CHARACTERIZATION OF ANXIOLYTIC COMPOUNDS FROM *ANNONA MURICATA* LEAF EXTRACT: A COMPUTATIONAL AND EXPERIMENTAL APPROACH

by

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ABSTRACT OF THE THESIS

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Annona muricata is a perennial tree found in most tropical areas of the world, including Western Africa, Central and South America and Southeast Asia. It has been used around the world medicinally by several cultures. Some of these cultures use *A. muricata* as an anxiolytic tea given to unruly patients. Traditional anxiolytic uses of *Annona muricata* in medicine have long existed, without knowledge of the active compound or compounds. We aim to scientifically support and extend these traditional uses by characterizing the bioactive compound(s) within the leaf extract. The active structures can then be modified to provide potentially new classes of active drugs. The anti-anxiety effects of *A. muricata* seen in traditional medicine were characterized by using a set of widely-accepted behavioral models of anxiolytic effects in mice. Partial phytochemical profiling done through ultra-high performance liquid chromatography (UHPLC) and gas chromatography-mass spectrometry (GC-MS) has identified a list of compounds that comprise the different fractions of the leaf extract. Through the mouse behavioral investigations, an active fraction has been determined to have a sedative effect, and through a dose-response study, an anxiolytic-like activity has been determined for the same fraction. Further fractionation of the extract and subsequent mouse behavioral studies have resulted in the discovery of smaller groups of potentially active compounds

that can be fully profiled and modeled using Computer-Aided Drug Discovery (CADD). The use of mouse behavioral models of anxiolytic effects and the chromatographic analysis of the leaf extract allowed the identification of active fractions in aqueous extract. Both sedative and anxiolytic-like concentrations of the extract's polar components were demonstrated, and further profiled. The profiled chemical compounds can be modeled to better suggest which compounds may provide the bioactive effects *in vivo*.

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

1. Introduction

Ethnobotany is the study of the symbiotic relationship between humans and plants, more specifically on the ways that humans use plants in everyday life. Ethnobotanical species are used as sources of food and medicinal remedies. Across the world, it is still common for plants to be used as a major component in natural medicines. Medicinal plants have also been found to be the number one source of biologically active compounds, and many documented medicinal plants have been scientifically proven to have therapeutic applications (Borris, 1996; Carlini, 2003; Faustino et al, 2010).

Ethnobotany is an important branch of science because many cultures, including many Western cultures, are looking for better alternatives to the already existing synthetic medicines. In addition to the drawback of having adverse side effects and frequent addiction with therapeutic usage, synthetic medicines also lose effectiveness over time as the biological system builds a tolerance to it, requiring different doses or even different drugs for the same effect. This opens up the possibility of using natural resources, such as plants, to see if a better alternative may exist in the natural world. Out of the 250,000-500,000 plants that exist on this planet, only 1-10% have been studied to determine if any potential medicinal value exists (Borris, 1996). This statistic supports the idea that plant species need to be studied and their medicinal values determined.

Another benefit of ethnobotany is that many of the compounds that have already been discovered are secondary metabolites, meaning that they are not directly involved with the plant's metabolic processes (de Souza et al, 2009). Therefore, harvesting these compounds for mass production will not destroy or alter the plant's normal processes, but will allow for a steady renewable resource to support man's needs for treatment.

Although widely used in Benin, West Africa as an anti-anxiety treatment, *Annona muricata* can be found all over the world as a treatment for a wide variety of ailments. *A. muricata* is a deciduous tree that belongs to the Annonaceae family, and it produces a heart-shaped, highly aromatic fruit. The fruit's nectar is commonly used in smoothies and yoghurts, giving this plant yet another cultural use (Lutchmedial et al, 2004). *A. muricata* is known in the United States as "soursop," in Benin as "chap-chap," and in South America as "graviola," "guanabana," and "pawpaw" (Adewole et al, 2008). Soursop is believed to have originated in Central or South America and spread across the world into all major tropical climates, including Western Africa and Southeast Asia. This spread is most likely because all parts of this tree are used in natural medicine spanning the tropics. Natural medicine exploits the bark, leaves, roots, fruit, and fruit seeds of the plant (Onimawo, 2002; de Souza et al, 2009). Parts of *A. muricata* have been used to treat ailments such as cancer (including prostate and liver cancers), diabetes mellitus, and have elicited an anti-viral effect against Herpes simplex virus-1 (Atawodi, 2011; Adewole et al, 2008; Padma et al, 1998). Extracts of soursop, specifically the leaf extract, have exhibited strong antioxidant properties, with a high success rate in capturing free radicals, and have also exhibited anti-inflammatory and antinociceptive properties (Lim, 2012; de Sousa et al, 2010). Various parts of the plant have been used for hypertension, or as a vasodilator, and as an antispasmodic (Lim, 2012).

Potentially one of the most important uses of soursop that has not yet, at least until now, been scientifically investigated is its use as an anti-anxiety treatment. Although often overlooked by society, anxiety disorders are among the most common neurological disorders in the world. In the United States, 15-26 million Americans suffer from one or

more of these disorders annually (Greenberg et al, 1999). Generalized anxiety disorder (GAD), is a prime example of an anxiety disorder that plagues humanity. GAD is characterized by obsessive, chronic worrying, and typically requires long-term treatment. Anxiety disorders are not only a problem because of the chronic worrying, but also because there is a high correlation between anxiety disorders and increased rates of alcohol abuse, marital problems, and suicide attempts (Iosifescu, 2010). The four main anxiety disorders are GAD, panic disorder, obsessive compulsive disorder, and post-traumatic stress disorder (PTSD), and they can be caused by a number of things, such as dietary deficiencies, hormonal changes, traumatic experiences, life stressors, aging, and genetics (Alramadhan et al, 2012; Bandelow et al, 2013).

Current synthetic, short-term anxiety treatments are costly, and may come with many undesired, adverse effects (Lakhan et al, 2010). Benzodiazepines are typically prescribed to patients of anxiety disorders, either instead of other treatments that include selective serotonin reuptake inhibitors (SSRIs) and serotonin-norepinephrine reuptake inhibitors (SNRIs), or in combination with these medications for quick relief that the other treatments do not provide (Bandelow et al, 2013). However, their usage is often accompanied with development of a strong dependence, as well as anterograde amnesia, impaired spatial and motor awareness and coordination (Maremmani et al, 2013). Therefore, many Americans have begun looking into nutraceutical supplements as a remedy for anxiety, with approximately 40% turning to herbal supplements or other alternative medicine (Barnes et al, 2008). This statistic, along with the statistic that less than 10% of the world's plant population has been examined for medicinal value, show,

more than ever, the importance of moving forward into this new frontier of natural medicine (George et al, 2012).

In studying anxiety and developing treatments for it, behavioral mouse models are often used to examine the effects of the anxiolytic agent *in vivo*. One such model, the Light/Dark Conflict Box test, is based on the innate aversion of mice to sources of bright light. It allows more anxious mice to spend a larger percentage of time in a dark area, and allows less anxious mice to explore a bright space (Crawley et al, 1980; Bourin et al, 2003). The most widely used model, the Elevated Plus-Maze test, is considered the most well-established behavioral mouse model in terms of assessing anxiety and motor behavior (Pellow et al, 1986; Foyet et al, 2012). Another model, the Open Field Exploration test, looks at the environment exploration and general locomotive activity of the mice (Prut et al, 2003). All of these approaches use the natural anxiety of mice to gauge if the treatments have any effect in lowering the anxiety of the animal.

This study was conducted to investigate the use of *A. muricata* as an anti-anxiety treatment, and to identify the bioactive compounds within the leaf extract. Three different behavioral mouse model assays were used in this study to determine which fractions of the extract exhibited an anxiolytic or sedative effect, as well as to determine the dose responsive effect of different concentrations of the active fractions. In addition, the extract fractions were administered intraperitoneally (IP) and orally to the mice to determine the effect of metabolism on the efficacy of the active fractions.

In developing new pharmaceuticals, the clinical trials can, and most likely will, get costly. Therefore, it is a benefit to be able to simulate a compound's biological activity before synthesizing and spending all of the money on model assays.

Cheminformatics, an emerging and progressing new computational science, provides the researcher an opportunity to sift through large amounts of data to determine if a specific compound will likely act in a way that would be beneficial for pharmaceutical utilization. This subject is currently a main aspect in today's pharmaceutical industry in terms of drug discovery (Jorgensen, 2004). Once biologically active fractions have been found from the leaf extract of *A. muricata*, they can each be modeled to see how likely they are to be causing the bioactive effect. These compounds can be checked to see if they optimally obey certain necessary parameters for drug development, such as Lipinski's Rule of 5. This allows the researcher to see if the molecule has certain characteristics that are indicative of drug-like behavior. Some of these rules include a limit on molecular weight, as well as relative solubility and lipophilicity (Akella et al, 2010). Even if the bioactive compounds are not optimally drug-like, they can still be chemically modified, as long as the structure important for its bioactive effect is not covered or altered.

2. Materials and Methods

2. 1 Plant Material Collection and Initial Phytochemical Profiling

The plant material (leaves) was collected at Abomey-calavi in Benin (West-Africa) with the following geographical location characterization: latitude (06°27'0"N), longitude (02°21'0"E); and an altitude of 12 m. The collection site was characterized by an average of 80% year round high hygrometry with a subequatorial climate characterized by two rainy and two dry seasons. The total annual pluviometry reaches 1200-1300 mm of water. The ambient temperature is relatively high (26.6°C) with a thermal amplitude of 7°C. The soil is ferrallitic, deep, well-drained and without concretions on rock sedimentary.

Plant material was dried and ground into a powder and was stored at 4°C until extraction. Phytochemical profiling was performed through a multi-solvent based fractionation of the leaf extract and followed using UHPLC and GC-MS. The powdered leaf material (7.011 g) was extracted three times with hexane and subsequently three times with methanol containing 1% glacial acetic acid. Both fractions were dried under vacuum using a rotary evaporator. The hexane-extract yielded 196 mg of residue (2.79%) and the methanol extract yielded 907 mg of residue (12.9%).

The methanol extract was fractionated by liquid/liquid partitioning between water and ethyl acetate. Both fractions were dried under vacuum using a rotary evaporator. The water fraction yielded 514 mg of material (56.7% of the methanol extract) and the ethyl acetate fraction yielded 343 mg (37.8% of the methanol extract).

2.2 Phytochemical screening by Gas Chromatography-Mass Spectrometry (GC-MS)

20 mL of d solvent (hexane or methanol) was added to 800 mg of plant material in a 50 mL Falcon test tube. Samples were extracted by vortexing for 60 min at 500 rpm. The mixture was allowed to settle for another 60 minutes, and 1 mL of sample was transferred into a vial for GC-MS analysis. The sample was analyzed with a Shimadzu GC2010 gas chromatograph coupled to a Shimadzu QP2010-Plus mass spectrometer. A Shimadzu SHRXI-5MS column (length = 30.0 m, film thickness = 0.25 µm, diameter = 0.25 mm) using a linear gradient from 70 °C to 315 °C with an increase of 10 °C per minute to separate the compounds. Data was recorded using GC-MS solution ver. 2.61 software. Major peaks were tentatively identified by comparison to the NIST 2008 library.

2.3 Identification of Bioactive Fraction from Leaf Extract of *A. muricata*

Five samples resulting from the fractionation of the extract were tested in the first behavioral mouse model: (i) plant material still containing all of the extract (positive control), (ii) plant material in which all fractions had previously been extracted (negative control), (iii) a hexane fraction, containing most nonpolar components of the extract, (iv) an ethyl acetate fraction, containing most of the amphipathic components of the extract, and (v) a water fraction, containing most of the polar components of the extract. All fractions were dissolved in phosphate buffer saline (PBS). Solutions from fractions were all made at 5 mg/kg (stock concentration). The stock solutions were diluted further with PBS to obtain solutions for administration at 2.5 mg/kg. Diazepam (positive control) solution was administered at a 2 mg/kg dose only (dissolved in PBS with 1% Tween 80).

2.4 Identification of Bioactive Fraction within Polar Components of Leaf Extract Through Preparative HPLC

Powdered leaves of *A. muricata* were extracted with hexane. All plant material that remained was extracted with methanol + 1% glacial acetic acid. The extract was then partitioned between butanol and water + 1% glacial acetic acid. The butanolic extract was then partitioned between ethyl acetate, methanol, and water + 1% glacial acetic acid, to give three fractions. The water extract was then separated into six fractions using preparative HPLC. Preparative HPLC was performed on a C18 column using a stepped gradient with a flow rate of 15 mL/min. Fractions were collected manually at timed intervals as shown in Table 1. The fractionation was done on a Shimadzu CBM-20A controller and a CTO-20AC oven at 40° C with the solvents water with formic acid and methanol with formic acid. The resin of each fraction was dissolved in PBS for administration to the mice.

2.5 Oral Dose Response Curve of Crude Polar Extract Determination

To determine the optimal concentration for oral administration of the polar extract, 15.27 g of plant material was crushed and extracted twice in methanol and twice in 2-propanol. The extracted material was combined and dissolved in PBS + 1% Tween 80 to ensure most polar compounds would be present in the treatment and the solution would be solubilized for absorption in the animals.

2.6 Animal Housing, Identification of Bioactive Fraction, and Dose Reponse Curve of Polar Fraction and Diazepam, Oral Administration of Potentially Bioactive Fractions, Oral Dose Response Curve of Crude Polar Extract

For all behavioral models, C57 BL/6 mice were purchased from Hilltop Lab Animals, Inc. (Scottsdale, PA). All mice were housed in a reverse light-dark cycle (12 hours/12 hours), with lights on at 19:00. They were given food and water *ad libitum* and the temperature of the facility was maintained at 22.2- 23.3 ºC. Testing was only performed during the dark half of the cycle, starting at about 10:00.

In the first two behavioral bioassays, 24 mice were individually housed and all ID numbers were determined randomly to separate mice into groups. The list of treatments for the second behavioral bioassay can be seen in Table 2. Treatment schedules were also randomly assigned using a die. Treatment and testing schedules can be seen in Tables 3-7 for the first and second assays, respectively. All three behavioral models were utilized in the first and second behavioral assay. After the light/dark box conflict test trials were completed, five mice were sacrificed due to prolapsed intestines. The remaining mice were re-organized into three groups with the same ID numbers, and the positive control solution was no longer used as a treatment. This is justified by the use of diazepam,

which already acts as a positive control. All mice were weighed prior to testing and all injection volumes were 30 mL/kg. All procedures were approved by the Rutgers Institutional Animal Care and Use Committee (IACUC) in a facility accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC).

For the third behavioral bioassay, 20 mice were housed four in each cage. ID numbers were determined randomly as described above. No groups were made for this assay. The list of treatments and treatment schedule of the third behavioral assay can be seen in Tables 8 and 9. Only the Open Field Exploration test was utilized. The fourth mouse assay was also run using 20 mice, separated into four groups of five. Each group received four different concentrations of the same treatment to identify any dose responsive effects. The four treatments were the positive control from the prior bioassay (RUTWS-1001), diazepam, and the two most potentially bioactive fractions of the polar components, as suggested from the prior bioassay (RUTWS-1004 and RUTWS-1005). For water fraction treatments, the four concentrations in descending order are as follows: 2.5 mg/kg, 1 mg/kg, 0.313 mg/kg, and 0 mg/kg (PBS as a negative control). For the generation of a diazepam dose response curve, the concentrations chosen were as follows: 0.9 mg/kg, 0.3 mg/kg, 0.1 mg/kg, and 0 mg/kg (PBS as a negative control). Treatment schedules can be seen in Table 10. The Open Field Exploration test was the only model utilized. To determine the differences in routes of administration, the animals in the fourth behavioral assay were treated orally.

The fifth behavioral bioassay was run with 8 mice to determine an oral dose response curve for the entire polar extract of *A. muricata* leaves. Each mouse was orally

given each of the following concentrations for dose responsive effect determination: 180 mg/kg, 90 mg/kg, 45 mg/kg, and 0 mg/kg (PBS; negative control). The Open Field Exploration and Light/Dark Conflict Box tests were utilized in this assay. Treatment and testing schedules can be seen in Tables 11 and 12.

2.7 Light/Dark Conflict Box Test

2.7.1 Identification of Bioactive Fraction from Leaf Extract of *A. muricata*

Two boxes are separated by a wall in the middle. One box is painted white and left uncovered, while the other box is painted black with a removable cover on top (Figure 1). Each mouse received four treatments: (i) PBS as negative control, (ii) diazepam (positive control; 2 mg/kg solution), (iii) low concentration of fraction (2 mg/kg), and (iv) high concentration of fraction (5 mg/kg). Mice were given an IP injection and set in the testing room to acclimatize, then placed into the testing box and recorded for three minutes with a Kodak 100 Sport camera. All videos were watched and the following parameters were measured: (i) amount of time spent in the open, light box (converted into a percentage of time), (ii) latency into the dark box, or how long until the mouse moved into the dark box after the test began, (iii) time spent in the middle of the light box, (iv) number of rears, and (v) number of transitions between boxes.

2.7.2 Dose Reponse Curve of Polar Fraction and Diazepam, Oral Dose Response Curve of Crude Polar Extract

The testing apparatus remained the same for the second and fifth behavioral bioassays. Different concentrations of the polar fraction and diazepam were administered to the mice as treatments. Mice were placed into the testing apparatus 30 minutes post-

injection and recorded for five minutes. The same parameters were measured during the video analysis.

2.8 Elevated Plus-Maze Test

An elevated platform in the shape of a plus sign was suspended 45 cm off the ground. Each arm was 5 cm wide and 30 cm long. The walls of the two arms that were considered to be closed were approximately 20 cm high, while the walls of the two arms that were considered to be open were 2.5 cm high (Figure 2). This model was utilized in the first two assays. The treatments were the same as above. All mice were placed into the testing area 30 minutes post-injection, and were recorded with the same camera for five minutes. All videos were watched and the following parameters were measured: (i) crosses into a new arm, or how many times all four paws crossed from the center of the platform into a new arm, whether open or closed, (ii) amount of time spent in the open arms, and (iii) number of rears.

2.9 Open Field Exploration Test

A large, open square (76 cm x 76cm) was constructed with 30 cm walls all around. A grid of 16 smaller squares was drawn onto the testing area (each square was 19 cm x 19 cm) (Figure 3). This model was utilized in all behavioral assays. The treatments for the first two bioassays were the same as above. For the identification of the bioactive fraction within the polar components of the extract, each mouse was randomly assigned two treatments: one sedative concentration and one anxiolytic concentration. All mice were placed into the testing area 30 minutes post-injection, and were recorded with the same camera for five minutes. All videos were watched and the following parameters were measured: (i) locomotion, or how many times all four paws crossed over one of the

gridlines, (ii) time spent in the center four squares as opposed to the outside of the grid, (iii) number of rears, and (iv) time spent in the four corner squares out of the entire amount of time spent around the edges of the grid.

2.10 Statistical Analyses

The following statistical tests were applied to all of the data from each test: analysis of variance (ANOVA), the Tukey test, and Dunnett's multiple comparison test. Statistical difference was concluded if $p \leq 0.05$.

2.11 Chemical Similarity Analysis

We performed chemical similarity analysis between the 51 compounds from plant extract and the 67 NIH Mental Health Drugs. First, 186 two dimensional chemical descriptors were calculated from the molecular structures of all the compounds by using Molecular Operating Environment (MOE) software. Then the chemical similarity between each of two compounds could be presented as the MOE chemical descriptor distance. Since it is not feasible to directly visualize the compounds in a 186 dimensional space, we performed a Principal Component Analysis (PCA) by using all the descriptor values of the NIH Mental Health Drugs and the plant extract compounds. The top three principal components (57% explained variance of all 186 MOE descriptors) could be used to generate a 3-D plot that gives us a direct visualization of the current MOE chemical space of all the compounds.

3. Results

3.1 Phytochemical Profiling of *A. muricata* leaf extract

Through GC-MS and UHPLC, we identified a list of compounds in the extracts of *A. muricata* leaves. Compounds that have already been identified and approved as NIST

compounds have been listed in Table 13. Many compounds in the extract have not yet been identified. Phytochemical studies on *A. muricata* revealed a broad range of biological activities such as: the production of approximately 82 acetogenins from 10 different groups, including muricin I, muricin H, cis-annomontacin, cis-corossolone, and annocatalin; a number of alkaloids, including reticulin, coreximine, coclarine, and anomurine; the essential oils β-caryophyllene, δ-cadinene, epi-α-cadinol, and α-cadinol (Lim, 2012; Adewole et al, 2008; de Sousa et al, 2010; Liaw et al, 2002). Other substances, such as flavonols, polyphenols, and flavones have also been isolated from the *A. muricata* extract (George et al, 2012). Chromatograms representing the phytochemical profiling of *A. muricata* leaf extract can be seen in Figures 4-8.

3.2 Determination of Active Fraction from Crude Extract through Behavioral Mouse Model

The results of the IP administration of the different fractions of *A. muricata* crude leaf extract on parameters tallied in the Light/Dark Conflict Box test, the Elevated Plus-Maze test, and the Open Field Exploration test are presented here. All statistical differences were found using Dunnett's Multiple Comparison Test.

3.2.1 Light/Dark Conflict Box Test

The parameters presented are the effects of the fractions on percent of time spent in the light box as well as number of rears ($F_{74} = 8.612$, $p<0.0001$) (Fig 9). No statistical difference was observed between treatments through this approach.

3.2.2 Elevated Plus-Maze Test

The parameters presented are the effects of the fractions on time spent in the open arms (F_{66} = 3.379, p=0.0038), the number of crosses into a new arm, and the number of rears (F_{67} = 14.39, p<0.0001) (Fig. 10). Statistical differences were found between the effect of the water (polar) fraction on the number of crosses into new arms and the number of rears (F_{67} = 6.287, p<0.0001).

3.2.3 Open Field Exploration Test

The parameters presented are the effects of the fractions on the time spent in the center of the grid (F_{67} = 5.661, p<0.0001), the time spent in the corners of the grid, locomotion of the mouse around the grid, and the number of rears (Fig. 11). Statistical difference was found using Dunnett's Multiple Comparison test between the effects of the lower concentration (2 mg/kg) of the water (polar) fraction on the amount of time spent in the corners of the grid (F_{69} = 13.75, p<0.0001).

3.3 Dose Response Curve Determination for the Active Water (Polar) Fraction

The results of the administration of the water (polar) fraction and diazepam for dose response determination in the Light/Dark Conflict Box test, the Elevated Plus-Maze test, and the Open Field Exploration test are presented here. All statistical differences were found using Dunnett's Multiple Comparison test. All figures are presented together in Figure 12.

3.3.1 Light/Dark Conflict Box Test

The parameter presented is the percent of time spent in the light box. Both dose response curves appear to be biphasic in shape. No statistical difference in behavior has been found between the varying concentrations.

3.3.2 Elevated Plus-Maze Test

The parameter presented is the percent of time spent in the open arms of the testing apparatus. Both dose response curves appear to be biphasic in shape. No statistical difference in behavior has been found between the varying concentrations of the water fraction.

3.3.3 Open Field Exploration Test

The parameter presented is the percent of time spent in the center four squares of the grid. Both dose response curves appear to be biphasic in shape. Statistical difference was found between the treatment of the lowest concentration of the water fraction (0.313 mg/kg) and the other treatments.

3.4 Extraction and Preparative HPLC

The fractionation procedure of the water fraction using preparative HPLC can be seen in Figure 13. A list of fractions used to determine biological efficacy of these sub fractions and other isolated fractions from other non-water solvents can be found in Table 8.

3.5 Determination of Active Fraction within Water (Polar) Fraction of Crude Extract

The results of the administration of the various extracted and fractionated fractions of the water fraction in the Open Field Exploration test are presented here. Both sedative and anxiolytic concentrations were used as determined by the dose response curves for the same testing approach. Statistical differences between the lower (or anxiolytic) concentrations are indicated in Figure 14.

3.6 Chemical Similarity Analysis

Since lipophilicity (logP), water solubility (logS), and molecular weight are three important factors that affect the oral bioavailability of drug molecules, we generated the 3D plot for the 51 compounds from *A. muricata* extract samples and 67 NIH mental health drugs (Fig. 15A). Furthermore, we performed PCA of the chemical descriptors as described above. After PCA with the 186 MOE descriptors for all the compounds, we selected the first three most important principal components to generate a three-dimensional plot (Fig. 15B) for these 118 (51 *A. muricata* extract and 67 metal health drug) compounds. These two plots could be viewed as two chemical spaces covered by the existing metal health drug molecules and the compounds analyzed in this study. There is only one outlier (escitalopram, CAS 128196-01-0) of NIH mental health drugs within these two chemical spaces. As a selective serotonin reuptake inhibitor, escitalopram has a chemical structure unrelated to that of other SSRIs or of tricyclic, tetracyclic, or other available antidepressant agents. On the other hand, several of our identified compounds from *A. muricata* extract samples are chemically similar to the available NIH mental health drug molecules on these two different chemical spaces, indicating the potentials of these compounds to be successful future drug candidates.

4. Discussion

The main goal of this study has been to isolate and identify the active compound(s) that provide the anxiolytic effects of the *Annona muricata* leaf extract, and compare these compounds to preexisting NIH mental health drugs to determine their potential of becoming new anti-anxiety drugs. Through the first behavioral mouse bioassay, it was determined that the active fraction of the crude extract was the water (polar) fraction, as shown by the statistical difference in the data of the water fraction as a

treatment compared to the other fractions (Figures 10B, 10C, 11D). Although the treatment did not exhibit an expected anxiolytic effect, it exhibited a similar effect to diazepam. Diazepam has a well-known biphasic dose response curve, which means that it exhibits anxiolytic effects at certain concentrations, but may exhibit sedative effects at a higher concentration. The concentration chosen for the first behavioral assay was high enough to be in the realm of sedative effects. Our results seem to accomplish our secondary goal of supporting traditional administration of this extract with scientific data; in some cultures, such as that of Benin, West Africa, the leaf material is boiled in water and consumed as a tea (Atawodi, 2011). This is supported by the first behavioral bioassay because when boiled with water as the solvent (to make tea), the polar compounds will be released from the leaves into solution.

Once the water fraction was determined to be the active one, it became important to determine if the active fraction was able to exhibit anxiolytic effects at a lower concentration. A dose response curve utilizing all three models from the first bioassay was created for the water fraction and diazepam for comparison. All of the parameters presented by this study do exhibit a curve that appears biphasic in shape. The shape of the dose response curves for diazepam validate the model, and the shape of the curves for the water fraction show that anxiolytic effects can be reached at a lower concentration. A statistically significant anxiolytic effect for the water fraction is seen in Figure 12E, giving a concentration that could be used in the subsequent assays as an established anxiolytic concentration.

After validating the ability of the water fraction to exhibit anxiolytic effects, further fractionation and profiling was done to increase the chance of finding the active

compound(s) within the fraction. Another behavioral mouse bioassay was performed, using a positive control water fraction (RUTWS-1001), the six fractions listed in Table 1, and the three other fractions from extraction of the leaf material, as seen in Table 8. The Open Field Exploration test was the only model utilized for this assay, and no statistical difference was seen between treatments. However, fraction 4 (RUTWS-1005) seems to have exhibited the desired anxiolytic and sedative effects, as seen in Figure 16. This fraction, as well as the fraction before it and the positive control (RUTWS-1004 and -1001, respectively) were compared to diazepam in a dose response curve of oral administration.

Oral administration of the fractionated extract is able to reveal whether the metabolism plays an important role in the efficacy of the active compound(s). The first bioassay using oral administration did not show any statistical difference between concentrations of the treatments. When drugs are administered to an organism orally, it must first undergo first pass metabolism before it takes its effect (Pond et al, 1984). We believe that the concentrations utilized were high enough for i.p. administration, where the compounds do not undergo first pass metabolism through the liver, but not high enough for oral administration. We have extracted all potential polar components of the extract and are performing another model with higher concentrations of the treatments for oral administration.

After performing the chemical similarity analysis between the compounds identified from the exact samples and the current available mental health drug molecules, we believe these compounds have a high possibility to be successful drug candidates in the future. Based on the original active *A. muricata* plant components identified in this

study, we will use optimized rational drug design procedure to create novel drug candidates with similar or higher activity but more suitable pharmaceutical properties.

5. Future Directions

Future directions for this project include:

- Complete phytochemical profiling and identification of all compounds within the subfraction of the aqueous extract.
- Modeling of identified compounds against preexisting NIH mental health drugs.
- Pharmacophore modeling of potentially bioactive compounds against preexisting NIH mental health drugs.
- Structure-based modeling of potentially bioactive compounds against 5-HT_{1A} receptor
- Synthesis of potentially bioactive compounds for bioassay experimentation
- Behavioral mouse bioassays to determine which compound(s) elicit anxiolytic effect (done alone and in conjunction with other potentially active compounds).

6. Tables and Figures

Table 1. Collection times of fractionated polar fraction through preparative HPLC.

Fraction	Collection time (mins)	Solvent
1	0-3	MeOH in Water + 0.1% Formic acid
2	3-8	MeOH in Water + 0.1% Formic acid
3	8-16	MeOH in Water + 0.1% Formic acid
4	16-24	MeOH in Water + 0.1% Formic acid
5	24-32	MeOH in Water + 0.1% Formic acid
6	32-40	MeOH in Water + 0.1% Formic

Table 2. Treatment list for second behavioral mouse bioassay for dose response determination. Code to the left of the numerical concentration represents randomly selected running code for treatments.

H_2O Fraction Concentrations (mg/kg)	Diazepam Concentrations (mg/kg)
W1: 2.5	D1: 2.0
W2: 0 (PBS; negative control)	D2: 1.0
W3: 0.313	D3: 0.25
W4: 5.0	D4: 0 (PBS; negative control)
W5: 0.625	D5: 0.5
W6: 1.25	D6: 0.125

Table 3. First behavioral mouse bioassay treatment schedule for the Light/Dark Box Conflict test. Mice ID numbers are listed at the top of each table. **A** Group #1 (Werc Solution #5: Water fraction/polar components); **B** Group #2 (Werc Solution #1: Positive control); **C** Group #3 (Werc Solution #4: Ethyl acetate fraction/amphipathic components); **D** Group #4 (Werc Solution #3: Hexane fraction/nonpolar components). **Key:** C = PBS baseline, D = diazepam (2 mg/kg), 5H = water fraction (5 mg/kg), 5L = water fraction (2 mg/kg), 1H = positive control (5 mg/kg), 1L = positive control (2 mg/kg), 4H = ethyl acetate fraction (5 mg/kg), 4L = ethyl acetate fraction (2 mg/kg), 3H = hexane fraction (5 mg/kg), 3L = hexane fraction (2 mg/kg).

Trial #	**1.1**	**1.2**	**1.3**	**1.4**	**1.5**	**1.6**
1	C	C	C	C	C	C
2	D	5L	5H	5H	5H	5L
3	5H	5H	D	5L	D	D
4	5L	D	5L	D	5L	5H
Trial #	**2.1**	**2.2**	**2.3**	**2.4**	**2.5**	**2.6**
1	C	C	C	C	C	C
2	1L	D	D	1H	1L	D
3	1H	1H	1L	D	D	1H
4	D	1L	1H	1L	1H	1L
Trial #	**3.1**	**3.2**	**3.3**	**3.4**	**3.5**	**3.6**
1	C	C	C	C	C	C
2	4L	4L	4H	D	D	D
3	D	4H	4L	4L	4H	4L
4	4H	D	D	4H	4L	4H
Trial #	**4.1**	**4.2**	**4.3**	**4.4**	**4.5**	**4.6**
1	C	C	C	C	C	C
2	3H	3L	3L	3L	3H	3L
3	D	3H	3H	D	3L	3H
4	3L	D	D	3H	D	D

Table 4. First behavioral mouse bioassay treatment schedule for the Elevated Plus-Maze test and Open Field Exploration test. Mice ID numbers are listed at the top of each table. **A** Group #1 (Werc Solution #5: Water fraction/polar components); **B** Group #2 (Werc Solution #4: Ethyl acetate fraction/amphipathic components); **C** Group #3 (Werc Solution #3: Hexane fraction/nonpolar components). **Key:** See Table 3.

Trial #	**1.1**	**1.2**	**1.3**	**1.4**	**1.5**	**2.3**
1	C	C	C	C	C	C
2	D	5L	5H	5H	5H	D
3	5H	5H	D	5L	D	5L
4	5L	D	5L	D	5L	5H
Trial #	**3.1**	**3.2**	**3.3**	**3.4**	**3.5**	**3.6**
1	C	C	C	C	C	C
2	4L	4L	4H	D	D	D
3	D	4H	4L	4L	4H	4L
4	4H	D	D	4H	4L	4H
Trial #	**2.5**	**4.1**	**4.2**	**4.3**	**4.4**	**4.5**	**4.6**
1	C	C	C	C	C	C	C
2	3L	3H	3L	3L	3L	3H	3L
3	D	D	3H	3H	D	3L	3H
4	3H	3L	D	D	3H	D	D

Table 5. Testing schedule for first behavioral mouse bioassay. The number following the testing approach corresponds to the trial numbers in the preceding tables. **Key:** LD = Light/Dark Box Conflict test; OF = Open Field Exploration test; EP = Elevated Plus-Maze test.

Day	Testing Approach	Day	Testing Approach
1	LD 1	7	OF 3
2	LD 2	8	OF 4
3	LD 3	9	EP 1
4	LD 4	10	EP 2
5	OF 1	11	EP 3
6	OF 2	12	EP 4

Table 6. Treatment schedule for second behavioral mouse bioassay for dose response determination. Mice ID numbers are listed at the top of each table. Each group received two randomized treatments from the polar fraction of the extract and two randomized treatments of diazepam solutions. For treatment identification, refer to Table 2. **A** Group #1; **B** Group #2; **C** Group #3.

Trial #	**1.1**	**1.2**	**1.3**	**1.4**	**1.5**	**1.6**	**1.7**	**1.8**
1	C	C	C	C	C	C	C	C
2	W1	D1	D2	D1	W2	W2	W1	D2
3	D2	D2	W2	W1	W1	D1	W2	D1
4	D1	W2	D1	W2	D1	W1	D1	W1
5	W2	W1	W1	D2	D2	D2	D2	W2
Trial #	**2.1**	**2.2**	**2.3**	**2.4**	**2.5**	**2.6**	**2.7**	**2.8**
1	C	C	C	C	C	C	C	C
2	W4	W4	D3	D4	D3	W3	W4	W3
3	D3	D4	W4	W3	W3	D4	D3	D3
4	D4	W3	D4	W4	D4	W4	W3	D4
5	W3	D3	W3	D3	W4	D3	D4	W4
Trial #	**3.1**	**3.2**	**3.3**	**3.4**	**3.5**	**3.6**	**3.7**	**3.8**
1	C	C	C	C	C	C	C	C
2	W5	W5	W6	W6	D5	W5	D5	D6
3	D6	D6	D5	W5	W5	W6	D6	D5
4	D5	W6	D6	D5	W6	D5	W5	W5
5	W6	D5	W5	D6	D6	D6	W6	W6

Table 7. Testing schedule for second behavioral mouse bioassay for dose response determination. The number following the testing approach corresponds to the trial numbers in the preceding tables. **Key:** See Table 5.

Day	Testing Approach	Day	Testing Approach	Day	Testing Approach
1	EP 1	6	OF 2	11	LD 4
2	LD 1	7	LD 3	12	OF 5
3	EP 2	8	OF 3	13	EP 4
4	OF 1	9	EP 3	14	LD 5
5	LD 2	10	OF 4	15	EP 5

Table 8. Treatment list for third behavioral mouse bioassay. Fractions were obtained through preparative HPLC.

Treatment	Running Code
H_2O fraction (positive control)	RUTWS-1001
Fraction 1 from H_2O fraction	RUTWS-1002
Fraction 2 from H_2O fraction	RUTWS-1003
Fraction 3 from H_2O fraction	RUTWS-1004
Fraction 4 from H_2O fraction	RUTWS-1005
Fraction 5 from H_2O fraction	RUTWS-1006
Fraction 6 from H_2O fraction	RUTWS-1007
Butanolic extract from H_2O fraction	RUTWS-1008
EtOAc fraction from Butanol	RUTWS-1009
MeOH fraction from Butanol	RUTWS-1010

Table 9. Treatment schedule for third behavioral mouse bioassay. Mouse ID numbers are listed to the left. **Key:** C = PBS (negative control); for treatment identification, please refer to Table 8 (H refers to the higher concentration of the solution, 2.5 mg/kg, and L refers to the lower concentration, 0.313 mg/kg).

Mouse ID	Day 1	Day 2	Day 3	Day 4
1	C	RUTWS-1006H	C	RUTWS-1007L
2	C	C	RUTWS-1002L	RUTWS-1004H
3	C	RUTWS-1007H	C	RUTWS-1001L
4	C	C	RUTWS-1003H	RUTWS-1005L
5	C	RUTWS-1004L	RUTWS-1009H	C
6	C	RUTWS-1007H	RUTWS-1004L	C
7	C	C	RUTWS-1008H	RUTWS-1005L
8	C	RUTWS-1003H	C	RUTWS-1003L
9	C	RUTWS-1009L	C	RUTWS-1001H
10	C	C	RUTWS-1010L	RUTWS-1008H
11	C	C	RUTWS-1010H	RUTWS-1003L
12	C	C	RUTWS-1002L	RUTWS-1001H
13	C	RUTWS-1008L	C	RUTWS-1005H
14	C	RUTWS-1009H	RUTWS-1007L	C
15	C	RUTWS-1006H	RUTWS-1009L	C
16	C	C	RUTWS-1010L	RUTWS-1002H
17	C	RUTWS-1006L	RUTWS-1005H	C
18	C	C	RUTWS-1006L	RUTWS-1002H
19	C	RUTWS-1001L	C	RUTWS-1010H
20	C	RUTWS-1004H	RUTWS-1008L	C

Table 10. Treatment schedule for fourth behavioral mouse bioassay. Mice ID numbers are listed at the top of each table. **A** Group #1: RUTWS-1004 (Fraction #3 from H_2O fraction); **B** Group #2: Diazepam; **C** Group #3: RUTWS-1001 (H_2O fraction; positive control); **D** Group #4: RUTWS-1005 (Fraction #4 from H_2O fraction). **Key:** C = PBS (negative control); H = high concentration of treatment; M = middle concentration of treatment; L = low concentration of treatment.

Trial #	**1**	**2**	**3**	**4**	**5**
1	C	C	C	C	C
2	3H	C	C	3H	3M
3	C	3M	3H	3M	3L
4	3L	3H	3M	C	3H
5	3M	3L	3L	3L	C
Trial #	**6**	**7**	**8**	**9**	**10**
1	C	C	C	C	C
2	C	DM	C	DL	DH
3	DM	DH	DL	DH	C
4	DL	DL	DH	C	DM
5	DH	C	DM	DM	DL
Trial #	**11**	**12**	**13**	**14**	**15**
1	C	C	C	C	C
2	C	1H	1L	C	1M
3	1L	1L	C	1M	1H
4	1M	C	1M	1H	1L
5	1H	1M	1H	1L	C
Trial #	**16**	**17**	**18**	**19**	**20**
1	C	C	C	C	C
2	4L	C	4L	4H	4M
3	C	4H	4M	4M	4L
4	4M	4L	C	C	4H
5	4H	4M	4H	4L	C

Table 11. Testing schedule for the fifth behavioral mouse bioassay. The number following the testing approach represents the trial number as seen in Table 12. **Key:** See Table 5.

Day	Testing Approach
1	OF 1
2	LD 1
3	OF 2
4	LD 2
5	OF 3
6	LD 3

Table 12. Treatment schedule for the fifth behavioral mouse bioassay. Mice ID numbers are listed at the top of the table. **Key:** C = PBS, PL = low concentration (45 mg/kg), PM = middle concentration (90 mg/kg), PH = high concentration (180 mg/kg).

Trial #	1	2	3	4	5	6	7	8
1	C	C	C	C	C	C	C	C
2	PM	PL	PH	PM	PL	PL	PH	PL
3	PL	PH	PM	PH	PH	PM	PM	PH

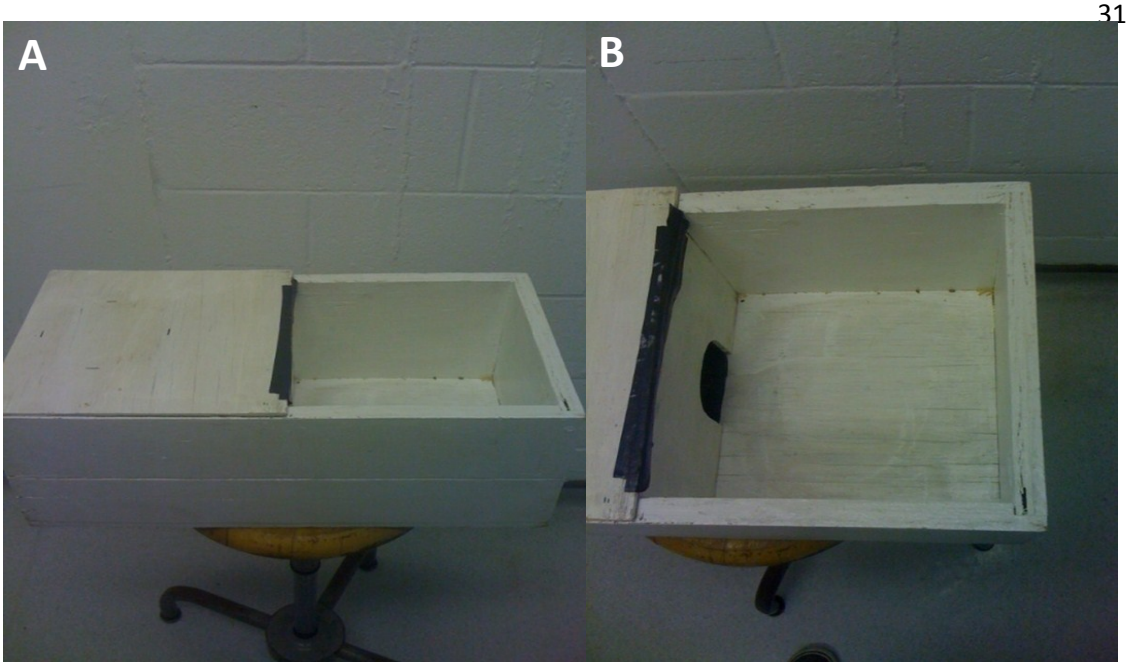


Figure 1. Photograph of the Light/Dark Conflict Box test apparatus. **A** The entire apparatus, **B** Close-up of the light box.

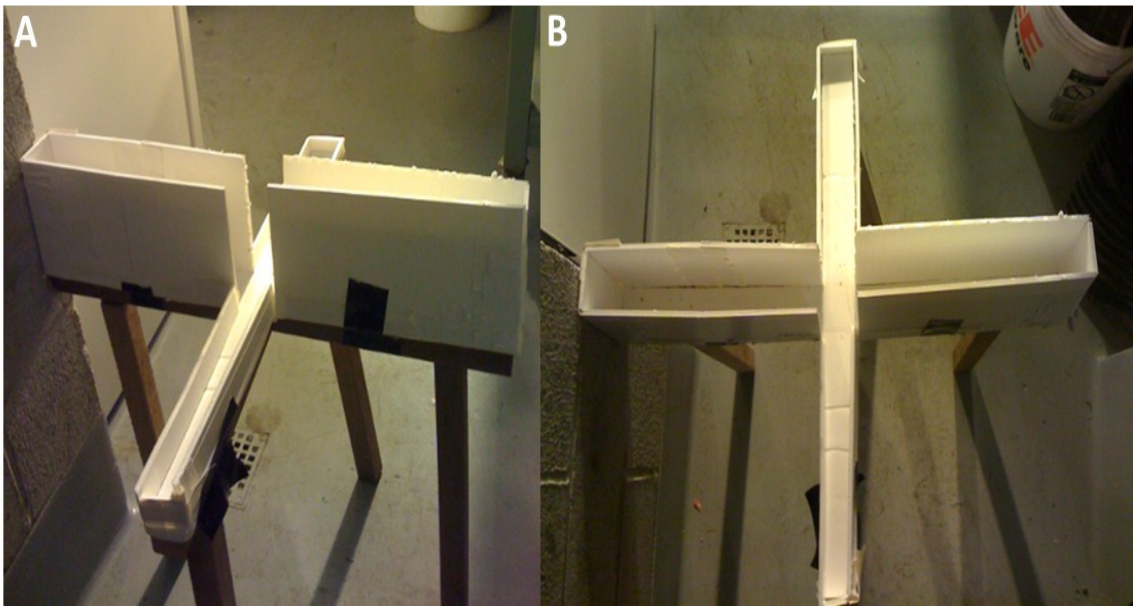


Figure 2. Photograph of the Elevated-Plus Maze test apparatus.

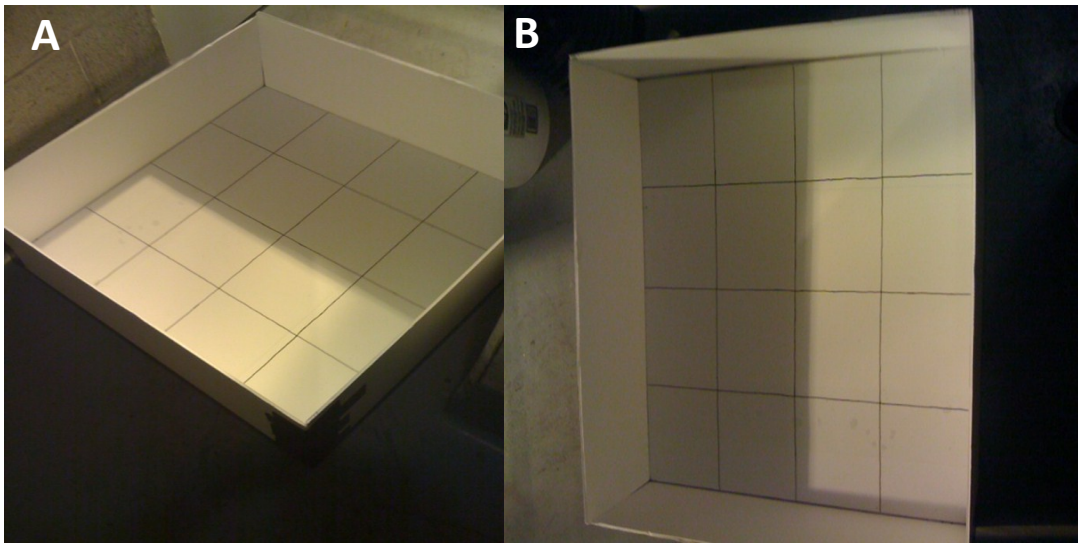


Figure 3. Photograph of the Open Field Exploration test apparatus.

Table 13. Identification of Compounds in the *Annona muricata* Leaf Extract*

Number	Name of Compounds from *A. muricata extracts* by **Tentative** NIST Identification
1	11,14,17-Eicosatrienoic acid methyl ester
2	1-Octacosanol
3	2-ethyl-1-decanol
4	2-Methoxy-4-Vinylphenol
5	3,5-Dihydroxy-6-methyl-2,3-dihydro-4H-pyran-4-one
6	3,7,11,15-Tetramethyl-2-hexadecen-1-ol
7	3-Hexadecanone
8	3-Tetradecanone
9	4-Isopropyl-1,3-cyclohexanedione
10	Alpha-Tocopherol
11	Alpha-Monopalmitin
12	Beta or gamma Tocopherol
13	Coumaran
14	Delta-Tocopherol
15	Erucyl amide
16	Long linear hydrocarbon or fatty acid derivative
17	Oleic acid
18	Palmetic acid
19	Palmitic acid methyl ester
20	Phytol
21	Reticuline
22	Stearic acid
23	Stearic acid methyl ester

*Along with more than 100 other unknown compounds comprised of steroids, long chain aliphatics, isoquinolines and other types of structural moieties.

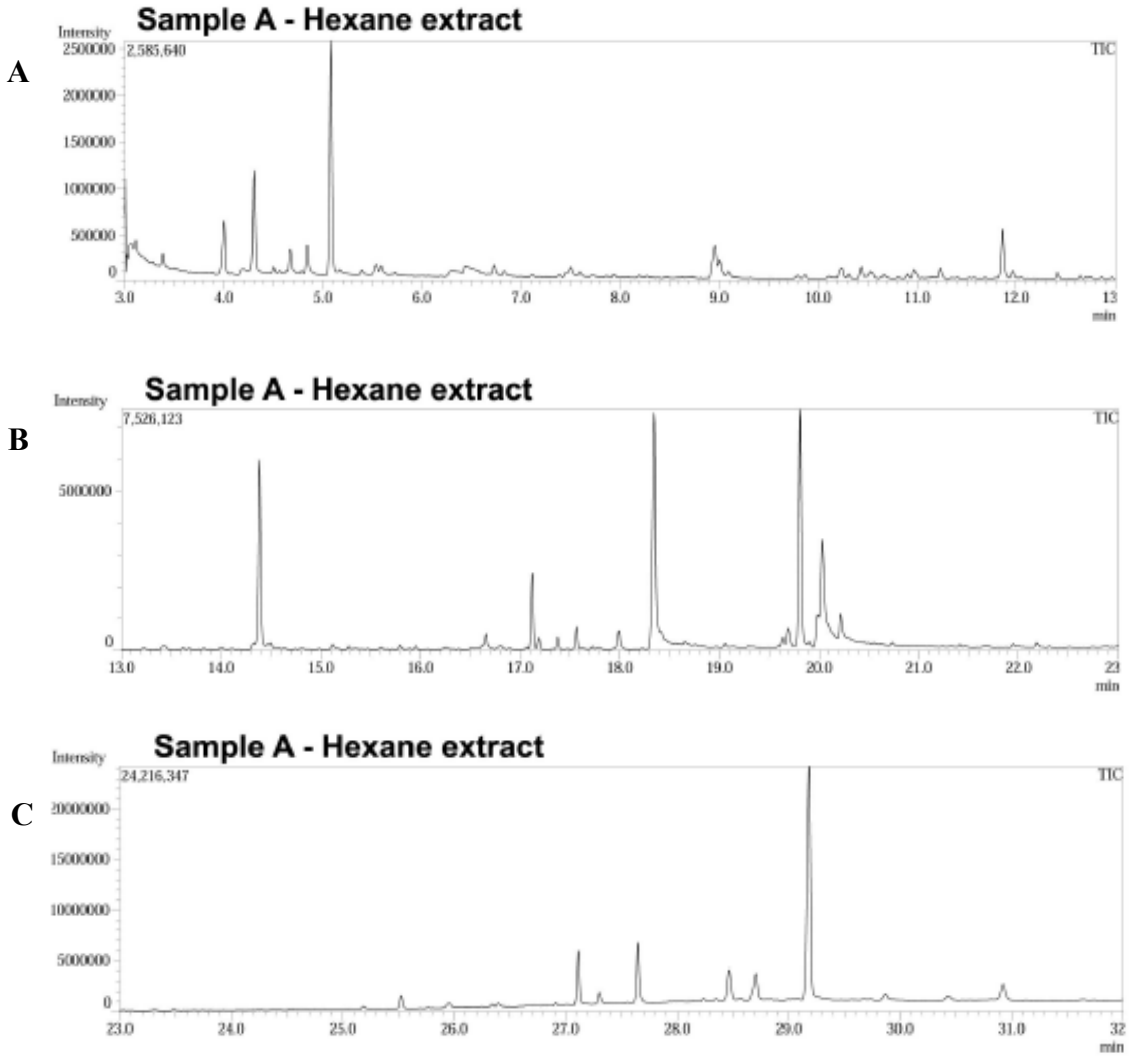


Figure 4. GC-MS Chromatogram of Hexane extraction of *A. muricata* leaves. **A** 3-13 minutes section. **B** 13-23 minutes section. **C** 23-32 minutes section.

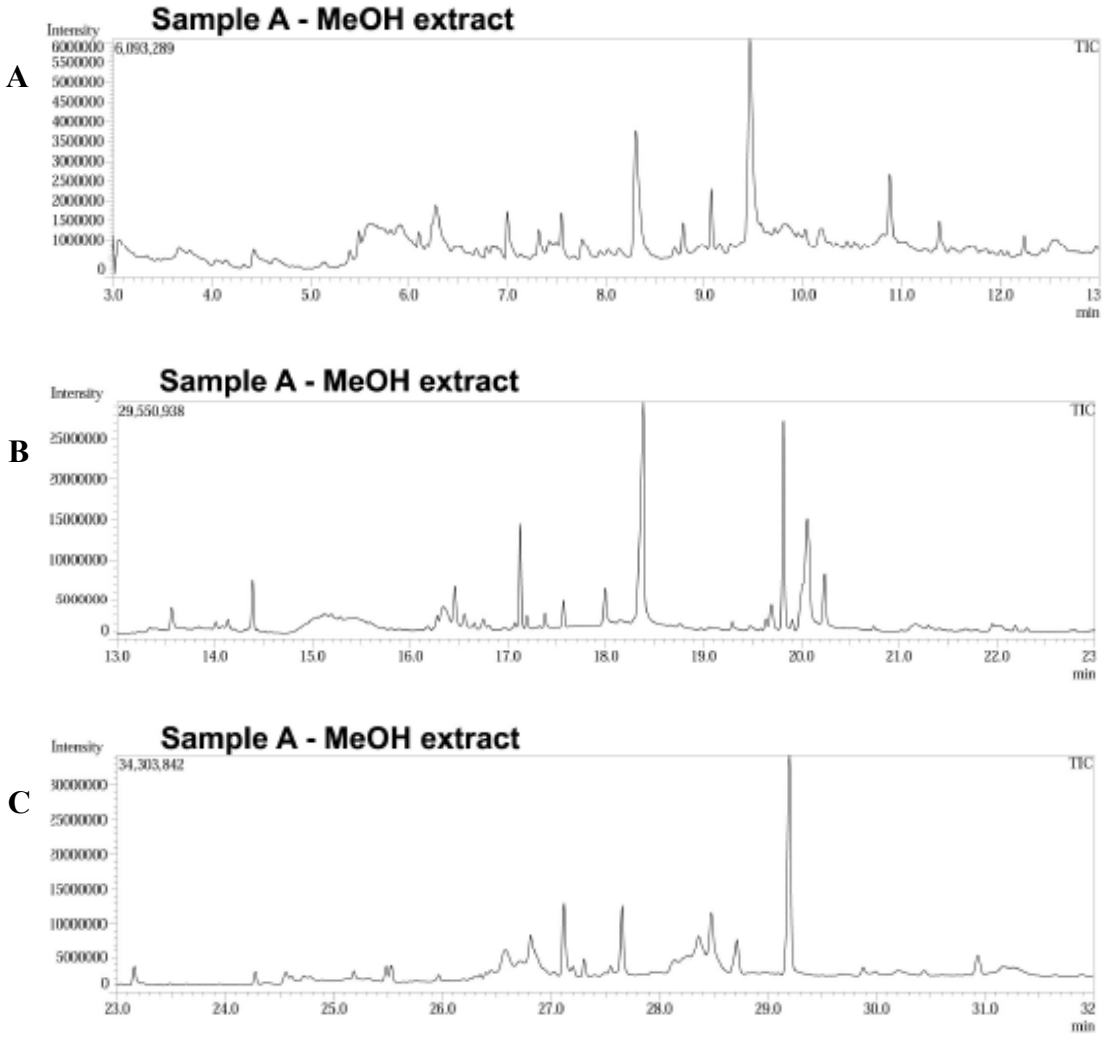


Figure 5. GC-MS Chromatogram of Methanol extraction of *A. muricata* leaves. **A** 3-13 minutes section. **B** 13-23 minutes section. **C** 23-32 minutes section.

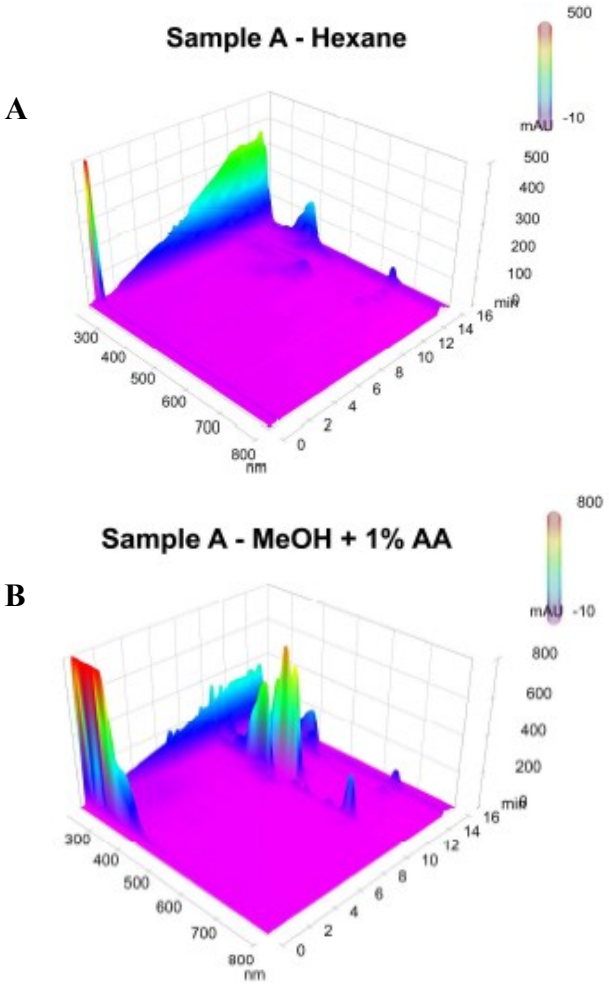
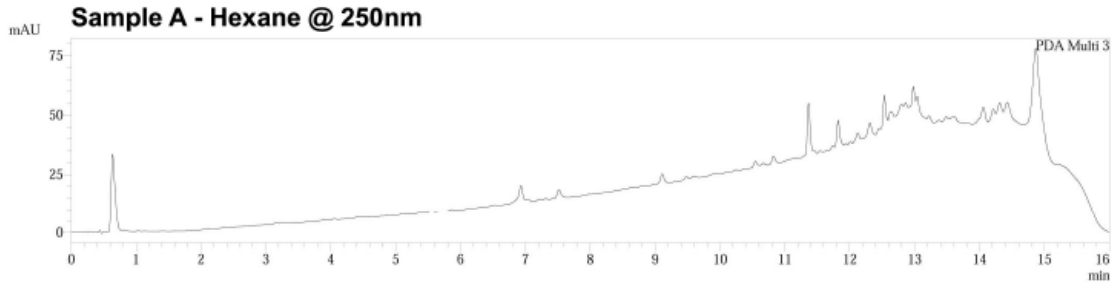
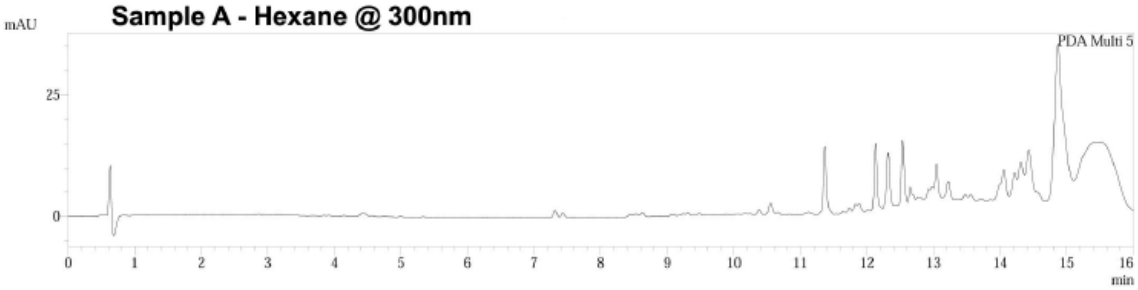


Figure 6. 3D UV-VIS Chromatogram of *A. muricata* leaf extract. **A** Hexane extract. **B** Methanol + 1% glacial acetic acid extract.

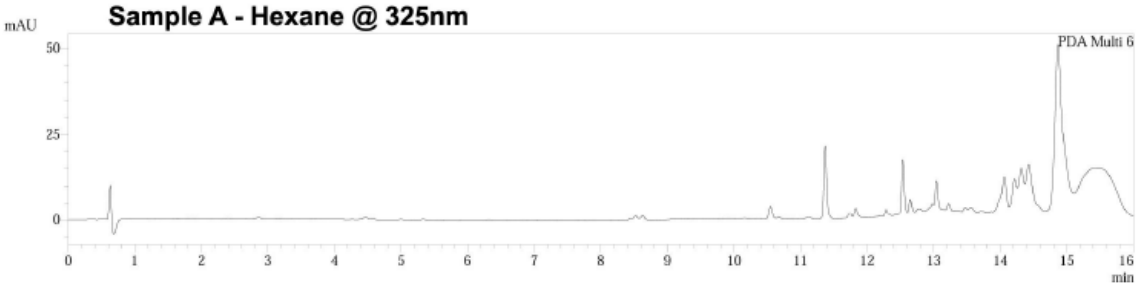
A


Sample A - Hexane @ 250nm
mAU
PDA Multi 3
min

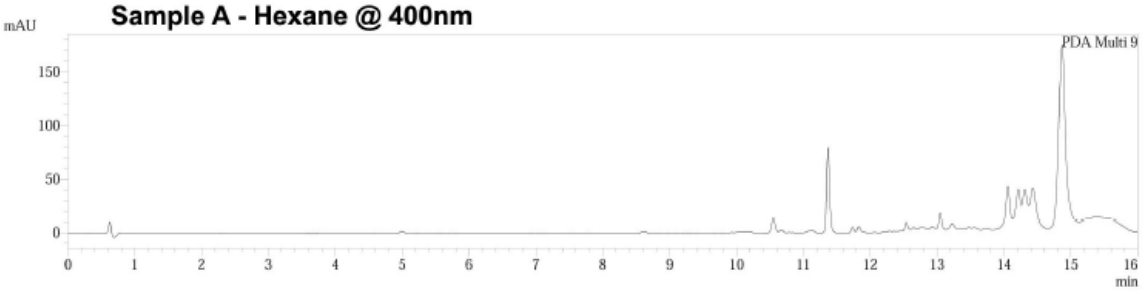
B


Sample A - Hexane @ 300nm
mAU
PDA Multi 5
min

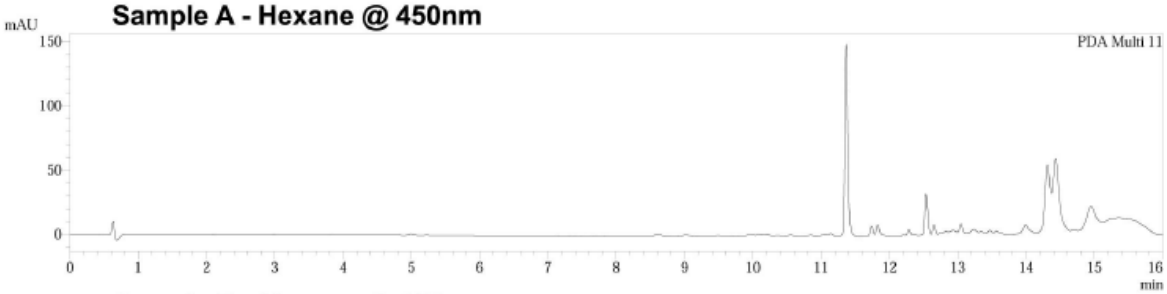
C


Sample A - Hexane @ 325nm
mAU
PDA Multi 6
min

D


Sample A - Hexane @ 400nm
mAU
PDA Multi 9
min

E


Sample A - Hexane @ 450nm
mAU
PDA Multi 11
min

F

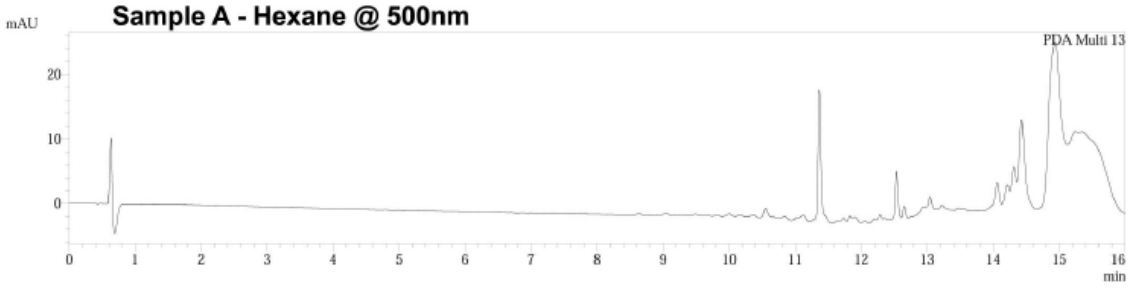
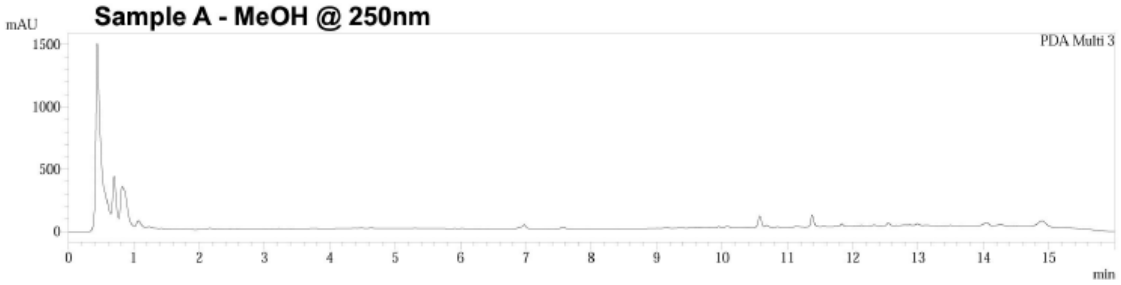
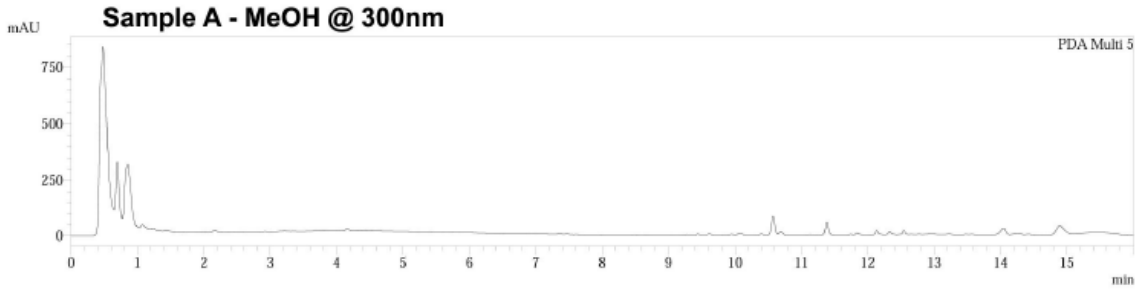


Figure 7. Extracted Chromatogram of hexane extract (minutes 0-16) at the following wavelengths: **A** 250 nm **B** 300 nm **C** 325 nm **D** 400 nm **E** 450 nm **F** 500 nm.

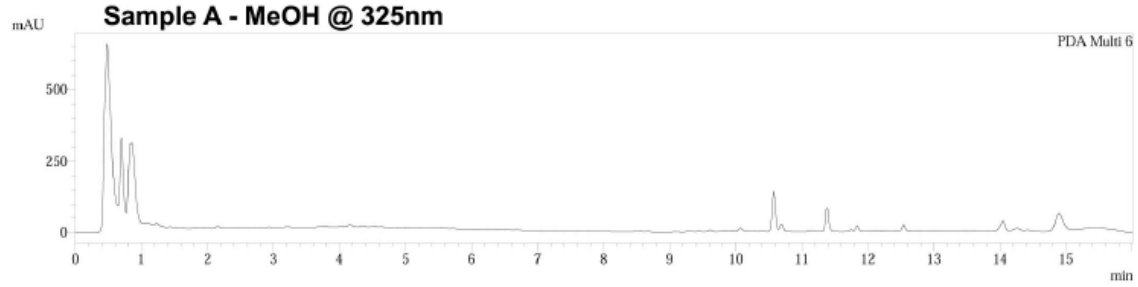
A


Sample A - MeOH @ 250nm
mAU
PDA Multi 3
1500
1000
500
0
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
min

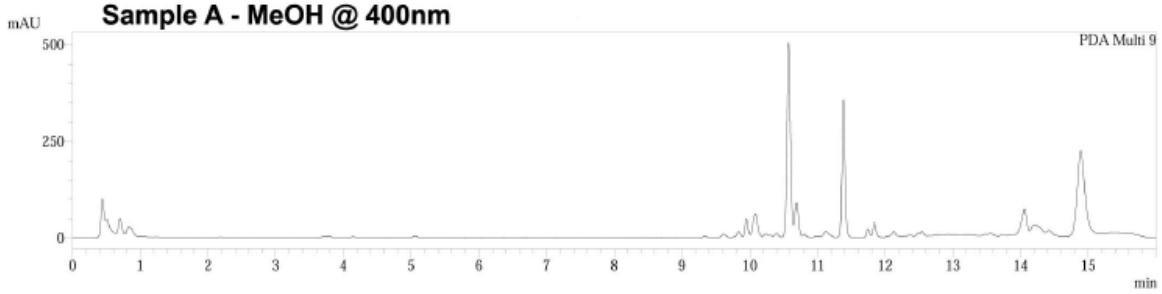
B


Sample A - MeOH @ 300nm
mAU
PDA Multi 5
750
500
250
0
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
min

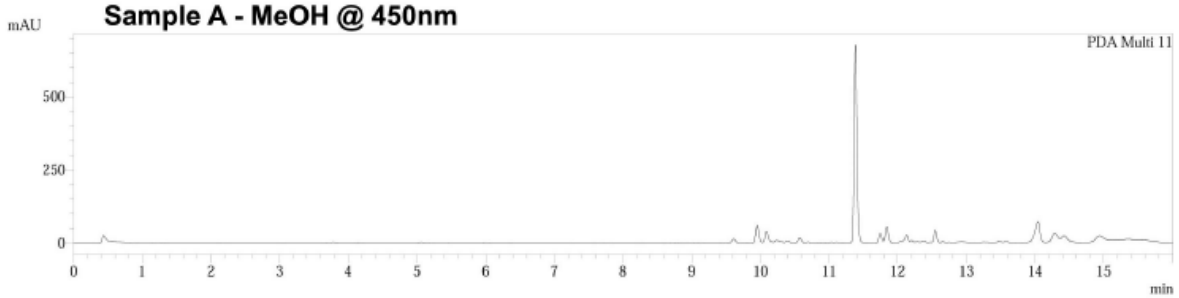
C


Sample A - MeOH @ 325nm
mAU
PDA Multi 6
500
250
0
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
min

D


Sample A - MeOH @ 400nm
mAU
PDA Multi 9
500
250
0
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
min

E


Sample A - MeOH @ 450nm
mAU
PDA Multi 11
500
250
0
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
min

F

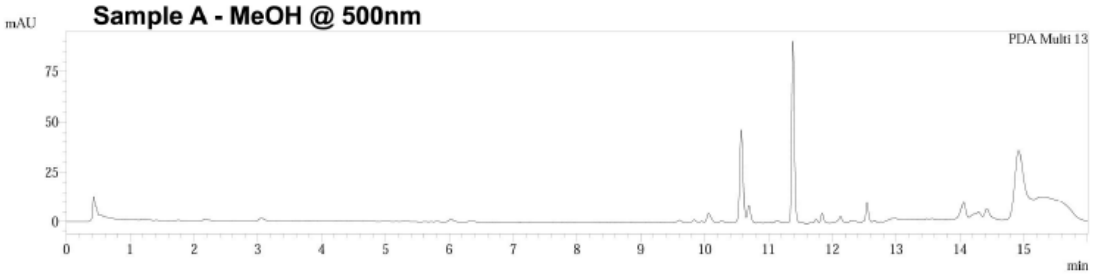


Figure 8. Extracted Chromatogram of methanol extract (minutes 0-16) at the following wavelengths: **A** 250 nm **B** 300 nm **C** 325 nm **D** 400 nm **E** 450 nm **F** 500 nm.

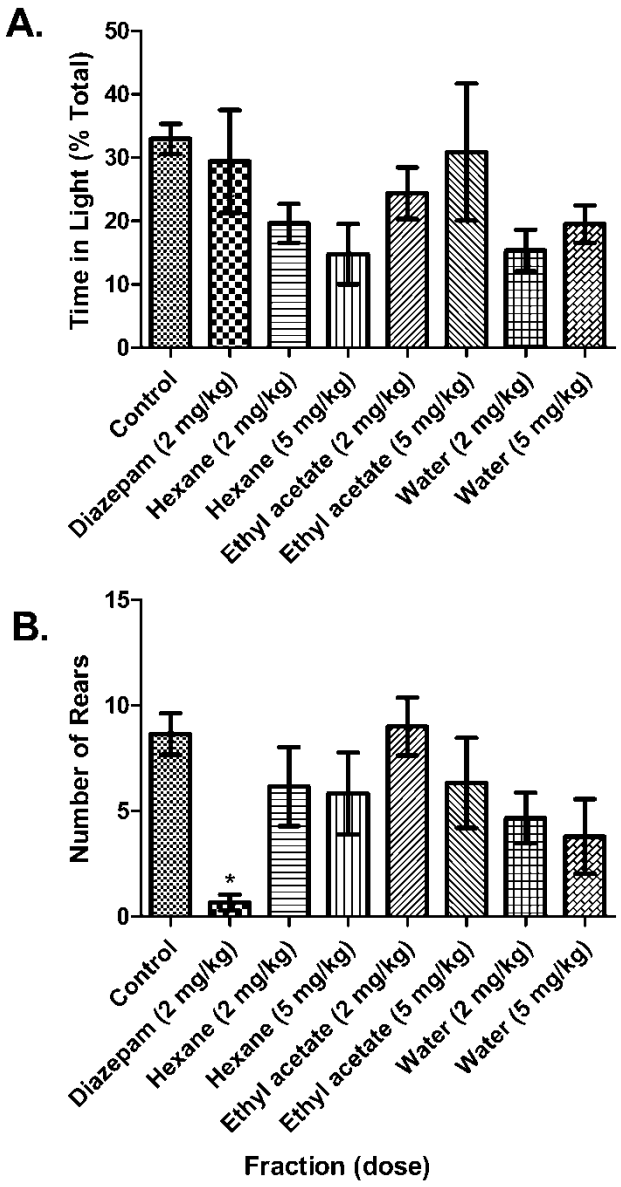


Figure 9. Effects of solvent extraction fractions from *Annona muricata* on behavior of mice in the light/dark conflict box. **A** Percent of total time spent in the light. Mice were separately placed on the lighted side of the box and the amount of time spent on the lighted side was tallied. **B** Rearing during test in the Light:Dark Box. Mice were separately placed on the lighted side of the box and the numbers of rears in a 3-min test were tallied. Values are means ± S.E.M. for 6 animals. Asterisk indicates a significant difference from controls at a significance level of $P<0.05$.

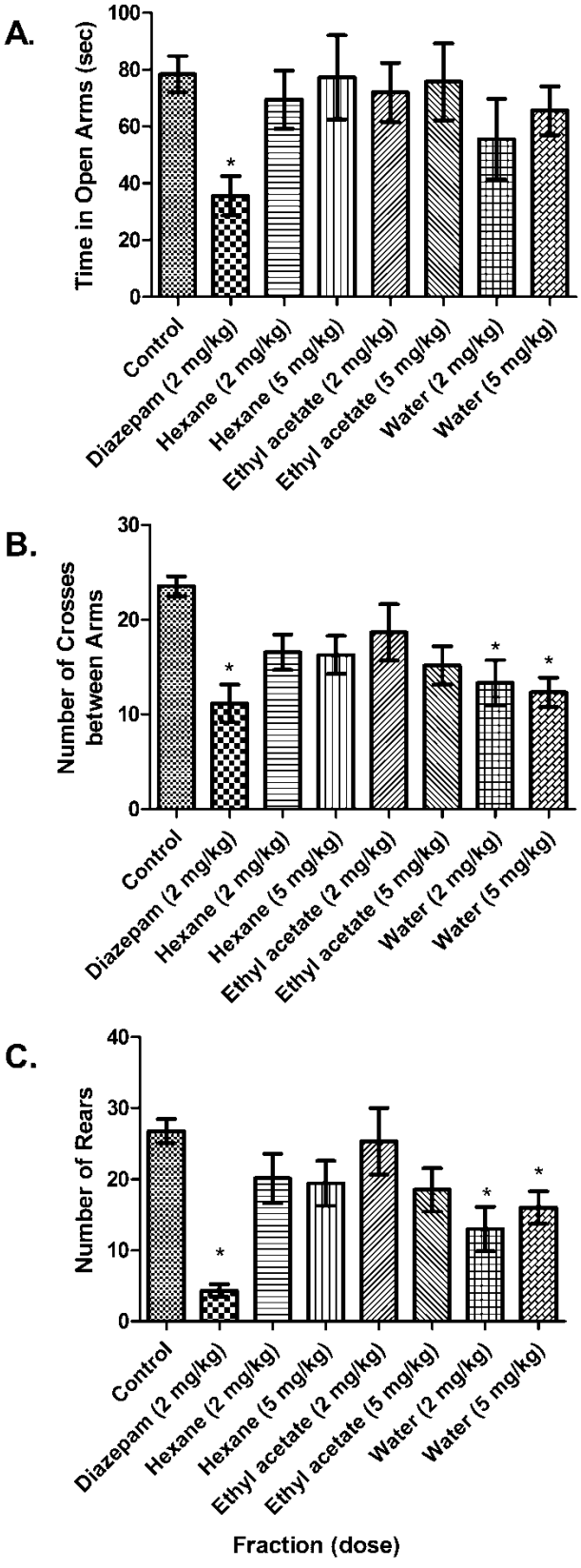

A.
Time in Open Arms (sec)
100
80
60
40
20
0
*
Control
Diazepam (2 mg/kg)
Hexane (2 mg/kg)
Hexane (5 mg/kg)
Ethyl acetate (2 mg/kg)
Ethyl acetate (5 mg/kg)
Water (2 mg/kg)
Water (5 mg/kg)
B.
Number of Crosses between Arms
30
20
10
0
*
*
*
Control
Diazepam (2 mg/kg)
Hexane (2 mg/kg)
Hexane (5 mg/kg)
Ethyl acetate (2 mg/kg)
Ethyl acetate (5 mg/kg)
Water (2 mg/kg)
Water (5 mg/kg)
C.
Number of Rears
40
30
20
10
0
*
*
*
Control
Diazepam (2 mg/kg)
Hexane (2 mg/kg)
Hexane (5 mg/kg)
Ethyl acetate (2 mg/kg)
Ethyl acetate (5 mg/kg)
Water (2 mg/kg)
Water (5 mg/kg)
Fraction (dose)

Figure 10. Effects of solvent extraction fractions from *Annona muricata* on behavior of mice in the elevated plus-maze. **A** Time spent in the open arms of the elevated plus-maze. Mice were separately placed in the center of the apparatus and the amount of time spent on in the open arms was tallied. Values are means ± S.E.M. for 6 animals. **B** Crossings between the open and closed arms of the elevated plus-maze. Mice were separately placed in the center of the apparatus and the crossings were tallied. Values are means ± S.E.M. for 6 animals. **C** Number of rears. Number of rears were tallied over the 5-min test. Values are means ± S.E.M. for 6 animals. Asterisks indicate significant differences from controls at a significance level of $P<0.05$.

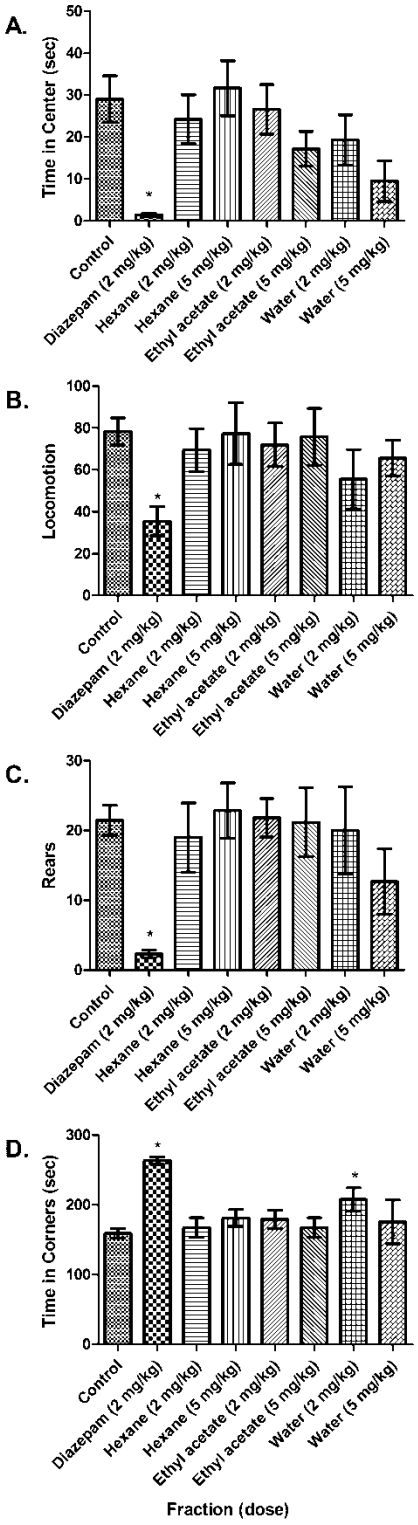

A.
Time in Center (sec)
50
40
30
20
10
0
*
Control
Diazepam (2 mg/kg)
Hexane (2 mg/kg)
Hexane (5 mg/kg)
Ethyl acetate (2 mg/kg)
Ethyl acetate (5 mg/kg)
Water (2 mg/kg)
Water (5 mg/kg)
B.
Locomotion
100
80
60
40
20
0
*
Control
Diazepam (2 mg/kg)
Hexane (2 mg/kg)
Hexane (5 mg/kg)
Ethyl acetate (2 mg/kg)
Ethyl acetate (5 mg/kg)
Water (2 mg/kg)
Water (5 mg/kg)
C.
Rears
30
20
10
0
*
Control
Diazepam (2 mg/kg)
Hexane (2 mg/kg)
Hexane (5 mg/kg)
Ethyl acetate (2 mg/kg)
Ethyl acetate (5 mg/kg)
Water (2 mg/kg)
Water (5 mg/kg)
D.
Time in Corners (sec)
300
200
100
0
*
*
Control
Diazepam (2 mg/kg)
Hexane (2 mg/kg)
Hexane (5 mg/kg)
Ethyl acetate (2 mg/kg)
Ethyl acetate (5 mg/kg)
Water (2 mg/kg)
Water (5 mg/kg)
Fraction (dose)

Figure 11. Effects of solvent extraction fractions from *Annona muricata* on behavior of mice in the open field exploration test. **A** Time spent in the center of the open field. Mice were separately placed in the center of the box and the amount of time spent on in the central 4 squares was tallied. Values are means ± S.E.M. for 6 animals. **B** Locomotion. Mice were placed separately in the center of the box and the number of times all four paws crossed a gridline was tallied. Values are means ± S.E.M. for 6 animals. **C** Number of rears in open field exploration test. Mice were placed separately in the center of the box and the number of rears was tallied. Values are means ± S.E.M. for 6 animals. **D** Time spent in the corners of the open field. Mice were separately placed in the center of the box and the amount of time spent on in the corner squares was tallied. Values are means ± S.E.M. for 6 animals. Asterisks indicate significant differences from controls at a significance level of $P<0.05$.

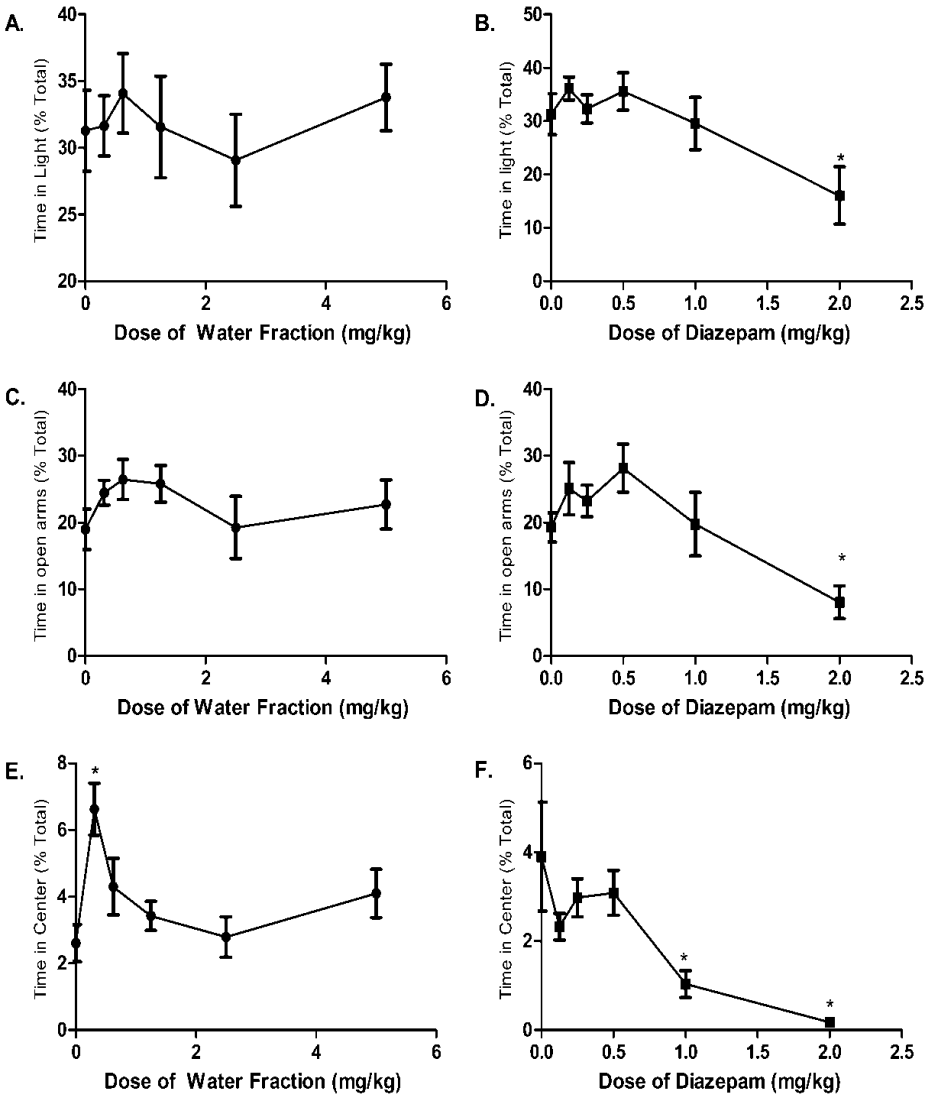

A.
Time in Light (% Total)
Dose of Water Fraction (mg/kg)
B.
Time in light (% Total)
Dose of Diazepam (mg/kg)
C.
Time in open arms (% Total)
Dose of Water Fraction (mg/kg)
D.
Time in open arms (% Total)
Dose of Diazepam (mg/kg)
E.
Time in Center (% Total)
Dose of Water Fraction (mg/kg)
F.
Time in Center (% Total)
Dose of Diazepam (mg/kg)

Figure 12. Dose response curves for effect of the water fraction (left column) and diazepam (right column) on three measures of anxiolytic like and sedative behavior (by row). **A** Effect of water fraction on percent of time spent in the light box of the Light/Dark Conflict Box test. Mice were separately placed into the light box and the amount of time spent in the light box was tallied. **B** Effect of diazepam on percent of time spent in the light box of the Light/Dark Conflict Box test. **C** Effect of the water fraction and **D** diazepam on percent of time spent in the open arms of the Elevated Plus-Maze test. Mice were separately placed into the center of the testing apparatus and the amount of time spent in the open arms was tallied. **E** Effect of the water fraction and **F** diazepam on percent of time spent in the center of the grid in the Open Field Exploration test. Mice were separately placed into the center of the testing apparatus and the amount of time spent in the center of the grid was tallied. All values are means ± S.E.M. for 8 animals. Asterisks indicate significant differences from controls at a significance level of $P<0.05$.

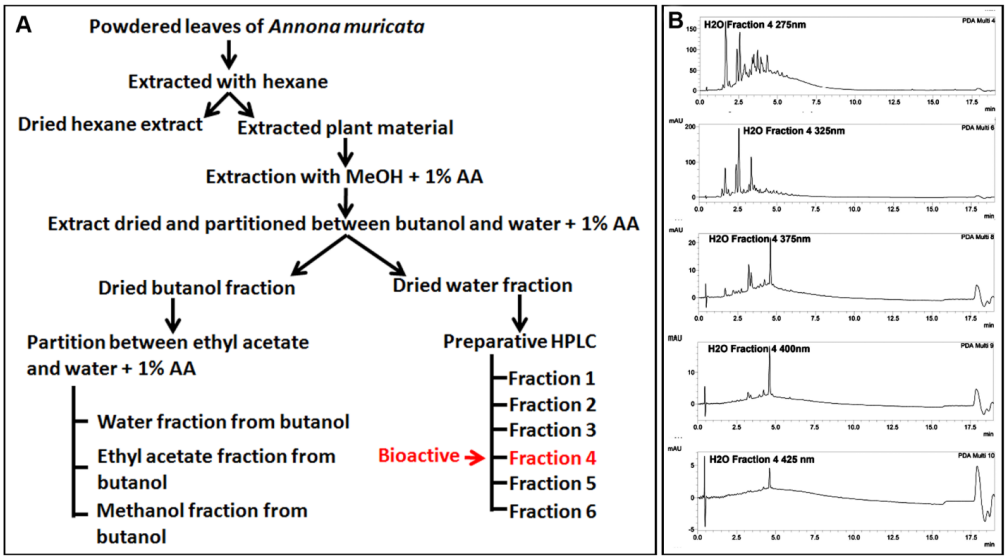


Figure 13. *Annona muricata* extraction flowchart (**A**) and the chromatogram of anxiolytic bioactive water fraction 4 (**B**) showing multiple peaks (compounds) that might be responsible for the activity.

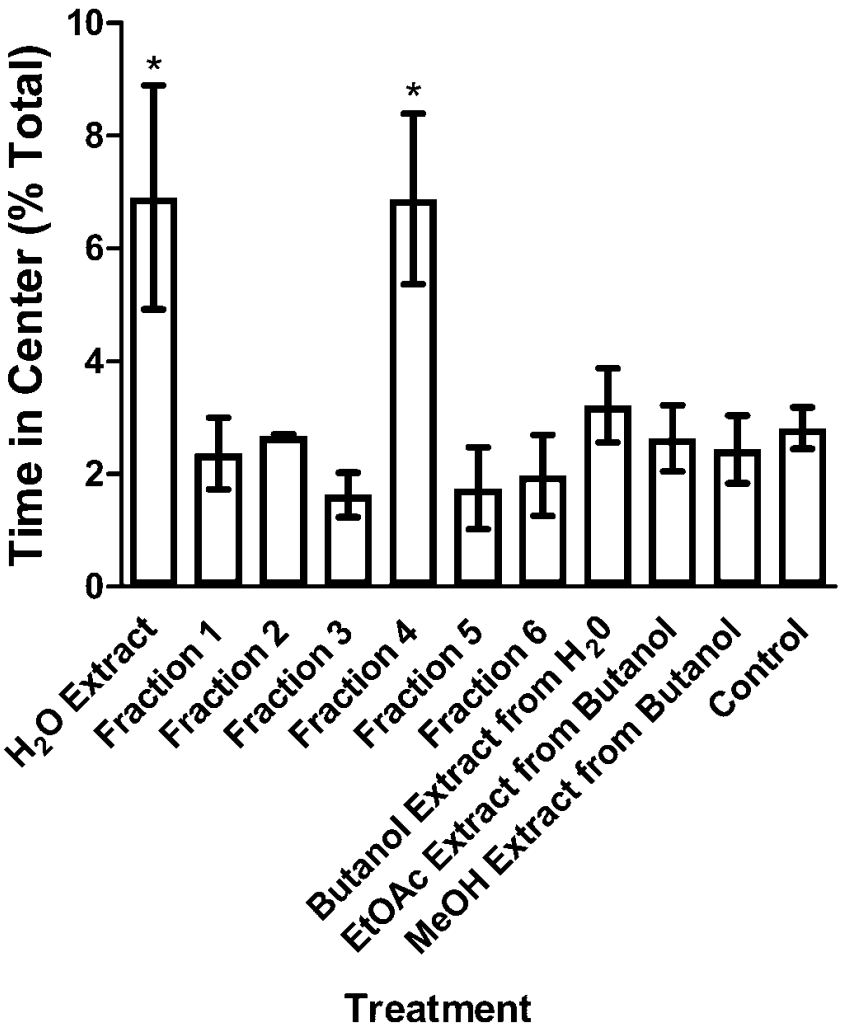
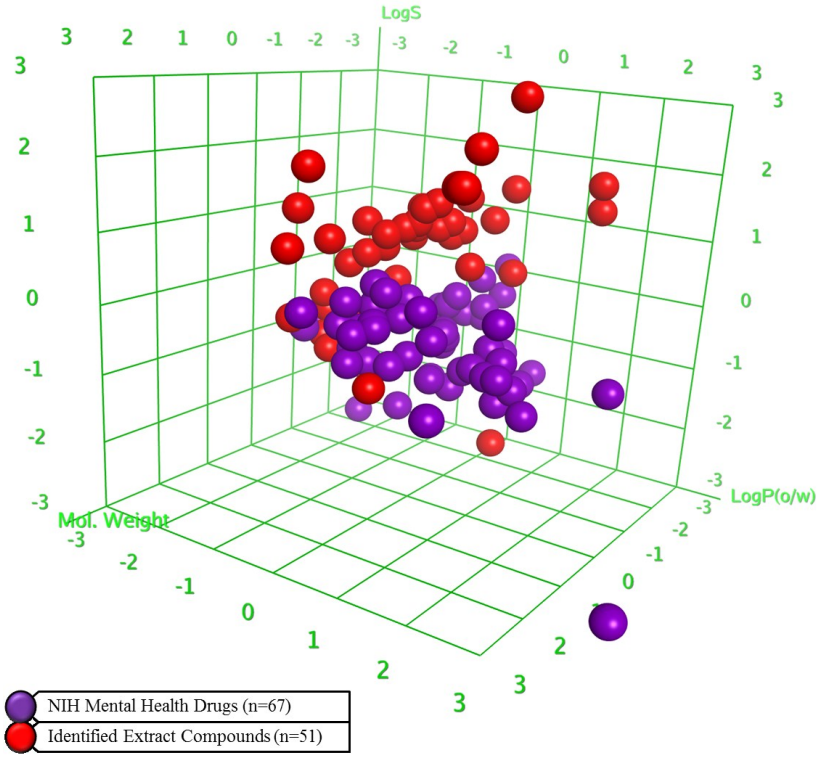

Time in Center (% Total)
10
8
6
4
2
0
*
*
H2O Extract
Fraction 1
Fraction 2
Fraction 3
Fraction 4
Fraction 5
Fraction 6
Butanol Extract from H20
EtOAc Extract from Butanol
MeOH Extract from Butanol
Control
Treatment

Figure 14. Effect of fractions from the water extract of *A. muricata* leaf on percent of time spent in the center four squares in the open field exploration test. An aqueous extract of the leaves was subjected to HPLC chromatography and further extracted against butanol, ethyl acetate (EtOAc) and methanol (MeOH). Values are means ± S.E.M. for 6 animals. Asterisks indicate significant differences from controls at a significance level of $P<0.05$.


LogS
Mol. Weight
LogP(o/w)
NIH Mental Health Drugs (n=67)
Identified Extract Compounds (n=51)

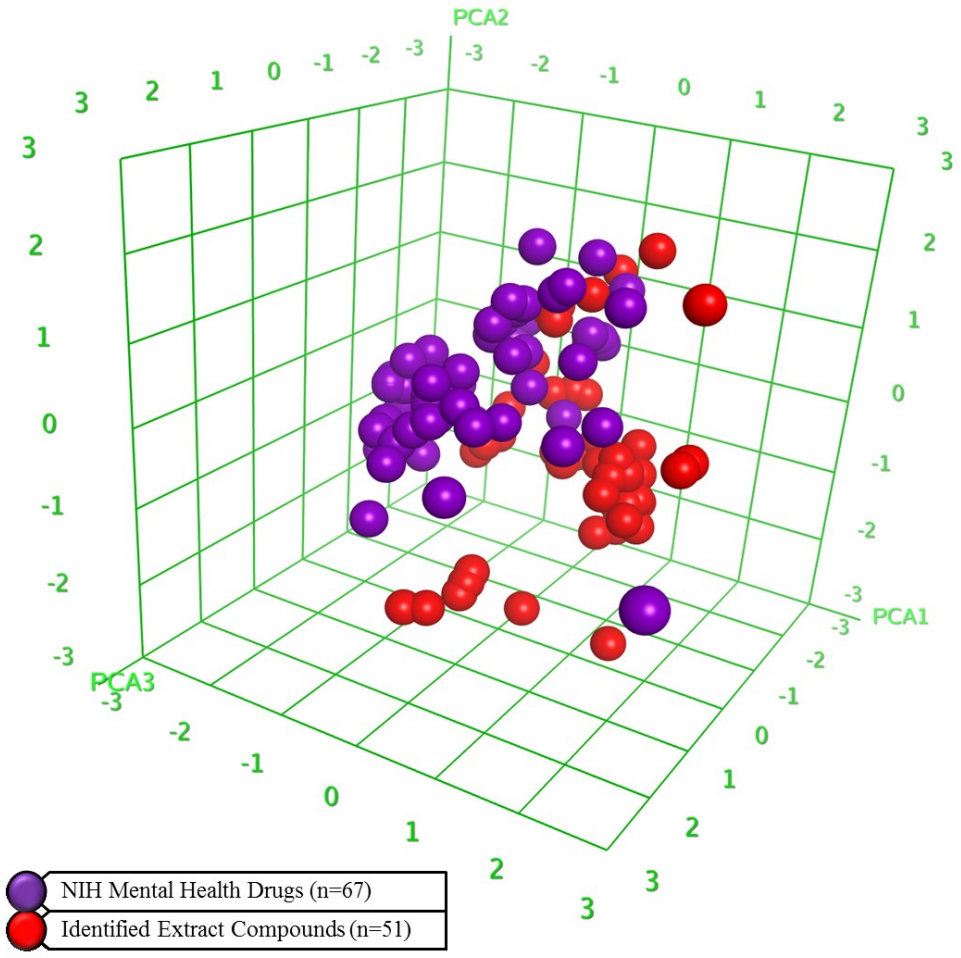


Figure 15. The chemical space of the identified compounds from *A. muricata* leaf extract (red) and the NIH mental health drugs (purple) shown as **A** the 3-D Plots of LogP (o/w), water solubility (LogS), & molecular weight and **B** the first three principle components of (57% explained variance) 186 two dimensional MOE descriptors using MOE® 2011.

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