes. From 30 min to 35 min, solvent A 0%:100% B and continued to 40 minutes. From 40 to 42 minutes, 10% A:90% B and run to 52

minutes. The flow rate was 0.75 mL/min throughout. Detection was monitored via diode array detector between 200 nm - 520 nm wavelength. All HPLC-UV analyses were carried out with 15 μ L injection volumes. Compounds (1) and (2) were monitored at 212 nm. In addition, compound (3) was monitored at 210, (4) at 256 nm, (8) at 264 nm, and (10) at 260 nm. The concentration of these compounds was quantified based on the standard curves.

Preparation of extracts

For analysis by HPLC

Lab-Neat formulation: Following manufacturer's production instructions, a 30% ethanolic extract (16 liters) was added to cover and saturate 2 kilos of dried plant material, and set aside for three weeks with occasional (apx. every 72 hours) stirring. 9 liters of the ethanolic extract were recovered, dried to 1.2 L through *in vacuo*, and sequentially partitioned with n-hexanes, ethyl acetate and n-butanol.

Odlyi-Neat, Manufacturer's formulation: 15 mL of the formulation neat, with ethanol removed and dried down to 10 mL, was subject to liquid-liquid partitioning sequentially with n-hexanes, ethyl acetate and n-butanol. The resultant n-butanol fraction was dried *in vacuo* and resulted in a 96.4 mg residue. Dried residues of these products were weighed and reconstituted to the appropriate concentration using HPLC-grade methanol.

Lab-bu and Odlyi-bu test samples were generated by bringing up from dry weight in 30% HPLC-grade methanol and 70% DI-water a 250 mg/mL stock concentration, from which 150, 100, 75, 50, 25, and 10 mg/mL concentrations were derived. In addition, 500 mg/mL concentrations were made separately and also tested. Samples were centrifuged prior to injection in HPLC, and all samples were injected with a 15 μ L injection volume. Each sample was injected in triplicate and a linear calibration curves ($r^2 = 0.99$) constructed by plotting the mean peak area percentage against concentration. The presence of compounds (1)

and (2) was determined with the use of 5-point calibration curves for standard compounds. Stock solutions were stored at 4°C (see Figure 37 -47).

Identification of the isolated compounds

For isolation and identification of standard compounds

Lab-Neat formulation was prepared as described above. The butanol-soluble fraction (Lab-Butanol) was subjected to silica gel gravity column chromatography and eluted with chloroform and methanol (19:1 CHCl₃:MeOH to 1:1 CHCl₃:MeOH), affording four major fractions. Fraction E resulting from 5:1 CHCl₃:MeOH elution was further purified using Sephadex LH-20 resin eluted with methanol. Fractions were evaluated using analytical HPLC.

Semi-preparative HPLC afforded the isolation of marker compounds (1) and (2). In addition, additional purification afforded compounds 3-11. Detailed analysis of the 1D and 2D NMR data allowed for the construction of the structure of Compound (1) and (2). Compounds 3-11 were identified based on ¹H spectra and ¹³C and comparison with prior literature [62]. Details on this work are reported elsewhere (see Chapter 2).

Standard Preparation

Stock solutions of the isolated GA standards, compound (1) and compound (2), were prepared as follows: 1-3 mg standard was accurately weighed. Next, a mixture of 30% HPLC-grade methanol and 70% filtered, deionized water was added and the solution was serial diluted to volume with the same solvent to afford samples of 1.00, 0.500, 0.250, 0.125, 0.0625, 0.03125, and 0.015625 mg/mL concentrations respectively. Each sample was injected in triplicate and a linear five-point calibration curve ($r^2 = 0.99$) was constructed by plotting the mean

peak area percentage against concentration. Each stock solution was stored at 4°C. In addition, compounds **(3)**, **(4)**, **(8)**, and **(10)** were also tested though results were not reproduced in triplicate. Stock solutions of these compounds were prepared at concentrations relevant to each compound, as follows: **(3)** at 3.5, 1.75, 0.875, and 0.4375 mg/mL; **(4)** at 1.9, 0.950, 0.475, 0.238, and 0.119 mg/mL; **(8)** at 0.4, 0.2, 0.1, 0.05, 0.025 mg/mL; **(10)** at 0.55, 0.275, 0.138 mg/mL (see Figures 42-47).

Detection Limit and Quantitation Limit

The limits of detection (LOD) and quantitation (LOQ) were determined based on the standard deviation of the response and the slope, which is one of the LOD and LOQ measurements in the ICH guidelines [63]. The LOD and LOQ were calculated as described as follows:

$$\text{LOD } (\mu\text{g mL}^{-1}) = 3.3 \sigma/S$$

$$\text{LOQ } (\mu\text{g mL}^{-1}) = 10 \sigma/S$$

where σ = the standard deviation of the response, and

S = the slope of the calibration curve.

To calculate σ , a calibration curve of the sample with compound that had a concentration similar to the LOD, LOQ was studied. The standard deviation of the y-intercepts of the regression lines was used as σ . For compound **(1)**, LOD was calculated as 0.13 mg/mL, and LOQ as 0.39 mg/mL (see Table 11). For compound **(2)**, LOD was calculated as 0.18 mg/mL and LOQ as 0.56 mg/mL. LOD and LOQ were not determined for compounds **(3)**, **(4)**, **(8)**, and **(10)**.

Results and Discussion

In the current study, a method was developed to analyze the content of the GAs, compound (1) and (2), novel botanical identifiers and potentially the health-relevant principles of the *Iresine celosia* botanical formulation. Initially, four samples were analyzed: Lab-Neat, a lab-generated 30% ethanolic extract, Odyli-Neat, the manufacturer's own 30% ethanolic extract, and butanol partitions of these fractions, Lab-Butanol and Odyli-Butanol. These samples were analyzed and the GAs quantified by HPLC-DAD. However, because 30% ethanolic crude extract's marker compounds were well below limits of detection in the form sold to patients, and saturated when the material was dried and brought up to detectable concentrations, further purification was required to standardize the extracts. Further purification steps are standard when dealing with complex mixtures, and in this case partitioning with hexane and ethyl acetate and finally butanol was required. With these defatting steps accomplished, the butanol fractions were analyzed for their presence of the two marker compounds.

The content of guanidine alkaloids within the complex botanical formulation of *Iresine celosia* is quite small. Compound (1) comprised 0.032% by weight of the Lab-bu formulation, and 0.0032% of the Odyli-bu formulation, and compound (2) comprised 0.4% of the Lab-bu formulation, and 0.04% of the Odyli-bu formulation. The Lab-bu fraction appeared to have almost ten times as much of compounds (1) and (2) as did the Odyli-bu fraction. Lab-bu fraction at 50 mg/mL concentration contained amounts of compounds (1) and (2) comparable to the Odyli-bu fraction at 500 mg/mL (see tables 12, 13, 18, 19).

Additionally, compounds (3), (4), (8), and (10) hydroxygalegine, uracil, 2'-deoxythymidine, and anisic acid respectively were also quantified in the Lab-bu

formulation, but were below the limits of detection for the Odyli-bu formulation (see Figures 50-53). Compound (3) comprised 0.002% by weight of the Lab-bu formulation, compound (4) comprised 0.004%, compound (8) comprised 0.0008%, and compound (10) comprised 0.001% of the Lab-bu formulation.

The lack of relevant marker compounds is said to be the major limiting factor hindering the widespread adoption of quality control approaches for botanical supplements [30]. To allow for documentation that products meet manufacturer's specifications and that products contain what their labels declare, analytical method development is needed. These methods define a level of quality that can be verified with each batch production.

Most notable among the compounds isolated from the Odyliresin™ botanical formulation are two novel cyclic guanidine alkaloids unique to *Iresine celosia*, the formulation's sole component. The novelty of these molecules within the botanical specimen, and the abundance with which they are found in the formulation among other reasons allow for the use of these compounds as good markers to identify and authenticate both the *Iresine celosia* plant and the Odyliresin™ formulation. In addition, while guanidine alkaloids are found in a handful of plant species, only one other plant is known to possess 2-substituted imidazolines, making them structurally novel identifiers [64-70]. The Odyliresin™ botanical formulation also possess a number of nucleosides (among them, 2'deoxy-uridine, 2'deoxy-thymidine, uracil and adenine), along with some additional small molecule structures (3-indole-carboxylic acid, anisic acid, and phenylacetamide).

Biologically active ingredients that represent the "health-relevant principle components" of the formulation are also preferred as marker compounds [30], although this point is elsewhere contested as unnecessary [56]. *In silico* screening

suggests that the GA marker compounds may also play a role in the biological activity of the extract when used for promotion of prostate health. Docking experiments revealed that the new compounds bind to the human androgen receptor, both wild type and mutant forms relevant in prostate-cancer progression. Further testing in LNCaP AR-sensitive cell lines showed favorable activity of the compounds in the 12.5-50 μ M range, while leaving PC3, AR-independent cell lines otherwise unchanged (see Chapter 2). Like other botanical structures EGCG and atraric acid, these compounds may also act as anti-androgens. Thus, due to their novelty and potential health-relevant component for the species in question, and the relative abundance with which they are found in the plant, these compounds can be used as unique identifiers to the botanical specimen, and could potentially be used to quantify the potency of what is likely the health-relevant principle within the extract.

The isolation of single active principles from botanical natural product dietary supplements like the Odyliresin™ formulation of *Iresine celosia* will allow for further evaluation of this formulation's safety when used in humans and efficacy when used as a remedy to promote health of the prostate, and authentication of the formulation's ingredients using the presence of chemical marker compounds in the commercial product.

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Perspective on the Regulatory and Scientific Evaluation of Botanical Dietary Supplement Safety

Abstract

The release of the Draft Guidance for Industry, Dietary Supplements: New Dietary Ingredient Notification and Related Issues in July 2011 marked a turning point for the dietary supplement industry. The document outlined recommendations for the new dietary ingredient (NDI) submission process, including a battery of pre-clinical tests required for submission prior to introduction of a product into interstate commerce, and with it defined a level of scientific testing heretofore unprecedented in the industry. As regulatory requirements to provide extensive toxicological testing data on botanical dietary supplements increase, without a corresponding increase in resources for companies called upon to provide such data or for regulators of the industry, *in silico* screening of natural product structures presents a resource-conscious strategy that could play a role in the risk assessment of botanical ingredients. Based on knowledge of structural features of compounds from botanical natural products known to be toxic, new compounds for which little or no toxicological data exists could be screened for similar functional groups and structural alerts generated. This information could then be used to guide chemical and toxicological testing resources to where they are needed most, as well as streamline the regulatory review of these products. In this work, a review of regulation of the dietary supplement industry since 1994 was conducted, with emphasis on the development of current thinking on the interpretation of the NDI notification system. A survey of use of *in silico* methodology for chemical risk

assessment is also discussed, as well as how such a method may be applied to botanical natural product structures in particular.

The Regulatory Landscape

In the U.S. marketplace alone, consumers continue to spend increasing amounts on botanical dietary supplements, from \$3 billion in 1996 to \$5 billion in 2010 [71]. More widespread use of herbal products has resulted in increased attention to the safety of these products by consumers in the market place, manufacturers, and regulatory bodies. The passage of the Dietary Supplement Health and Education Act (DSHEA) in October 1994 authorized the U.S. Food and Drug Administration (FDA) to extend its authority to monitor and evaluate the safety, quality and labeling of dietary supplements. Core elements of the amendment to the Food, Drug and Cosmetic Act resulting from the passage of DSHEA include enhanced reach of the FDA to monitor dietary safety and quality, require pre-market notification, conduct labeling oversight, and post-market surveillance. Of these, the ability to monitor safety and quality through the use of pre-market notification has caused the greatest difficulty in compliance for botanical dietary supplements manufacturers.

To specify, monitoring dietary supplement safety and quality includes the authority to impose an immediate ban of products that pose an imminent hazard, impose requirements for Good Manufacturing Principles (GMP), inspect facilities, and collect adverse event reports associated with dietary supplement use. Instituting pre-market notification requirements works to prohibit any new dietary ingredient (NDI) for which there is inadequate information from entrance into interstate commerce. For those ingredients that are considered new, manufacturers

must provide reasonable assurance that it does not present a significant or unreasonable risk of illness or injury. However, debate over what constitutes a new dietary ingredient and the information needed to provide such a reasonable assurance is a current point of discussion among manufacturers, trade groups, and regulators of dietary supplements.

The dietary supplement industry has been operating under the New Dietary Ingredient (NDI) submission process since the 1994 passage of DSHEA. When DSHEA was signed into law, the legislation modified the Federal Food, Drug and Cosmetic Act (FDCA) by adding section 413, which outlines requirements to market a new dietary ingredient or a dietary supplement that includes a new dietary ingredient [72, 73]. The term new dietary ingredient is defined as “a dietary ingredient that was not marketed in the United States before October 15, 1994 and does not include any dietary ingredient which was marketed in the United States before October 15” [74]. Specifically, this statute requires the manufacturer or distributor of a NDI, or of the dietary supplement that contains the NDI, to submit a premarket notification to FDA at least 75 days before introducing the supplement into interstate commerce or delivering it for introduction into interstate commerce, unless the NDI and any other dietary ingredients in the dietary supplement “have been present in the food supply as an article used for food in a form in which the food has not been chemically altered” [75]. Providing proof for this claim, however, is quite specific. Sales records, manufacturing records, commercial invoices, magazine advertisements, mail order catalogues, and sales brochures are all acceptable forms of evidence; affidavits alone are not [76]. Supposing these forms of proof can be supplied, they apply only if the currently marketed dietary supplement formulation has been in no way

chemically altered from the product as it existed prior to 1994. In addition, because no official list of dietary ingredients that can be considered “grandfathered” is recognized by regulatory authorities, it is rarely the case that supplement manufacturers are able to prove that their product is not new, even if it has a history of use as a traditional botanical medicine. Thus, most botanical dietary supplements are subject to the NDI submission process.

For those dietary supplements that must complete the NDI submission process, a new level of scientific rigor and premarket testing is outlined in the Draft Guidance document, heretofore unprecedented in the industry. Although the NDI submission provision has existed since the 1994 passage of DSHEA, recent debate in the dietary supplement community has centered around whether or not recent enforcement efforts have added greater rigor to the provision than was originally intended in the law. This point of debate matters to manufacturers of dietary supplements because the level of scientific testing outlined in recent legislation and regulatory guidance documents rises to a level of rigor that may not be appropriate for dietary supplements, and so cost-prohibitive as to put many dietary supplement companies out of business.

It has been pointed out elsewhere that the provisions required for premarket testing of dietary supplements and the wording copies almost directly from provisions outlined for the testing of food additives [77]. This is a significant point of contention, since the passage of DSHEA can be viewed historically as a battle between regulators and supplement manufacturers about whether or not dietary supplements could be considered and thus recalled as illegal food additives (for which prior market testing must demonstrate the safety with scientific evidence) or their own distinct regulatory class, neither food, food additive nor

drug, but dietary supplements (which do not require premarket proof of safety, only a reasonable expectation of safety, which lays the burden of proof that a product is unsafe upon regulators in order to issue a recall) [78]. Ultimately, the view that dietary supplements were not food additives and did not require proof of safety, but only a reasonable expectation of safety, carried the day and DSHEA was written and passed to reflect this thinking. DSHEA specifically noted that dietary supplements are not to be treated as food additives. This was pointed out in the 1994 law because such testing is not necessarily appropriate for the supplements being tested. Thus, an NDI Draft Guidance that has copied pre-market testing provisions from food additive premarket safety testing guidance documents would be out of line with DSHEA.

In addition, as the majority of the dietary supplement industry is comprised of small companies, employing 20 or fewer, such testing may be cost prohibitive [79]. Even if the industry were to comply by providing what amounts to millions of dollars in pre-market chemical characterization and toxicological testing for each new dietary ingredient brought to market, it is unclear that the regulatory authorities have the manpower to cull and make use of the information submitted. At the time of commentary on the NDI submission process in 1997, the agency estimated the number of NDI notifications that would be filed annually would be between zero and 12 [80]. However, the fact that there are “only” 700 submissions annually even though there are an estimated 55,600 dietary supplement products on the market is now a figure used as an indication that the industry has been lax with compliance [81].

This change in agency thinking indicates that a shift has occurred toward a more stringent NDI submission policy, requiring more scientific information prior

to product launch. Helping manufacturers clear the hurdle of regulatory compliance with an enhanced scientific framework may present a new area of opportunity for contract laboratories, including natural products laboratories housed in academia that specialize in analytical chemistry and pharmacology, and are willing to work as contract laboratories under an academic umbrella. Understanding the development of current thinking on the NDI provision may help to clarify potential avenues whereby information required for NDI submission, or what amounts to preclinical testing of botanicals, could be accomplished in a cost-effective, resource-conscious manner.

A Reasonable Expectation of Safety

In September 1997, FDA issued a Final Rule on Premarket Notification for a New Dietary ingredient [82], to clarify the “procedure by which a manufacturer or distributor of dietary supplements or of a new dietary ingredient is to submit under the Food Drug and Cosmetic Act the information on which it has concluded that a dietary supplement containing an NDI will reasonably be expected to be safe” [83]. Although additional clarification is outlined in the rule, manufacturers and distributors of dietary supplements still required guidance on a number of issues related to new dietary ingredients and requirements for notification submissions. On January 4, 2011, the Food Safety Modernization Act (FSMA) was signed into law [84]. The legislation included in section 113 required that 180 days after enactment of FSMA, the FDA issue a guidance document on NDIs. FSMA directed the guidance to include provisions that clarify when an ingredient is considered an NDI and when the manufacturer or distributor of a dietary ingredient is required to provide the required information under section 413 of the

FDCA [85]. FSMA also directed FDA to include the evidence needed to document the safety of NDIs as well as appropriate methods for establishing the identity of NDI. On June 21, 2011, one of the original sponsors of DSHEA, Senator Hatch (R-UT), alongside Senator Harkin (D-IA) submitted a letter to Congress calling for the release of the guidance document to “provide clarity, predictability, and certainty to dietary supplement manufacturers and the public on FDA’s interpretation and expectation related to the marketing of NDIs.” In July 2011, the Draft Guidance for Industry: Dietary Supplements: New Dietary Ingredient Notifications and Related Issues was released for public comment [86]. If FDA is able to meet all of its program priorities, a final NDI guidance is anticipated for 2014 [87].

The draft guidance is presented in a question-and-answer format to help industry determine when a dietary ingredient is new, deciding when premarket safety notification is necessary, and what type of information is needed for a complete notification. While the statement that any dietary ingredient marketed in the US before October 15, 1994 is exempt from the need to file an NDI seems straight forward enough, the information required to prove that a dietary ingredient was marketed before that time is very specific. The dietary ingredient must have (1) been sold or offered for sale (2) as a dietary supplement, in bulk as a dietary ingredient for use in dietary supplements, or as an ingredient in a blend or formulation of dietary ingredients for use in dietary supplements (3) in the United States (4) before October 15, 1994, and (5) not have undergone any changes in manufacturing processes that would alter the chemical composition of the ingredient or (6) changed the composition of materials used to make the ingredient [88]. To establish that marketing took place in the U.S., the identity

(e.g., chemical or botanical name) and form (e.g. ground herb, water extract, oil) of the marketed ingredient, and whether the ingredient was marketed as a dietary ingredient or for some other purpose, must be included in the evidence used. Valid forms of evidence include written business reports, promotional materials, or press reports with a contemporaneous date prior to October 15, 1994. Sales records, manufacturing records, commercial invoices, magazine advertisements, mail order catalogues and sales brochures are all acceptable; affidavits alone are not. In short, the requirements to prove that a dietary ingredient is “grandfathered”, and does not require a NDI notification, is extensive and very difficult to achieve, thus requiring that most dietary supplements on the market today submit NDI.

Within the new dietary ingredient notification, filers are asked to include information that makes the case to the FDA that there exists for their products a standard of safety, defined as a “reasonable certainty of no harm.” The NDI Guidance states,

The NDI safety standard is different than the standard for food additives, drugs, pesticides, and other FDA-regulated products. Recommendations in guidance documents that are tailored to the safety assessment needs of other FDA-regulated products may not always be appropriate for dietary ingredients and dietary supplement...You should use your own best judgment in compiling scientific evidence that provides a basis to conclude that the NDI that is the subject of your notification will reasonably be expected to be safe when used under the conditions recommended or suggested in the labeling of the dietary supplement in the notification....You must provide the information that forms the basis on which you have concluded that a dietary supplement containing the NDI will reasonably be expected to be safe under the supplement's labeled conditions of use (21 U.S.C. 350b(a)(2)). In general, this information should include an adequate history of safe use, safety studies, or both [88].

This “reasonable expectation of safety” is phrased in the FDCA Sec 402(f)(1)(B) as a “reasonable assurance that such (an) ingredient does not present a significant or unreasonable risk of illness or injury.”

In outlining what sorts of information may be used in the NDI submission in order to reach such a conclusion, the draft guidance introduces a scientific framework, involving animal and human safety studies that are required when history of use data and literature are inadequate [89]. These include an extensive battery of toxicological testing used in the submission of new drug applications.

For a botanical supplement like *Iresine celosia*, the NDI is intended for daily chronic use, and has a documented history of safe intermittent use, and the proposed use of the NDI leads to intake levels that are the same as or less than the levels consumed historically, the following types of data are recommended [90]:

- (1) A three-study genetic toxicity (gentox) battery (bacterial mutagenesis, *in vitro* cytogenetics, and *in vivo* mammalian test) that includes a test for gene mutations in bacteria, either an *in vitro* mouse lymphoma thymidine kinase+/- gene mutation assay (preferred) or another suitable *in vitro* test with cytogenetic evaluation of chromosomal damage using mammalian cells, and an *in vivo* test for chromosomal damage using mammalian hematopoietic cells;
 - (2) a 14-day range-finding oral study to establish a maximum tolerated dose (MTD) in an appropriate animal model;
 - (3) a 90-day sub-chronic oral study in the same species as the range-finding study to establish an MTD and a No Observed Adverse Effect Level (NOAEL) for use in calculating the margin of safety;
 - (4) a multi-generation rodent reproductive study (minimum two generations); and
 - (5) a teratology study (rodent or non-rodent);
- except that the latter two studies are not needed if the product is labeled as not for use by women of childbearing age, pregnant or lactating women, and children 13 and younger.

The cost of this battery of tests was calculated at a presentation by international analytical testing lab firm Eurofins Scientific Incorporated in collaboration with Spheris Incorporated on December 12, 2011 at the Food and Drug Law Institute in Washington D.C. The findings are summarized in Tables 20-21. For a botanical dietary supplement like *Iresine celosia* with only one new

dietary ingredient, supposing they are able to prove their historical use, estimates range anywhere between \$573,310 - \$690,600 for intermittent use, and \$2,198,310 - \$2,815,600 for chronic use. If they are unable to prove their historical use, testing costs can range anywhere between \$2,758,310 - \$3,382,500. These numbers represent starting points, of course, as additional testing may be needed (see Table 20-21). The Draft Guidance also goes on to mention that, “based on the nature of the NDI and the results of other testing special studies (e.g., carcinogenicity, ADME) may be needed to provide a reasonable expectation of safety. Other non-clinical studies to assess immunotoxicity and neurotoxicity should be conducted on a case-by-case basis, as appropriate.”

Table 20:
Safety Texting Recommendations
Documented Historical Use

	Intermittent		Daily Chronic		Cost
	Less Than	Greater Than	Less Than	Greater Than	
Two-Study Genetic Toxicity Battery Bacterial Mutagenesis (Ames) <i>In vitro</i> cytogenetic		x		x	\$4,310 - \$7,800 \$26,000 - \$34,000
14-Day Range-Finding Oral Study in Animals		x		x	\$50,000 - \$75,000
90-Day Sub-Chronic Oral Study in Animals		x		x	\$125,000 - \$179,800
One-Generation Rodent Reproductivity Study				x	\$220,000
Teratology Study		x		x	\$138,000 (Rat) \$164,000 (Rabbit)
One-Year Chronic Toxicity or Two-Year Carcinogenesis Study				x	\$1,500,000 - \$2,000,000 (Rat)
Single-Dose ADME Study in Animals		x			\$230,000 (Rat)
Repeat-Dose ADME Study in Animals				x	\$135,000 (Rat)

Total, Daily Chronic: \$2,198,310 - \$2,815,600

Total, Intermittent: \$573,310 - \$690,600

Per New Dietary Ingredient

Note: Based on the nature of the NDI and the results of other testing special studies (e.g., carcinogenicity, ADME) may be needed to provide a reasonable expectation of safety. Other non-clinical studies to assess immunotoxicity and neurotoxicity should be conducted on a case-by-case basis, as appropriate.

Table 21:
Safety Texting Recommendations
No History of Documented Historical Use

	Daily Chronic	Intermittent	Cost
Two-Study Genetic Toxicity Battery Bacterial Mutagenesis (Ames) <i>In vitro</i> cytogenetic <i>In vivo</i> mammalian test (micronucleus)	x	x	\$4,310 - \$7,800 \$26,000 - \$34,000 \$25,000 - \$31,900
14-Day Range-Finding Oral Study in Animals	x	x	\$50,000 - \$75,000
90-Day Sub-Chronic Oral Study in Animals	x	x	\$125,000 - \$179,800
Multi-Generation Rodent Reproductivity Study	x	x	\$525,000
Teratology Study	x	x	\$138,000 (Rat) \$164,000 (Rabbit)
One-Year Chronic Toxicity or Two-Year Carcinogenesis Study	x	x	\$1,500,000 - \$2,000,000 (Rat)
Single-Dose ADME Study in Animals	x	x	\$230,000 (Rat)
Repeat-Dose ADME Study in Animals	x	x	\$135,000 (Rat)

Total: \$2,758,310 - \$3,382,500

Per New Dietary Ingredient

Note: Based on the nature of the NDI and the results of other testing special studies (e.g., carcinogenicity, ADME) may be needed to provide a reasonable expectation of safety. Other non-clinical studies to assess immunotoxicity and neurotoxicity should be conducted on a case-by-case basis, as appropriate.

Currently, funding to conduct studies to evaluate the safety and efficacy of dietary supplements is extremely limited. “It is impractical to think that any government agency, institute, or private organization could provide sufficient funds to adequately test the safety of tens of thousands of dietary supplement products often marketed in combinations that may change in formulation from year to year” [91]. The burden to conduct such testing has fallen to dietary supplement companies. Those who cannot afford the extensive toxicological testing outlined in the NDI may be forced out of business. In addition, regulators do not have the manpower needed to sift through these documents and toxicological testing results, even should companies be able to afford them.

Nevertheless, in spite of historical use and a generally accepted perception that “natural” equates to safe, it is true that some natural product preparations possess compounds that are known to be harmful when ingested by humans at higher dosages, when taken over longer periods of time than is appropriate (exposure), when taken in combination with prescription drugs (herb-drug interactions), or used by certain high-risk patient populations (i.e., those with compromised kidney and liver function) [92, 93]. Thus, the challenge remains, to evaluate the safety of botanicals and preparations prior to introduction to interstate commerce, to remain conscious of resources available to manufacturers and regulators of dietary supplements, to test botanicals within a framework true to the intent of DSHEA, and to use best judgment to support a reasonable expectation of safety.

Understanding the Risk Profiles Presented by Herbal Medicines:

A Question for Pharmacognosy

While it is true that “natural” does not equate to safe, it is also true that the toxicity profiles of herbal medicines differ from those presented by toxic chemicals, food additives and synthetic pharmaceutical compounds. For this reason, they are and should continue to be regulated accordingly. When data was evaluated from 55 countries between 1968-1997 was evaluated, analysis published in the British Medical Journal noted that adverse events from herbal remedies “amount to only a tiny fraction of adverse events associated with conventional drugs held in the same (WHO) database” [11, 94]. Most of our risk profiling and test parameters concerning compounds we ingest grow out of the pharmaceutical paradigm that tests single molecular entities with potent and specific biological effects. However, herbals present a very different risk profile, both due to their dosing and due to specific presence or absence of structural features and functional groups. One major reason the safety profiles of dietary supplements and pharmaceuticals differ is because their chemistry differs.

The current risk assessment methodologies for chemicals regarding human toxicity endpoints are often derived from those for preclinical studies of pharmaceuticals. The methods for hazard assessment are largely the same for industrial chemicals, pesticides, and drug candidates. But this approach may not necessarily be appropriate for the risk assessment of herbal medicines now available as dietary supplements. Understanding and using the information we know about the action of phytochemicals present in herbal medicines, and the chemistry associated with their toxicity, is being under-utilized in the production of herbal dietary supplements, and their safety review by regulators.

For dietary supplement safety concerns unrelated to adulteration, contamination, or other manufacturing and quality control procedures, but rather those that are inherent to the botanical preparations themselves, it is important to understand the toxic liabilities associated with phytochemicals present in botanical preparations in particular. It is to be acknowledged that herbal medicines, in use, are usually complex mixtures whose dynamic pharmacology is the result of a biochemical matrix of molecules that act to carry secondary metabolites throughout the body. The biological action of complex botanical mixtures is thought to be the result of isolable single molecule principles. Their bioactivity, including toxicity, is the result of chemical structures and chemical properties. The scientific discipline and collected wisdom encompassing the study of the chemistry, pharmacology, and toxicology of these secondary metabolites and their use in complex preparations is known as pharmacognosy. The findings of the science of pharmacognosy can and should be applied to the evaluation of the safety of botanical dietary supplements.

In many cases, the active forms of secondary metabolites that are the basis of any rational phytotherapy are often prodrugs whose active form is generated through metabolic bioactivation *in vivo*, usually through Phase I metabolism. Thus, the active forms of molecules giving rise to favorable bioactivity, as well as toxic bioreactivity, is often the results of fleeting metabolites that fall into two broad categories: free radicals, and reactive electrophiles that are highly water soluble, and made more so by the oxidation, reduction, and hydrolysis resulting from Phase I biotransforming enzymes, cytochromes p450 (CYP enzymes). Due to the presence of reactive metabolites, the functional macromolecular structure of endogenous targets, like proteins and nucleic acids, can be altered *in vivo*. Their

effects range from chemoprevention in some cases, generally for weaker electrophiles with longer half lives, and mutagenesis and cell death in others, particularly for those that form briefly and whose targets are indiscriminate [11]. The resultant reactive intermediates can bind covalently to DNA and proteins, leading to organ toxicity and even carcinogenicity. In addition, some phytochemicals are shown to form reactive intermediates capable of irreversibly inhibiting various CYPs, which are critical to the metabolism and elimination of xenobiotics from the body, including therapeutic drugs. Less frequently, herbal compounds can also be converted to toxic or carcinogenic metabolites through Phase II metabolism enzymes that carry out reactions like glucoronidation sulfonation, acetylation, methylation, and glutathione conjugation.

The fleeting, reactive nature of these compounds pose considerable analytical challenges in attempts to determine their properties. It is understood that herbal medicines, often comprised of multiple active substances, undergo complicated fates *in vivo*. These fates can often be difficult to study, to follow and trace throughout the body, as intermediates responsible for biological reactivity are often fleeting in nature, and can be metabolized rapidly. For this reason, attempts to understand the absorption, distribution, and metabolism (ADME) and toxicity may best be accomplished by leveraging analysis of the structures isolated from phytochemicals, and evaluating their ability to form BRIs *in vivo*.

Screening natural product structures isolated from herbals used in dietary supplements for their likelihood to form such metabolites is an important step in identifying potential liabilities [15]. This screening can be conducted via a structure-based approach, in comparison with structures from herbal medicines that are known to be toxic, or converted *in vivo* to electrophilic mutagens and

carcinogens, and renal- or hepato-toxins. The study of reactive metabolites was pioneered in the 1940s through the work of James and Elizabeth Miller, who studied the mechanisms of action and metabolism of the carcinogenic azo dye 4-dimethylaminoazobenzene (DAB), formerly used as a food coloring [95]. Further study of the mechanisms of action and metabolic fate of a large number of carcinogens led to the identification of several chemical functional groups and substructures (Structural Alerts, SA) that are used to screen for toxic liabilities for pharmaceuticals, environmental toxins, and food additives to this day [16, 96]. Many functional groups in chemical structures are known to be associated with the formation of reactive metabolites, very often catalyzed by the CYP enzymes. It is important to keep in mind, however, not all compounds with such functional groups are toxic, since formation of reactive intermediates is limited by the ability of CYPs to activate them.

Through the consideration of the types of biologically active compounds that may be present in the plant, it is possible to make educated estimates of the potential hazard of any given botanical, and prioritize their level of concern to safety. In their 2005 publication, Dietary Supplements: A Framework for Evaluating Safety, the National Academies of Science encouraged the use of chemical relatedness, or similarities to known toxic chemical compounds when considering the safety of botanical dietary supplements and their chemical components [97]. In the absence of information about the activity of the ingredient in question in humans, animals, or *in vitro* experiments, it is scientifically acceptable and appropriate to use information about safety concerns of related substances to inform a decision about the associated risk of the dietary supplement ingredients and their constitutive phytochemistry.

While most botanical dietary supplements can be considered safe, we know the formation of reactive metabolites from compounds like saffrole (Sassafras oil), aristolochic acid (Aristolochiaceae family, especially genus *Aristolochia* and *Assarum*), methysticin (Kava Kava, *Piper methysticum*), and others have been reported [92]. Their toxicity arises primarily from the presence of six biological reactive intermediates, ranging in electrophilicity, from highly reactive and positively charged, to more stable, neutral electrophiles: carbocations, nitrenium ions, epoxides, quinones, quinone methides, and simple Michael acceptors (MAs). Other structural alert groups exist within natural products, including acetylenes, benzodioxoles, and terminal alkenes, among others (see Fig. 56). Of the 57 known structural alerts associated with mutagenicity and genotoxic carcinogenicity, 16 regularly occur within botanical natural products [11, 98].

Awareness of the presence of these compounds can be used in early product development, just as it is in early drug discovery, to evaluate the possibility of a given compounds becoming a metabolic liability. The need to assess the ability of a chemical to act as a mutagen or genotoxic carcinogen is one of the primary requirements in regulatory toxicology, as is assessing the ability for a compound to induce liver injury by inhibition of CYP enzymes [17, 21, 22, 24, 25].

Fig. 59: Common Chemical Structural Alert Groups from Natural Products

Leveraging Accumulated Knowledge and Existent Strategies

Structural alerts (SAs) for toxicities like mutagenicity and carcinogenicity, and CYP-related metabolism have been identified and codified into software programs by using existing knowledge, expert human judgment, bioassay data, and other modeling approaches [99]. These computer programs can assist in predicting the potential propensity for a chemical, or libraries of chemicals, to cause particular effects. A wide range of approaches and algorithms are incorporated into metabolism prediction software, with some using the structural features and physiochemical properties of test substrate compounds to predict the most likely metabolic sites. Some also incorporate docking studies and molecular dynamics simulation studies to assess P450-related pharmacological processes [100]. Apart from predicting only the possibility of particular sites of metabolism resulting from the biotransformation of a molecule, some software programs are designed to calculate the probability of a compound to be metabolized at a particular site, as well as other physiochemical parameters. Several proprietary, but few public, *in silico* methods are available for assessing ADMET properties.

Whatever method or algorithm is used, *in silico* toxicology, or the computational assessment of toxic liabilities, can allow researchers to evaluate a large number of chemicals, with consideration for a variety of endpoints, and ranges of exposure conditions considered simultaneously. Other benefits include a substantial reduction, replacement, and refinement in the need for biological and particularly animal toxicological testing in establishing the safety of chemical substances. In addition, because bioactive and bioreactive intermediates formed from phytochemicals are often fleeting intermediates produced *in vivo*, *in silico* work has the added advantage of exploring these compounds in simulated studies.

The strategy can be cost and time effective, and provides information that can guide appropriate further testing and the conscientious use of resources.

These programs are used by regulators throughout the FDA, the EPA, by industry and academic researchers alike to evaluate a wide variety of chemical substances [101-103]. For instance, regulators at the US Food and Drug Administration's Office of Food Additive Safety (OFAS), who administer the program that evaluates the safety information and industry submissions for various categories of food substances, including food additives, employ structure activity relationships (SAR) studies to evaluate the risk posed by new chemicals in these submissions [16]. It has been pointed out elsewhere that the framework wording and most of the scientific standards presented in the NDI draft guidance for dietary supplements are borrowed heavily from the food additive review process, so much so that the two "seem to be indistinguishable" [77, 104]. While the testing framework and scientific standards required for NDIs may be new to the dietary supplement industry, regulators and manufacturers have already encountered the challenges presented by these provisions to some extent within the framework of food additive regulation, and developed working solutions to these challenges. Computational screening for structural alerts generates threshold of concern that then guides appropriate toxicological testing. A similar strategy can and should be applied to the evaluation of dietary supplements, especially considering that dietary supplements are to be regulated less stringently than food additives, under DSHEA.

It is important to keep in mind that models are guides, not hard forecasts. While a great deal of computational work, with a vast amount of expert knowledge that allows for a broad scope evaluation of toxic liabilities associated

with structures, it must be kept in mind that all models, even animal and cell culture models, are approximations. *In silico* predictions are most often conducted alongside other testing strategies, and these data constitute an integrated testing strategy. Assessing the relative value of information derived from computational toxicology can and should be weighed alongside other, different pieces of available information that has been gathered when making an overall risk assessment, and when considering what other information may be needed to conduct a robust assessment.

Conclusion

The consumption of any botanical ingredient carries with it a certain degree of inherent risk to at least some segments of the human population, even for those plants used as foods or with a history of use for medicinal purposes.

In the absence of comprehensive human clinical trials that establish safety, scientific evidence for risk can be obtained by considering whether the plant constituents are compounds with established toxicity or closely related in structure to compounds with established toxicity [32].

Electrophilic structural alert groups commonly associated with natural product toxicity can be used to flag and identify potential natural product liabilities. Because neither regulatory bodies nor dietary supplement manufacturers have resources or time to conduct extensive toxicological testing, we can use structural features of natural product compounds to help us focus on areas of potential concern and prioritize efforts and resources. Computational capacity can be leveraged to flag and compile this data, and alert us to potential concerns. The basis of this work, however, is the isolation of chemical compounds present in laboratories of pharmacognosy. Their work plays a vital role in the

evaluation of potential toxicities associated with botanicals developed into dietary supplements by providing chemical structures needed for this type of risk assessment. Moreover, this data is required for new dietary ingredient (NDI) submission, and should be fully utilized when considering a justification for a reasonable expectation of safety. This helps both manufacturers and regulators leverage existing data necessary for NDI submissions to appropriately evaluate generalized potential risk profiles of botanical extract's constitutive chemistry, and prioritize needs for future toxicological testing.

DSHEA set out dietary supplements as a different level of premarket safety concern from pharmaceuticals or food additives, because the chemical levels of concern are different. Understanding and using the information we know about the action of phytochemicals present in herbal medicines, and the chemistry associated with their toxicity, is being under-utilized in the production of herbal dietary supplements, and their safety review by regulators. Neither manufacturers nor regulators have the resources available to do extensive pre-clinical testing of individual new dietary ingredients, which include extensive animal testing, and to review all the data that would be generated therefrom. The scientific understanding of the phytochemistry and resultant pharmacology of natural products should be better integrated in to the preliminary pre-market screening required by the NDI submission process, as it has been by other sectors of food and drugs, for which pre-market safety standards are more stringent. This information can and should be used to direct and guide the appropriate biological safety testing needed for herbal dietary supplements. Regulators and producers of pharmaceuticals, pesticides, and food additives have previously used structural alerts to help classify the safety or potential liability associated with compounds.

Such a strategy can and should be applied to dietary supplements, to assess the safety of their products, and to help prioritize their testing efforts.

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***In silico* ADME/TOX Evaluation of Compounds
Isolated from *Iresine celosia* and Related
Metabolites**

Introduction

The aim of this work was to evaluate the safety of isolated phytochemicals from the *Iresine celosia* Odyliresin™ formulation using *in silico* drug metabolism and toxicology simulation studies, to predict possible metabolites generated *in vivo*, and their possible toxicological endpoints using ADMET predictor proprietary software. Compounds isolated from *Iresine celosia* botanical extract were screened using Simulations Plus ADMET Predictor proprietary software, to develop recommended maximum-recommended therapeutic oral dose and estimate overall toxic risk profiles of the compounds, including mutagenesis, chromosomal aberrations, reproductive toxicity, and human liver adverse effects. Previously, the extracts have been standardized, and the presence of guanidine alkaloids that may represent the health-relevant principle of the extract, as quantified. This work outlines the findings of screening these compounds for ADME/Tox using proprietary systems pharmacology software. In addition, to evaluate the formulation's risk for potential herb-drug interactions, the ability of these compounds to inhibit CYP450 enzymes was also evaluated. Because many phytochemicals act as prodrugs, the generation of potentially bioactive or bioreactive (toxic) metabolites through Phase I biotransformation was also evaluated, and compared with botanical secondary metabolites known to cause toxicity. *In silico* screening of individual compounds isolated from the formulation suggest very little CYP inhibition a low level of safety concern overall for the compounds evaluated. Overall, these data support a reasonable expectation of safety when using this preparation as directed.

Methods

Phytochemicals isolated from *Iresine celosia* and their CYP450-produced biological metabolites were screened for their potential toxicity using the ADMET predictor proprietary software, a modeling tool from Simulations Plus. The ADMET Predictor Metabolism Module was used to determine whether compounds screened would be substrates for the nine most common CYP isoforms included in the model (2A6, 2B6, 2C8, 2E1, 1A2, 2C9, 2C19, 2D6 and 3A4), whether an atom within a molecule could be a metabolic site, and what the predicted metabolite species could be. Metabolic site prediction models were trained on an extensively curated and updated version of the Accelrys Metabolite database along with published datasets of sites of metabolism and general review articles. The data is curated and updated to reflect more recent publications, and false positives removed when further literature investigations revealed a particular reaction in question to be mediated by a different CYP or through a non-CYP-enzyme. Published literature has also gone on to validate sites of metabolism predicted by the model which were then experimentally verified, which suggests an added degree of confidence in the metabolism prediction models.

The ADMET Predictor Toxicity Module is used to predict toxicity relative to food products, pharmaceuticals, and environmental chemicals such as pesticides. The toxicity module offers information on a variety of end points, including estrogen and androgen receptor toxicity, chromosomal aberrations, a qualitative filter of mutagenicity in five strains of *Salmonella* bacteria with and without microsomal activation, human liver adverse effects, reproductive and developmental toxicity, maximum recommended therapeutic dose (MRTD),

carcinogenicity in rats and mice as TD50, and acute lethal toxicity in rats as LD50, among others.

Parent structures were input into ADMET Predictor using MedChem Designer. Analysis was run on these compounds with default settings, including running on pH 7.4 and pH 2. Output data containing toxicity, metabolism, and other physiochemical test results was exported to Excel for further analysis. Parent structures were then put into MedChem Designer, to generate metabolite structures and ADMET Predictor properties of metabolites taken. This data was also exported to Excel for further analysis.

Both models have been used extensively by a variety of companies within the pharmaceutical and chemical industries, as well as with the U.S. Food and Drug Administration with whom the company has recently developed collaborative agreement to further develop *in vitro-in vivo* correlation models to streamline regulatory review. These models are also licensed for academic use at steeply discounted rates, making them cost-effective for academic laboratories. These models have been extensively curated, are updated regularly, and do include some natural products in their data set. However, because natural products are very difficult to track as they are metabolized throughout the body, the natural product specific dataset that could inform metabolic site predictions and ADME toxicity modules like the ones used are quite limited. Thus, one draw back to the model that may exist is its training on pharmaceutical and environmental pesticide compounds, for which there is a greater wealth of metabolic and toxicity data. In addition, the model is unable to predict the formation of metabolites with short half-lives, such as the formation of epoxides. While it can make predictions about a compound's ability to be metabolized by UGT-enzymes, it is primarily focused

on the prediction of metabolites formed through the nine CYP-isoforms listed above. Should a compound be metabolized by one of these enzymes, then undergo a Phase I reaction like sulfonation prior to being further metabolized by one of the CYP-enzymes included in the model, the model will be unable to make this logical leap. Thus, study and awareness of examples of known natural product metabolism were studied and also applied when evaluating results from both the Metabolism and Toxicity modules.

Internal cross-validation of the model was conducted using a set of botanical natural product structures known to inhibit CYP450 enzyme function, or to form biologically reactive intermediates, derived primarily from the Botanical Safety Handbook and other resources [12-14, 92].

Results

Enzymes primarily responsible for the metabolism of these GA compounds are CYP219, 2C19, and 2E1. Compound 1 and Compound 3 can be expected to be a substrate to CYP2C19 and 2E1. Compound 2 can be expected to be a substrate to CYP2C19 but not to 2E1. Compound 1 is also likely to be metabolized by CYP2D6. Compound 1 and 3 may be further metabolized through glucoronidation via the UGT2B7 enzyme.

Compound 1 and 3 may be contraindicated with use alongside protein pump inhibitors, as 2C19 is responsible for clearing this class of drugs. Because of further glucoronidation via UGT2B7, these compounds may be contraindicated with use in alcoholics and cancer patients. However, compounds 1 and 3 show very low likelihood of acting as mutagens. They may cause adverse liver events, but their maximum recommended therapeutic dose is high (above 3.16 mg/kg/day for a 60 kg-body weight/day), indicating less potential for overall toxicity.

For Compound 2, the maximum recommended therapeutic dose is lower (below 3.16 mg/kg/day for a 60 kg-body weight/day). On further inspection, however, when LD50 and TD50 values for rats and mice are examined, it is found that the compound in its current formulation is present in concentration well below the concentrations required to cause lethality and toxicity in 50% of the respective populations.

Overall, these compounds each present an overall ADMET risk of 3/24, indicating that they are relatively low in risk as compared with the World Drug Index training set.

Outlines of findings for the guanidine alkaloid compounds are discussed below.

Overall Simulation Summary for Compound 1:

Compound 1 can be expected to be a substrate to CYP2C19 and 2E1.

CYP2C19 is important in the clearance of several classes of exceptionally widely used drugs, such as the proton pump inhibitors (PPIs) like clopidogrel, phenytoin and S-mephenytoin, as well as barbiturates, benzodiazepines, SSRIs, and the antimalarial proguanil. Possible contraindication with use alongside PPI (protein pump inhibitors). This compound may be a CYP2E1 substrate (60% likelihood). Very few drugs are cleared by 2E1, although it generates reactive oxygen species without substrate present, and are thus implicated in liver malfunction in alcoholics and cancer patients because of these reactive oxygen species. It is also likely that this compound is further metabolized through glucoronidation via the UGT2B7 enzyme. This enzyme is responsible for conjugation of bile acids, catechol estrogens, morphine and naproxen, hydroxymidazolam, gemfibrozil, AZT and many other drugs. Midazolam is also a substrate, and structurally similar to this compound, with both sharing an imidazo- ring. Possible contraindication with use in alcoholics, and cancer patients may be advised. Compound 1 does not appear to pose a liability to overall CYP-function (CYP_RISK: 0).

Maximum recommended therapeutic oral dose (TOX_MRTD), based on mg dosage/60kg-body weight/day, is above 3.16 mg/kg/day, indicating less potential for overall toxicity. LD50 for lethal acute rat (TOX_RAT) in mg/kg oral dose that would be lethal to 50% of rats was 768.94 mg/kg/day, requiring a 46.14 gram oral dose for a 60kg-body weight/day. TD50 or oral dose required to induce tumors in 50% of rats after exposure over a standard lifetime (TOX_BRM_Rat), measured in mg/kg/day oral dose, is 1.95 mg/kg/day. This would require a human equivalent oral dose of 117 mg/day of the pure compound. In mice (TOX_BRM_Mouse), the number is 75.25 mg/kg/day, equating to a 4.52 gram oral dose each day of the single compound. TOX_Code Xr indicates that this compound may present a risk

in terms of carcinogenicity in chronic rat studies, because TOX_BRM_Rat is below 4 mg/kg/day oral dose. For a 60 kg body weight, that would amount to 240 mg/kg/day of the pure compound. With MRTD above 3.6 mg/kg/day, a LD50 calling for 46 gram/day oral dose, and a TD50 oral dose of pure compound at 117 mg/kg/day, the compound in its current formulation is so dilute as to be presumed to be safe because it is present in an average daily dose in concentrations well below the concentrations above.

With an overall mutagenicity score representing the results of virtual Ames testing (TOX_MUT_Risk) of 1, on a score of 0-6 (with or without microsomal activation), this compound is not likely to present a serious mutagenic risk. Out of the ten virtual Ames tests conducted, this compound flagged one: an elevated risk for a metabolite of the pure compound likely to cause mutagenesis in TA1535 strain of *Salmonella typhimurium* (TOX_MUT_m1535). Other qualitative risk estimations for toxic liabilities may also include elevated risk for reproductive toxin (TOX_REPR) at 72%, elevated risk for chromosomal aberration (TOX_CABR) at 79%.

Human liver adverse effect as the likelihood of causing elevation in the levels of a variety of liver enzymes, including elevated alkaline phosphatase enzyme (TOX_AlkPhos) at 98%, elevated GGT enzyme (TOX_GGT) at 86%, elevated LDH enzyme (TOX_LDH), elevated SGOT (TOX_SGOT) at 99%, and elevated SGPT enzyme at 74%. Thus, extremely concentrated doses of this compound may cause cardiac toxicity, chromosomal aberration, and reproductive toxicity. Elevation at higher doses may result in increased levels of alkaline phosphatase (98%), GGT (86%), LDH, SGOT (99%), and SGPT (74%) liver enzymes. The binary value, yes/no, TOX_Code HP indicates that a combination of liver enzymes levels would rise as a result of this compound, and that this compound could result in hepatotoxicity. Further indication of liver damage or disease, by the presence of higher levels of enzyme, the binary yes/no value,

TOX_Code SG indicates increased levels in serum glutamic pyruvic transaminase (SGPT), now known as alanine aminotransferase (ALT) may be elevated in the presence of compound 1. Thus, this compound may cause increased levels of enzymes involved with hepatotoxicity.

The model does not indicate that the compound would be toxic to the androgen receptor or estrogen receptor in rats (TOX_AR_Filter and TOX_AR_Filter).

With an overall ADMET_RISK composite score for all of these models, on a scale of 0-24, this compound scored 3.

BRIs present in Compound 1:

Through CYP2E6, the oxidation of the secondary alcohol to a ketone, a Simple Michael acceptor product is formed (metabolite 4, M4). This intermediate may be a possible mutagen (TOX_Code Mut), TOX_MUT_Risk 2 (out of 0-6).

ADMET results:

CYP2C19 Substrate: Yes

CLint: 0.034

Intrinsic clearance constants for predicted sites of CYP2C19 mediated metabolism expressed in uL/min/mg microsomal protein.

CYP2E1 Substrate: Yes (60%)

MET_UGT2B7: Yes, 97%.

Qualitative model of a glucoronidation by the UDP glucuronosyltransferase 2B7 enzyme. Responsible for conjugation of bile acids, catechol estrogens, morphine and naproxen, hydroxymidazolam, gemfibrozil, AZT and many other drugs.

Midrazolam is also a substrate, and structurally similar to this compound, with both sharing an imidazo- ring.

Results: Further metabolism of this molecule via glucoronidation via the UGT2B7 enzyme is predicted.

CYP_RISK: 0

ADMET Code for metabolic liability - a computational filter developed by using a refined subset of the WDI.

TOX_MRTD: Above 3.16 (65%)

A qualitative assessment of the maximum recommended therapeutic dose administered as an oral dose in mg/kg/day.

TOX_MUT_Risk: 1

Represents the results of virtual Ames testing. Predicts overall mutagenicity by counting the number of “Positive” mutagenicity predictions. On a score of 0-6 (with or without microsomal activation).

Result: We would not expect compound 1 to act as a mutagen.

TOX_MUT_Code: m4

Summarizes output of TOX_MUT_* models from the program. Accounts for the compound's metabolites.

Results: Metabolite 4 (m4) may be a possible mutagen (TOX_Code Mut), with a TOX_MUT_Risk of 2 out of a 0-6 scale.

TOX_MUT_1535: Negative

Qualitative assessment of mutagenicity of the pure compound in TA1535 strain of *S. typhimurium*.

TOX_MUT_m1535: Positive (50%)

Qualitative assessment of mutagenicity of the compound and its microsomal rat liver metabolites in TA1535 strain of *S. Typhimurium*.

TOX_Risk: 3

Score from 0-7, indicates the number of toxicity problems a compound might have. An overall toxic liability score, derived from World Drug Index (this includes natural product structures). Score exceeds 2 for ~10% focused WDI.

Results: This compound may present a low-moderate toxicological risk as a single chemical entity. Dosing, formulation, and carrier vehicle chemistry should be considered.

TOX_Code: Xr, HP, SG

Results:

Xr: This compound may present a risk in terms of carcinogenicity in chronic rat studies.

HP: this compound may present a risk as a hepatotoxin.

SG: serum glutamic pyruvic transaminase (SGPT), now known as alanine aminotransferase (ALT) test. Measured to see if the liver is damaged or diseased, by presence of higher ALT levels. This compound may cause elevated levels of ALT.

TOX_Rat: 768.94 mg/kg/day oral dose LD50

TOX_BRM_RAT: 1.95 mg/kg/day oral dose TD50

Predicts the TD50 value of a particular compound in units of mg/kg/day. The TD50 is the dose of a substance administered orally to rats over the course of their lifetimes that results in the appearance of tumors in 50 percent of their population.

TOX_BRM_MOUSE: 75.252 mg/kg/day oral dose TD50

Similar TD50 value for mouse model.

TOX_CABR: Toxic (79%)

Qualitative estimation of triggering the mutagenic chromosomal aberrations.

TOX_REPR: Toxic (72%)

Qualitative estimation of reproductive / developmental toxicity.

TOX_AlkPhos: Elevated (98%)

Human liver adverse effect as the likelihood of causing elevation in the levels of Alkaline Phosphatase enzyme.

TOX_GGT: Elevated

Human liver adverse effect as the likelihood of causing elevation in the levels of GGT enzyme.

TOX_SGOT: Elevated (99%)

Human liver adverse effect as the likelihood of causing elevation in the levels of SGOT enzyme.

TOX_SGPT: Elevated (74%)

Human liver adverse effect as the likelihood of causing elevation in the levels of SGPT enzyme.

TOX_AR_Filter: Non-toxic to AR

Qualitative assessment of the androgen receptor toxicity in rats.

TOX_ER_Filter: Non-toxic to ER

Qualitative assessment of the estrogen receptor toxicity in rats.

Overall ADMET_Risk: 3

On a scale of 0-24. Summarizes all other ADMET Risk/Code models - a computational filter developed by Simulations Plus Inc. using a refined subset of the WDI.

Overall Simulation Summary for Compound 2:

Compound 2 can be expected to be a substrate to CYP2C19 but not to 2E1. CYP2C19 is important in the clearance of several classes of exceptionally widely used drugs, such as the proton pump inhibitors (PPIs) like clopidogrel, phenytoin and S-mephenytoin, as well as barbiturates, benzodiazepenes, SSRIs, and the antimalarial proguanil. Possible contraindication with use alongside PPI (protein pump inhibitors). Compound 2 does not appear to pose a liability to overall CYP-function (CYP_RISK: 0).

Maximum recommended therapeutic oral dose (TOX_MRTD), based on mg dosage/60kg-body weight/day, is below 3.16 mg/kg/day, indicating some potential for overall toxicity. On further inspection, however, LD50 and TD50 values are still quite high. LD50 for lethal acute rat (TOX_RAT) in mg/kg oral dose that would be lethal to 50% of rats was 721.84 mg/kg/day, requiring a 43.31 gram oral dose for a 60kg-body weight/day. The TD50 or oral dose required to induce tumors in 50% of rats after exposure over a standard lifetime (TOX_BRM_Rat), measured in mg/kg/day oral dose, is 3.86 mg/kg/day. This would require a human equivalent oral dose of 231.6 mg/day of the pure compound. In mice (TOX_BRM_Mouse), the number is 91.36 mg/kg/day, equating to a 5.48 gram oral dose each day of the single compound. TOX_Code Xr indicates that this compound may present a risk in terms of carcinogenicity in chronic rat studies, because TOX_BRM_Rat is below 4 mg/kg/day oral dose. For a 60 kg body weight, that would amount to 240 mg/kg/day of the pure compound. The compound in its current formulation is so dilute as to be presumed to be safe because it is present in an average daily dose in concentrations well below concentrations cited above.

With an overall mutagenicity score representing the results of virtual Ames testing (TOX_MUT_Risk) of 1, on a score of 0-6 (with or without microsomal

activation), this compound is not likely to present a serious mutagenic risk. Out of the ten virtual Ames tests conducted, this compound flagged one: an elevated risk for a metabolite of the pure compound likely to cause mutagenesis in TA1535 strain of *Salmonella typhimurium* (TOX_MUT_m1535). Other qualitative risk estimations for toxic liabilities may also elevated risk for chromosomal aberration (TOX_CABR) at 71%, but not a risk as a reproductive toxin (TOX_REPR) at 80%.

Human liver adverse effect as the likelihood of causing elevation in the levels of a variety of liver enzymes, including elevated alkaline phosphatase enzyme (TOX_AlkPhos) at 98%, elevated GGT enzyme (TOX_GGT) at 86%, elevated LDH enzyme (TOX_LDH), elevated SGOT (TOX_SGOT) at 90%, and elevated SGPT enzyme at 74%. Thus, extremely concentrated doses of this compound may cause cardiac toxicity, chromosomal aberration, and reproductive toxicity. Elevation at higher doses may result in increased levels of alkaline phosphatase (98%), GGT (86%), LDH, SGOT (99%), and SGPT (74%) liver enzymes. The binary value, yes/no, TOX_Code HP indicates that a combination of liver enzymes levels would rise as a result of this compound, and that this compound could result in hepatotoxicity. Further indication of liver damage or disease, by the presence of higher levels of enzyme, the binary yes/no value, TOX_Code SG indicates increased levels in serum glutamic pyruvic transaminase (SGPT), now known as alanine aminotransferase (ALT) may be elevated in the presence of compound 2. Thus, this compound may cause increased levels of enzymes involved with hepatotoxicity.

The model does not indicate that the compound would be toxic to the androgen receptor or estrogen receptor in rats (TOX_AR_Filter and TOX_ER_Filter).

With an overall ADMET_RISK composite score for all of these models, on a scale of 0-24, this compound scored 3.

BRIs present in Compound 2:

Through CYP2C19, a potential simple Michael Acceptor is formed (metabolite 4, M4). This intermediate may be a possible mutagen (TOX_Code Mut), TOX_MUT_Risk of 3 (out of 0-6).

ADMET results:

CYP2C19 Substrate: Yes

CLint: 0.0145

Intrinsic clearance constants for predicted sites of CYP2C19 mediated metabolism expressed in uL/min/mg microsomal protein.

CYP2E1 Substrate: Non-substrate

MET_UGT2B7: Yes, 97%.

Qualitative model of a glucoronidation by the UDP glucuronosyltransferase 2B7 enzyme. Responsible for conjugation of bile acids, catechol estrogens, morphine and naproxen, hydroxymidazolam, gemfibrozil, AZT and many other drugs.

Midazolam is also a substrate, and structurally similar to this compound, with both sharing an imidazo- ring.

Results: Further metabolism of this molecule via glucoronidation via the UGT2B7 enzyme is predicted.

CYP_RISK: 0

ADMET Code for metabolic liability - a computational filter developed by using a refined subset of the WDI.

TOX_MRTD: Below 3.16 (56%)

A qualitative assessment of the maximum recommended therapeutic dose administered as an oral dose in mg/kg/day.

TOX_MUT_Risk: 1

Represents the results of 10 virtual Ames tests. Predicts overall mutagenicity by counting the number of “Positive” mutagenicity predictions. On a score of 0-6 (with or without microsomal activation).

Result: We would not expect compound 2 to act as a mutagen.

TOX_MUT_Code: m4

Summarizes output of TOX_MUT_* models from the program. Accounts for the compound’s metabolites.

Results: Metabolite 4 (m4) may be a possible mutagen (TOX_Code Mut), with a TOX_MUT_Risk of 2 out of a 0-6 scale.

TOX_MUT_1535: Negative

Qualitative assessment of mutagenicity of the pure compound in TA1535 strain of *S. Typhinmurium*.

TOX_MUT_m1535: Positive (58%)

Qualitative assessment of mutagenicity of the compound and its microsomal rat liver metabolites in TA1535 strain of *S. Typhinmurium*.

TOX_Risk: 3

Score from 0-7, indicates the number of toxicity problems a compound might have. An overall toxic liability score, derived from World Drug Index (this includes natural product structures). Score exceeds 2 for ~10% focused WDI.

Results: This compound may present a low-moderate toxicological risk as a single chemical entity. Dosing, formulation, and carrier vehicle chemistry should be considered.

TOX_Code: Xr, HP, SG

Results:

Xr: This compound may present a risk in terms of carcinogenicity in chronic rat studies.

HP: this compound may present a risk as a hepatotoxin.

SG: serum glutamic pyruvic transaminase (SGPT), now known as alanine aminotransferase (ALT) test. Measured to see if the liver is damaged or diseased, by presence of higher ALT levels. This compound may cause elevated levels of ALT.

TOX_Rat: 721.84 mg/kg/day oral dose LD50

TOX_BRM_RAT: 3.86 mg/kg/day oral dose TD50

Predicts the TD50 value of a particular compound in units of mg/kg/day. The TD50 is the dose of a substance administered orally to rats over the course of their lifetimes that results in the appearance of tumors in 50 percent of their population.

TOX_BRM_MOUSE: 91.36 mg/kg/day oral dose TD50

Similar TD50 value for mouse model.

TOX_CABR: Toxic (71%)

Qualitative estimation of triggering the mutagenic chromosomal aberrations.

TOX_REPR: Non-toxic

Qualitative estimation of reproductive / developmental toxicity.

TOX_AlkPhos: Elevated (98%)

Human liver adverse effect as the likelihood of causing elevation in the levels of Alkaline Phosphatase enzyme.

TOX_GGT: Elevated (70%)

Human liver adverse effect as the likelihood of causing elevation in the levels of GGT enzyme.

TOX_SGOT: Elevated (90%)

Human liver adverse effect as the likelihood of causing elevation in the levels of SGOT enzyme.

TOX_SGPT: Elevated (74%)

Human liver adverse effect as the likelihood of causing elevation in the levels of SGPT enzyme.

TOX_AR_Filter: Non-toxic to AR

Qualitative assessment of the androgen receptor toxicity in rats.

TOX_ER_Filter: Non-toxic to ER

Qualitative assessment of the estrogen receptor toxicity in rats.

Overall ADMET_Risk: 3

On a scale of 0-24. Summarizes all other ADMET Risk/Code models - a computational filter developed by Simulations Plus Inc. using a refined subset of the WDI.

Overall Simulation Summary for Compound 3:

Compound 3 can be expected to be a substrate to CYP2C19 and 2E1.

CYP2C19 is important in the clearance of several classes of exceptionally widely used drugs, such as the proton pump inhibitors (PPIs) like clopidogrel, phenytoin and S-mephenytoin, as well as barbiturates, benzodiazepenes, SSRIs, and the antimalarial proguanil. Possible contraindication with use of this compound alongside PPI (proton pump inhibitors). This compound may be a CYP2E1 substrate (60% likelihood). Very few drugs cleared by 2E1, although it generates reactive oxygen species without substrate present, and are thus implicated in liver malfunction in alcoholics and cancer patients because of these reactive oxygen species. It is also likely that this compound is further metabolized through glucoronidation via the UGT2B7 enzyme. This enzyme is responsible for conjugation of bile acids, catechol estrogens, morphine and naproxen, hydroxymidazolam, gemfibrozil, AZT and many other drugs. Midazolam is also a substrate, and structurally similar to this compound, with both sharing an imidazo- ring. Possible contraindication with use in alcoholics, and cancer patients may be advised. Compound 3 does not appear to pose a liability to overall CYP-function (CYP_RISK: 0).

Maximum recommended therapeutic oral dose (TOX_MRTD), based on mg dosage/60kg-body weight/day, is above 3.16 mg/kg/day, indicating less potential for overall toxicity. LD50 for lethal acute rat (TOX_RAT) in mg/kg oral dose that would be lethal to 50% of rats was 985.77 mg/kg/day, requiring a 59.15 gram oral dose for a 60kg-body weight/day. The TD50 or oral dose required to induce tumors in 50% of rats after exposure over a standard lifetime (TOX_BRM_Rat), measured in mg/kg/day oral dose, is 15.23 mg/kg/day. This would require a human equivalent oral dose of 913.8 mg/day of the pure compound. In mice (TOX_BRM_Mouse), the number is 38.27 mg/kg/day, equating to a 2.30 gram

oral dose each day of the single compound. The compound in its current formulation is so dilute as to be presumed to be safe.

With an overall mutagenicity score representing the results of virtual Ames testing (TOX_MUT_Risk) of 0, on a score of 0-6 (with or without microsomal activation), this compound is not likely to present a serious mutagenic risk. No risk for chromosomal aberration (TOX_CABR) at 94%, or as a reproductive toxin (TOX_REPR) at 75% was found.

Human liver adverse effect as the likelihood of causing elevation in the levels of a variety of liver enzymes, including elevated alkaline phosphatase enzyme (TOX_AlkPhos) at 98%, elevated GGT enzyme (TOX_GGT) at 86%, elevated LDH enzyme (TOX_LDH), elevated SGOT (TOX_SGOT) at 76%, and elevated SGPT enzyme at 66%. Thus, extremely concentrated doses of this compound may cause cardiac toxicity, chromosomal aberration, and reproductive toxicity. Elevation at higher doses may result in increased levels of alkaline phosphatase (98%), GGT (86%), LDH, SGOT (76%), and SGPT (66%) liver enzymes. The binary value, yes/no, TOX_Code HP indicates that a combination of liver enzymes levels would rise as a result of this compound, and that this compound could result in hepatotoxicity. Further indication of liver damage or disease, by the presence of higher levels of enzyme, the binary yes/no value, TOX_Code SG indicates increased levels in serum glutamic pyruvic transaminase (SGPT), now known as alanine aminotransferase (ALT) may be elevated in the presence of compound 3. Thus, this compound may cause increased levels of enzymes involved with hepatotoxicity.

The model does not indicate that the compound would be toxic to the androgen receptor or estrogen receptor in rats (TOX_AR_Filter and TOX_ER_Filter).

With an overall ADMET_RISK composite score for all of these models, on a scale of 0-24, this compound scored 3.

BRIs present in Compound 1:

None indicated.

ADMET results:

CYP2C19 Substrate: Yes

CLint: 12.9

Intrinsic clearance constants for predicted sites of CYP2C19 mediated metabolism expressed in uL/min/mg microsomal protein.

CYP2D6 Substrate: Yes (98%)

CLint: 5.0

MET_2D6_Inh: Yes

CYP2E1 Substrate: Yes (58%)

MET_UGT1A6: Yes, 58%.

Qualitative model of a glucoronidation by the UDP glucuronosyltransferase 1A6 enzyme. Metabolizes small, planar, and phenolic chemicals.

Results: Further metabolism of this molecule via glucoronidation via the UGT1A6 enzyme is predicted.

CYP_RISK: 0

ADMET Code for metabolic liability - a computational filter developed by using a refined subset of the WDI.

TOX_MRTD: Above 3.16 (56%)

A qualitative assessment of the maximum recommended therapeutic dose administered as an oral dose in mg/kg/day.

TOX_MUT_Risk: 0

Represents the results of 10 virtual Ames tests. Predicts overall mutagenicity by counting the number of “Positive” mutagenicity predictions. On a score of 0-6 (with or without microsomal activation).

Result: We would not expect compound 3 to act as a mutagen.

TOX_Risk: 2

Score from 0-7, indicates the number of toxicity problems a compound might have. An overall toxic liability score, derived from World Drug Index (this includes natural product structures). Score exceeds 2 for ~10% focused WDI.

Results: This compound may present a low-moderate toxicological risk as a single chemical entity. Dosing, formulation, and carrier vehicle chemistry should be considered.

TOX_Code: HD, HP, SG

Results:

HD: This compound possesses hydrogen bond donors.

HP: this compound may present a risk as a hepatotoxin.

SG: serum glutamic pyruvic transaminase (SGPT), now known as alanine aminotransferase (ALT) test. Measured to see if the liver is damaged or diseased, by presence of higher ALT levels. This compound may cause elevated levels of ALT.

TOX_Rat: 985.77 mg/kg/day oral dose LD50

TOX_BRM_RAT: 15.23 mg/kg/day oral dose TD50

Predicts the TD50 value of a particular compound in units of mg/kg/day. The TD50 is the dose of a substance administered orally to rats over the course of their lifetimes that results in the appearance of tumors in 50 percent of their population.

TOX_BRM_MOUSE: 38.27 mg/kg/day oral dose TD50

Similar TD50 value for mouse model.

TOX_CABR: Toxic (71%)

Qualitative estimation of triggering the mutagenic chromosomal aberrations.

TOX_REPR: Non-toxic

Qualitative estimation of reproductive / developmental toxicity.

TOX_AlkPhos: Elevated (98%)

Human liver adverse effect as the likelihood of causing elevation in the levels of Alkaline Phosphatase enzyme.

TOX_GGT: Elevated (70%)

Human liver adverse effect as the likelihood of causing elevation in the levels of GGT enzyme.

TOX_SGOT: Elevated (90%)

Human liver adverse effect as the likelihood of causing elevation in the levels of SGOT enzyme.

TOX_SGPT: Elevated (74%)

Human liver adverse effect as the likelihood of causing elevation in the levels of SGPT enzyme.

TOX_AR_Filter: Non-toxic to AR

Qualitative assessment of the androgen receptor toxicity in rats.

TOX_ER_Filter: Non-toxic to ER

Qualitative assessment of the estrogen receptor toxicity in rats.

Overall ADMET_Risk: 3

On a scale of 0-24. Summarizes all other ADMET Risk/Code models - a computational filter developed by Simulations Plus Inc. using a refined subset of the WDI.

Conclusions

Phytochemicals isolated from *Iresine celosia* and their CYP450-produced biological metabolites were screened for their potential toxicity using the ADMET predictor proprietary software, a modeling tool from Simulations Plus. The software was also used to predict metabolites formed from chemical structures within the body, to analyze whether these structures are likely to go on to cause toxicity, and to determine the ability of these compounds to interfere with other drug substances, by way of their ability to inhibit Cytochrome P450 (CYP) drug metabolizing enzymes.

ADMET Predictor proved capable of predicting CYP sites of metabolism. ADMET Predictor was found unable to handle Michael addition reactions (including when these reactions were CYP mediated). ADMET Predictor unable to handle epoxides or arene oxides, because of the extremely brief and fleeting nature of these compounds, but it was correctly predict oxidation of phenyl ring at para position, which is the result of an epoxide-forming reaction. Most O- and N-dealkylations were also correctly predicted from test studies. Overall, the program accomplished what it sets out to do: to cover the most commonly observed potential sites of oxidation.

Based on the information obtained from *in silico* screening of compounds isolated from *Iresine celosia* formulation, a reasonable expectation of safety is warranted when using this preparation as directed.

Conclusion

Through a comprehensive phytochemical evaluation, phytochemical compounds present within the Odyliresin™ formulation have been isolated, and identified using a variety of chromatographic and spectroscopic techniques, including HR-MS, NMR, HPLC-UV, FT-IR and CD spectroscopy. Unique botanical marker compounds have been identified, which allows for authentication of the identity of the botanical and standardization of the formulation extract. Methods have been developed to isolate these compounds, and fingerprint profiles generated to characterize the extract. These compounds are structurally rare within the plant kingdom, and contain stereochemical complexity that derivatization and crystallization methods have failed to resolve. At present, we are using electronic circular dichroism (E-CD) and computer simulations to attempt to resolve the three stereocenters, with a total of 8 possible overall conformations.

Biological activity of these compounds has been screened using *in silico* docking experiments with the human androgen receptor (AR), prostate-cancer relevant mutant types and wild type varieties. This preliminary information supported further testing in vitro and the marker compounds did, in fact, prove active on the 12.5 to 50 μ M range. This data suggests that *I. celosia* may have implications in promoting prostate health. Additional testing of the compound for mechanism of action studies could be interesting. Based on comparable *in silico* docking results as EGCG and aristolochic acid, known antiandrogens, it is possible that these compounds may also work to competitively inhibit the binding of testosterone to the AR. However, further testing through receptor based competitive inhibition assays would be necessary.

These compounds were also screened for biological activity and reactivity, based on the presence of natural product specific biologically reactive intermediates. The compounds were further screened *in silico* using proprietary computational toxicology software, Simulations Plus. Sites of metabolism and CYP-inhibition were considered, as were mutagenicity, implications adverse liver function, and general dosing, among other considerations. Guanidine alkaloid compounds **1**, **2**, and even **3** are metabolized primarily by the CYP2C19, however, CYP219, 2E1 and 2D6 may also play a role in their clearance from the body. Phase II glucoronidation of these compounds is also likely, with UGT2B7 also becoming involved. Predicted metabolites were also screened for their reactivity. Further animal studies could be useful to confirm LD50 and TD50 values derived from computational simulation, in either rat or mouse. However, it is likely that the toxicity arising from the use of these individual compounds within concentrated doses of the extract is quite low. Therefore, animal studies conducted on a small scale and in one species may be sufficient to obtain the confirmatory information needed. Overall, a generalized evaluation of the safety and risk profile of the botanical's individual compounds was obtained, from which can be extrapolated a low level of risk when using this product as directed.

It is important to keep in mind that the formulation is not comprised solely of active principles, but that the formulation is a plant extract containing a variety of compounds, but health relevant and those that may be irrelevant. Similarly, the cell testing assays we conduct and docking simulations are simplified models that consider the individual structures themselves. Only by considering the totality of this information can we approximate the possible biological activity of the formulation in total. The study of complex botanical materials is its own

discipline, and attempts to find isolable principles and standardize these extracts currently represents the most useful strategy toward understanding their activity in humans. The natural variety inherent in preparations, in growing conditions, in species evolution from a chemocentric perspective, the complex matrix in which what are thought to be active principles are delivered to the body is often discounted, and can present challenges to the standardization of such preparations. Based on HPLC standardization of the Odyliresin™ formulation of *Iresine celosia*, it's difficult to assess whether these compounds would be present in enough quantity to affect a health outcome. However, synergistic effects of compounds within the extract may play a role in its biological activity. Such information could not be obtained from computational toxicology studies at this time, and in such a case, the thoughtful conduct of animal studies may prove helpful in further elucidating the formulation's modes of action.

Chemistry is the key to understanding natural products, and considering whether herbal medicines “work” and whether or not they are safe. At present, the advances in chemistry have far outpaced the development of clinical pharmacology and toxicology to understand the working of complex botanical mixtures. Because definitive pharmacological and toxicological results are hard to come by with respect to herbal medicines, some take this to mean that herbals have no actual health effect, and therefore no basis in therapy from a scientific perspective. Nevertheless, people continue to buy, ingest, and use herbal products. Chemistry present in herbal products becomes part of the larger biochemical milieu of items people ingest and substances to which people are exposed, which also includes food, water, and alcohol, too. Because this complex milieu is difficult to study, and at present only being tackled in the realm of systems

pharmacology; because chemistry is by far the most advanced scientific expression of ethnopharmacology — it seems reasonable to leverage what we know to be true about the chemistry and bioactivity/bioreactivity profile of these compounds and use it to consider compounds for which information is lacking, and distinguish thresholds of concern that may warrant further testing to ensure a reasonable expectation of safety.

Rare Guanidine Alkaloids from *Iresine celosia*

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Supporting Information Available

For Compound **1**

Figure S1. ¹H NMR spectrum of Compound (**1**) in (CD₃)₂S=O

Figure S2. ¹³C NMR spectrum of Compound (**1**) in (CD₃)₂S=O

Figure S3. COSY correlations of Compound (**1**) in (CD₃)₂S=O

Figure S4. COSY spectrum of Compound (**1**) in (CD₃)₂S=O

Figure S5: HSQC spectrum of Compound (**1**) in (CD₃)₂S=O

Figure S6: HMBC correlations (H → C) for Compound (**1**) in (CD₃)₂S=O

Figure S7: HMBC spectrum (H → C) of Compound (**1**), in (CD₃)₂S=O, unexchanged

Figure S8: HMBC spectrum (H → C) of Compound (**1**), proton exchanged with D₂O

Figure S9. NOESY correlations of Compound (**1**) in $(\text{CD}_3)_2\text{S}=\text{O}$

Fig. S10. NOESY spectrum of Compound (**1**) in $(\text{CD}_3)_2\text{S}=\text{O}$

Fig. S11. NOESY spectrum of (**1**) in $(\text{CD}_3)_2\text{S}=\text{O}$, with irradiated proton at 3.82 ppm

Fig. S12. NOESY spectrum of (**1**) in $(\text{CD}_3)_2\text{S}=\text{O}$, with irradiated proton at 3.46 ppm

Figure S13. ^1H NMR spectrum of Compound (**1**) in $(\text{CD}_3)_2\text{S}=\text{O}$, unexchanged

Figure S14. ^1H NMR spectrum of Compound (**1**) in $(\text{CD}_3)_2\text{S}=\text{O}$, exchanged with D_2O

For Compound **2**

Figure S1. ^1H NMR spectrum of Compound (**2**) in $(\text{CD}_3)_2\text{S}=\text{O}$

Figure S2. ^1H NMR spectrum of Compound (**2**) in (CD_3OD)

Figure S3. COSY correlations of Compound (**2**) in $(\text{CD}_3)_2\text{S}=\text{O}$

Figure S4. COSY spectrum of Compound (**2**) in $(\text{CD}_3)_2\text{S}=\text{O}$

Figure S5. ^{13}C NMR spectrum of Compound (**2**) in $(\text{CD}_3)_2\text{S}=\text{O}$

Figure S6: HSQC spectrum of Compound (**2**) in $(\text{CD}_3)_2\text{S}=\text{O}$

Figure S7: HMBC correlations ($\text{H} \rightarrow \text{C}$) of Compound (**2**) in $(\text{CD}_3)_2\text{S}=\text{O}$

Figure S8: HMBC spectrum ($\text{H} \rightarrow \text{C}$) of Compound (**2**) in $(\text{CD}_3)_2\text{S}=\text{O}$

Figure S9. ^1H NMR spectrum of Compound (**2**) in $(\text{CD}_3)_2\text{S}=\text{O}$, unexchanged

Figure S10. ^1H NMR spectrum of Compound (**2**) in $(\text{CD}_3)_2\text{S}=\text{O}$, exchanged with D_2O

Figure 5: Isolation scheme for *Iresine celosia*

Figure 6. ^1H NMR spectrum of Compound (1) in $(\text{CD}_3)_2\text{S}=\text{O}$, unexchanged

Figure 7. ^1H NMR spectrum (1) in $(\text{CD}_3)_2\text{S}=\text{O}$, exchanged with D_2O

Figure 8. COSY correlations of Compound (**1**) in (CD₃)₂S=O

Figure 9. COSY spectrum of Compound (**1**) in (CD₃)₂S=O

Figure 10. ^{13}C NMR spectrum of Compound (**1**) in $(\text{CD}_3)_2\text{S}=\text{O}$

Figure 11. HSQC spectrum of Compound (**1**) in $(\text{CD}_3)_2\text{S}=\text{O}$

Figure 12. HMBC correlations (H $\rightarrow$ C) for Compound (**1**) in $(\text{CD}_3)_2\text{S}=\text{O}$

Figure 13: HMBC spectrum (H $\rightarrow$ C) for Compound (**1**), in $(\text{CD}_3)_2\text{S}=\text{O}$, unexchanged

Figure 14: HMBC spectrum (H $\rightarrow$ C) for Compound (**1**), proton exchanged with D₂O

Figure 15. Key NOESY correlations of Compound (**1**) in $(\text{CD}_3)_2\text{S}=\text{O}$

Figure 16. NOESY spectrum of Compound (**1**) in $(\text{CD}_3)_2\text{S}=\text{O}$

Figure 17. NOESY Spectrum of Compound (1) in $(\text{CD}_3)_2\text{S}=\text{O}$,
with irradiated proton at 3.82ppm

Figure 18. NOESY spectrum of Compound (1) in $(\text{CD}_3)_2\text{S}=\text{O}$,
with irradiated proton at 3.46 ppm

Figure 19: IR Spectrum of Compound (1)

Figure 20: MS Spectrum for Compound (1)

MS Spectrum Peak List

<i>m/z</i>	<i>Calc m/z</i>	<i>Diff(ppm)</i>	<i>z</i>	<i>Abund</i>	<i>Formula</i>	<i>Ion</i>
242.15	242.1499	0.24	1	114901.5	C ₁₁ H ₂₀ N ₃ O ₃	(M+H) ⁺
243.1526	243.1528	-0.68	1	14650.9	C ₁₁ H ₂₀ N ₃ O ₃	(M+H) ⁺
244.1552	244.155	0.54	1	1705.4	C ₁₁ H ₂₀ N ₃ O ₃	(M+H) ⁺
245.153	245.1574	-18.3	1	228.5	C ₁₁ H ₂₀ N ₃ O ₃	(M+H) ⁺

Table 5. Spectral Assignments for Compound (2)

<u>Position</u>	<u>δ C (mult.)</u>	<u>δ H (mult., J in Hz)</u>	<u>COSY</u>	<u>HMBC</u>
1	18.17, 3H	1.73, s	H-4	C-5, C-3, C-2
2	138.45			H-5, H-4, H-1
3	117.42			H-5, H-4, H-1
4	41.87, 2H	3.95, dd (7.1)	H-6, H-1	C-3, C-2, C-4', C-4''
5	25.86, 3H	1.79, s	H-4	C-3, C-2, C-1
6		5.18, t (7.1)	H-4	C-5, C-1
3'	2H	8.05, s		
4'	159.14			H-4, H-4''
1''	12.87, 3H	1.14, d (7.1)	H-2''	C-5'', C-3'', C-2''
2''	43.73, 1H	2.56, m	H-3'', H-1''	C-5'', C-3'', C-1''
3''	54.53, 1H	4.11, m (10.1, 6.3)	H-4'', H-2''	C-5'', C-4'', C-1''
4''	50.88, 2H	3.72, dd (10.1)	H-4'', H-3''	C-3'', C-2''
		3.36, dd (10.1, 6.3)	H-4'', H-3''	C-3'', C-2''
5''	175.59			H-2'', H-1''
6''	1H	12.69, s

Figure 21. ^1H NMR spectrum of Compound (**2**) in $(\text{CD}_3)_2\text{S}=\text{O}$, unexchanged

Figure 22. ^1H NMR spectrum of Compound (**2**) in $(\text{CD}_3)_2\text{S}=\text{O}$, exchanged with D_2O

Figure 23. Key $^1\text{H} \rightarrow ^1\text{H}$ COSY correlations of Compound (**2**) in $(\text{CD}_3)_2\text{S}=\text{O}$

Figure 24. COSY spectrum of Compound (**2**) in (CD₃)₂S=O

Figure 25. ^{13}C NMR spectrum of Compound (**2**) in $(\text{CD}_3)_2\text{S}=\text{O}$

Figure 26: HSQC spectrum of Compound (**2**) in $(\text{CD}_3)_2\text{S}=\text{O}$

Figure 27: HMBC correlations (H $\rightarrow$ C) for Compound (**2**) in $(\text{CD}_3)_2\text{S}=\text{O}$

Figure 28: HMBC spectrum (H $\rightarrow$ C) for Compound (**2**) in $(\text{CD}_3)_2\text{S}=\text{O}$

Figure 29: IR spectrum of Compound (2)

Figure 30: MS Spectrum for Compound (2)

MS Spectrum Peak List

<i>m/z</i>	<i>Calc m/z</i>	Diff(ppm)	<i>z</i>	Abund	Formula	Ion
226.1547	226.155	-1.25	1	15759.5	C ₁₁ H ₂₀ N ₃ O ₂	(M+H) ⁺
227.158	227.1579	0.35	1	1856.1	C ₁₁ H ₂₀ N ₃ O ₂	(M+H) ⁺
228.1599	228.1602	-1.52	1	266.5	C ₁₁ H ₂₀ N ₃ O ₂	(M+H) ⁺

**Table 6. Spectral Assignments for Compound (3),
Hydroxygalegine**

Position	δ C (mult.)	δ H (mult., J in Hz)	COSY	HMBC
2	156.34			H-5
5	45.5, 2H	4.09, d (7.1)	H-10, H-6	
6	119.52, 1H	5.31, t (7.1)	H-10, H-5	H-8, H-5. H-10
7	140.28			H-8, H-5. H-10
8	60.53, 2H	4.12, s		H-6, H-10
10	20.97, 3H	1.81, s	H-6, H-5	H-6, H-8

Figure 31. ^1H spectrum of Compound (**3**) in CD_3OD

Figure 32. ^{13}C spectrum of Compound (**3**) in CD_3OD

Figure 33. COSY spectrum of Compound (**3**) in CD₃OD

Figure 34. HMBC spectrum of Compound (**3**) in CD₃OD

Figure 35. HSQC spectrum of Compound (**3**) in CD₃OD

Table 7. Ligand Binding Affinities for Control Structures with WT and Mutant Androgen Receptors

PBD ID#	Ligand	affinity kcal/mol	rmsd l.b.	Dist from best mode, rmsd u.b.
2YHD	testosterone	-11.2	0	0
		-8.9	1.472	6.158
		-8.4	1.214	6.579
1I37		-11.2	0	0
		-9.3	1.148	6.536
		-8.3	1.477	2.715
2Q7K		-11.8	0	0
2YHD	dihydrotestosterone	-10.2	0	0
		-9.4	1.529	2.897
		-9.4	1.145	6.123
1I37		-10.9	0	0
		-9.5	1.110	6.095
		-9.2	1.273	2.573
2Q7K		-11.3	0	0
		-10.1	1.150	6.093
		-9.9	1.180	2.544
2YHD	stigmaterol	-1.7	0	0
		-1.5	2.781	7.429
		-0.3	1.577	3.298
1I37		-1.6	0	0
		-1.5	2.782	7.433
		-0.3	2.838	7.497
2Q7K		-1.9	0	0
		-1.4	2.795	8.079
		-0.8	0.890	2.486
2YHD	betasitosterol	-2.3	0	0
		-1.1	2.796	7.498
		-0.5	2.777	7.364
1I37		-0.1	0	0
		-2.8	1.228	2.848
		-0.5	0	0
2Q7K		-1.2	2.881	7.442
		-1.2	2.792	7.508
		-6.1	0	0
2YHD	atraric acid	-5.9	1.547	1.859
		-5.8	2.259	5.216
		-6.3	0	0
1I37		-5.9	2.232	3.037
		-5.9	1.983	5.014
		-6.8	0	0
2Q7K		-6.7	0.665	1.158
		-6.2	1.905	5.012
		-6.8	0	0
2YHD	EGCG	-6.8	0.014	1.443
		-6.8	0	0
		-4.2	0	0
1I37		-4.1	0.005	1.443
		-6.0	0	0
		-6.0	0.030	1.443

Table 8. Ligand Binding Affinities for *I. celosia* Structures with WT and Mutant Androgen Receptors

PBD ID#	Ligand	affinity kcal/mol	rmsd l.b.	Dist from best mode, rmsd u.b.
2YHD	compound 1	-6.6	0	0
		-6.5	2.251	5.559
		-6.4	2.147	5.984
1I37		-6.8	0	0
		-6.8	1.507	1.875
		-6.6	1.947	6.062
2Q7K		-7.1	0	0
		-7.0	2.195	5.709
		-7.0	1.409	1.851
2YHD	compound 2	-6.8	0	0
		-6.8	3.537	5.840
		-6.6	3.611	5.954
1I37		-6.7	0	0
		-6.7	1.703	2.434
		-6.6	1.386	1.847
2Q7K		-7.1	0	0
		-7.0	3.599	5.995
		-7.0	3.234	5.757
2YHD	hydroxygalegine	-5.1	0	0
		-5.0	0.993	1.156
		-4.8	3.163	4.828
1I37		-5.3	0	0
		-5.2	1.052	1.527
		-4.9	3.516	4.812
2Q7K		-5.5	0	0
		-5.4	1.279	1.747
		-5.2	3.479	4.904
2YHD	tlatancuayin	-7.3	0	0
		-6.1	1.739	7.080
		-6.0	1.612	7.120
1I37		-6.3	0	0
		-5.7	1.792	6.695
		-5.7	1.215	2.555
2Q7K		-6.7	0	0
		-6.4	1.813	7.315
		-6.1	1.622	7.087
2YHD	drimane 2b	-7.3	0	0
		-7.2	1.389	5.513
		-7.1	1.364	5.244
1I37		-7.2	0	0
		-6.9	1.292	5.490
		-6.9	1.488	3.227
2Q7K		-7.6	0	0
		-7.6	1.375	5.516
		-7.3	1.353	3.00
2YHD	drimane 3	-8.5	0	0
		-8.1	1.575	5.636
		-8.1	1.708	5.957
1I37		-8.4	0	0
		-8.4	1.643	5.424
		-8.2	1.542	5.643
2Q7K		-9.0	0	0
		-9.0	1.534	5.641
		-8.7	1.622	5.463

Table 9. Ligand Binding Affinities for Natural Product Structures Associated with Prostate Health with WT and Mutant ARs

PBD ID#	Ligand	affinity kcal/mol	rmsd l.b.	Dist from best mode, rmsd u.b.
2YHD	apigenin	-8.6	0	0
		-8.2	2.102	3.740
		-8.1	2.037	6.748
1I37		-7.5	0	0
		-7.4	0.124	1.076
		-7.3	1.995	6.568
2Q7K		-9.1	0	0
		-9.0	0.032	1.074
		-8.8	2.091	3.658
2YHD	baicalein	-8.4	0	0
		-7.8	2.522	6.121
		-7.5	2.540	6.505
1I37		-8.0	0	0
		-7.9	2.337	6.491
		-6.5	1.723	3.319
2Q7K		-8.6	0	0
		-8.6	0.019	1.074
		-8.5	1.878	3.391
2YHD	emodin	-8.7	0	0
		-8.4	1.348	5.535
		-8.4	1.192	1.347
1I37		-8.2	0	0
		-8.0	1.473	5.560
		-6.8	1.116	3.221
2Q7K		-9.1	0	0
		-8.9	1.501	5.572
		-8.9	1.150	3.306
2YHD	genistein	-9.2	0	0
		-9.1	1.148	6.884
		-9.1	1.137	6.806
1I37		-9.3	0	0
		-8.8	1.469	6.965
		-8.3	1.546	7.143
2Q7K		-10.1	0	0
		-9.6	1.183	6.882
		-9.4	1.272	6.819
2YHD	icaritin	-7.7	0	0
		-7.2	1.365	1.720
		-5.7	2.073	7.059
1I37		-6.9	0	0
		-5.3	2.004	7.105
		-5.2	1.503	2.078
2Q7K		-7.2	0	0
		-6.1	1.457	1.930
		-5.7	2.025	7.057
2YHD	xanthohumol	-5.8	0	0
		-5.7	1.420	3.829
		-5.4	1.925	7.397
1I37		-2.9	0	0
		-2.9	2.038	3.288
		-2.8	12.33	14.592
2Q7K		-3.7	0	0
		-3.7	1.717	4.113
		-3.6	1.040	1.580

Figure 36: *In vitro* prostate cancer cell screening of lab-generated butanol extract against AR-positive LNCaP and AR-negative PC3 cells

Figure 37: *In vitro* prostate cancer cell screening of Compound (1) against AR-positive LNCaP and AR-negative PC3 cells

Figure 38: *In vitro* prostate cancer cell screening of Compound (2) against AR-positive LNCaP and AR-negative PC3 cells

Figure 39: Ethyl acetate (bottom) fraction from 30% ethanol extract of *Iresine celosia* overlaps strongly with the butanol partition (top)

Fig. 40: Procedure for the determination of compounds in lab-generated *Iresine celosia* extract and manufacturer's Odyliresin™ formulation

Table 10: Characteristics of the compounds isolated from the *Iresine celosia* lab formulation's butanol extract (Lab-bu)

	Compound Name	MW	peak(s) nm	Rt (min)
1	Compound 1	241.29	212	17.7
2	Compound 2	225.29	212	25.4
3	Hydroxygalegine	143.19	210	19
4	Uracil	112.09	256	21.5
5	Adenine	135.13	210, 260	5.6
6	3-indole-carboxylic acid	161.16	212, 282	30
7	2'-deoxy-uridine	228.20	210, 260	8.3
8	2'-deoxy-thymidine	242.23	264	11.8
9	2'-deoxy-5'-O-methyl-uridine	242.23	210, 260	14.8
10	4-methoxy-benzoic acid (anisic acid)	152.15	260	23.3
11	Phenylacetamide	135.07	212	30.2

Table 11: Calibration curve data for selected compounds isolated from the *Iresine celosia* lab formulation's butanol extract (Lab-bu)

Compound	Rt (min)	Slope (+SD)	r ²	LOD (mg/mL)	LOQ (mg/mL)
1	17.7	21.3 x 10 ⁷ (± 8)	0.99	0.13	0.39
2	25.4	4.4 x 10 ⁷ (± 1.7)	0.99	0.18	0.57
3	19	3.3 x 10 ⁷	0.99		
4	21.5	4.3 x 10 ⁷	0.99		
8	11.8	19.5 x 10 ⁷	0.99		
10	23.3	14 x 10 ⁷	0.99

Figure 41: Lab n-butanol (Lab-bu) extract at 212 nm

Figure 42: HPLC-UV chromatograms of *Iresine celosia* 30% ethanol extracts (D, F) and their butanol partitions (A, B, C, E) at various concentrations showing the presence of compounds (1-11)

Figure 43: Compounds (1), (2), (3) retention times compared with Lab-bu formulation (50 mg/mL)

Figure 44: Compounds (4), (8), (10) retention times compared with Lab-bu formulation (50 mg/mL)

Fig. 45: Calibration Curves for Compound (1) at concentrations 0.0626 to 1.0 mg/mL

Fig. 46: Calibration Curves for Compound (2) at concentrations 0.0626 to 1 mg/mL

Fig. 47: Calibration Curves for Compounds (3) at various concentrations

Fig. 48: Calibration Curves for Compounds (4) at various concentrations

Fig. 49: Calibration Curves for Compounds **(8)** at various concentrations

Fig. 50: Calibration Curves for Compounds **(10)** at various concentrations

Fig. 51: Calibration curve for presence of Compound (1) in Lab-bu formulation

Fig. 52: Calibration curve for presence of Compound (2) in Lab-bu formulation

Table 12: Calibration curve data for presence of Compound (1) in Lab-bu formulation

Formulation Conc.	C1 (mAU)	Compound Conc. $y = 213,227,870.70x + 31,411,604.31$	STDEV	RSD %
25	24,095,597		75,927	0.3
50	35,298,368	0.018	177,776	0.5
75	48,057,725	0.078	146,607	0.3
150	93,947,009	0.293	50,742	0.1
100	77,316,453	0.215		
500	114,027,640	0.387		

Table 13: Calibration curve data for presence of Compound (2) in Lab-bu formulation

Formulation Conc.	C2 (mAU)	Compound Conc. $y = 44,367,064.37x + 12,035,158.63$	STDEV	RSD %
25	15,861,869	0.086	33,011	0.2
50	22,053,873	0.226	2,788,877	12.6
75	36,997,866	0.563	4,930,550	13.3
100	47,774,367	0.806	10,840,923	15.4
150	71,661,551	1.344	2,871,870	4
500	108,202,498	2.168

Fig. 53: Calibration curve for presence of Compound (3) in Lab-bu formulation (single run)

Fig. 54: Calibration curve for presence of Compounds (4) in Lab-bu formulation (single run)

Fig. 55: Calibration curve for presence of Compound (8) in Lab-bu formulation (single run)

Fig. 56: Calibration curve for presence of Compound (10) in Lab-bu formulation (single run)

Fig. 57: Calibration curve for presence of Compound (1) in Odyli-bu formulation

Fig. 58: Calibration curve for presence of Compound (2) in Odyli-bu formulation

Table 14: Calibration curve data for presence of Compound (3) in Lab-bu formulation

<u>Conc.</u>	<u>C3</u>	$y = 32,909,431.43x + 34,707,557.30$	<u>STDEV</u>	<u>RSD%</u>
25	13,658,054		150,840	1.1
50	19,351,673		560,898	2.9
75	25,341,316		14,069	0.001
100	29,427,093		0	0
150	44,140,639	0.287	373,174	0.8
500	72,696,509	1.154	0	0

Table 15: Calibration curve data for presence of Compound (4) in Lab-bu formulation

<u>Conc.</u>	<u>C4</u>	$y = 43,120,226.10x + 4,947,652.33$	<u>STDEV</u>	<u>RSD%</u>
25	6,264,923	0.031	20,434	0.3
50	11,605,373	0.154	81,082	0.7
75	17,337,917	0.287	204,982	1.2
150	33,534,116	0.663	192,108	0.6

Table 16: Calibration curve data for presence of Compound (8) in Lab-bu formulation

<u>Conc.</u>	<u>C8</u>	$y = 194,620,399.2110x + 2,097,490.6430$	<u>STDEV</u>	<u>RSD%</u>
25	4,107,492	0.010	17,233	0.4
50	7,693,548	0.029	43,770	0.6
75	12,376,112	0.053	737,959	6.0
150	25,671,359	0.12	36,678	0.1

Table 17: Calibration curve data for presence of Compound (10) in Lab-bu formulation

<u>Conc.</u>	<u>C10</u>	$y = 140,436,619.22x + 12,351,658.00$	<u>STDEV</u>	<u>RSD%</u>
25	6,198,365		14,135	0.2
50	11,484,942		6,638	0.1
75	17,255,272	0.035	446,842	2.6
150	34,207,895	0.16	191,226	0.6

Table 18: Calibration curve data for presence of Compound (1) in Odyli-bu formulation

<u>Formul. Conc.</u>	<u>C1</u>	<u>Compound Conc.</u> $y = 44,367,064.37x + 12,035,158.63$	<u>STDEV</u>	<u>RSD</u> %
75	8,247,406		445,954	3.3
100	11,330,014		7,101	0.05
150	13,750,423		130,586	0.9
250	19,109,994		752,666	3.8
500	34,830,045	0.016	1,046,853	4.6

Table 19: Calibration curve data for presence of Compound (2) in Odyli-bu formulation

<u>Formul. Conc.</u>	<u>C2</u>	<u>Compound Conc.</u> $y = 44,367,064.37x + 12,035,158.63$	<u>STDEV</u>	<u>RSD</u> %
50	13,671,820	0.037	445,954	3.3
75	14,385,347	0.053	7,101	0.05
100	14,818,083	0.063	130,586	0.9
250	19,865,699	0.176	752,666	3.8
500	21,833,592	0.221	1,046,853	4.6

Figure 60: Prediction of toxicity outcomes for the following categories based on chemical structure

Figure 61: Predicted sites of metabolism for Compound 1

Figure 62: Predicted metabolites for Compound 1

Figure 63: Predicted sites of metabolism for Compound 2

Figure 64: Predicted metabolites for Compound 2

Figure 65: Predicted metabolites for Compound 3