ISOLATION OF BIOACTIVE COMPOUNDS FOUND IN TRADITIONAL CHINESE MEDICINAL PLANTS: SPECIES OF FOCUS - *STEPHANIA LONGA*

by
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ABSTRACT

ISOLATION OF BIOACTIVE COMPOUNDS FOUND IN TRADITIONAL CHINESE MEDICINAL PLANTS: SPECIES OF FOCUS - *STEPHANIA LONGA*

Use of natural products to help treat and cure various ailments has been practiced for centuries, long before the advent of modern medicine or pharmaceuticals. Notably, Traditional Chinese Medicine (TCM) has utilized the healing properties of herbs and plants for thousands of years to treat diseases, ranging from fever to infections to even cancer. One species of plant, *Stephania longa*, has demonstrated potential bioactivity against *Trypanosoma brucei,* a parasite that causes African Trypanosomiasis ("African sleeping sickness"). A bioassay guided fractionation of *S. longa* was conducted using various chemical purification methods. Isolation of four possible compounds showing inhibition activity against *T. brucei* was achieved. Further research is needed for the additional purification and eventual identification of the bioactive compounds.

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CHAPTER I: INTRODUCTION

Background

The use of naturally occurring products to treat and cure various diseases has had a significant impact on medicinal practices. Even before the development of modern medicine, components of animals, plants, and fungi have been utilized to help combat ailments ranging from cancer to viral infections. One of the most prominent and enduring practices of using natural products for medicinal applications is Traditional Chinese Medicine (TCM). Indigenous plants and herbs found on the coasts and mainland of China have been used for centuries to treat illnesses long before the advent of modern pharmaceuticals.[1] Initial research into the specific compounds found in the plants used in TCM have yielded the discovery of many bioactive compounds, including artemisinin (an antimalarial) and huperzine A (a treatment for Alzheimer's disease).[2]

Artemisinin, a drug used to treat malaria, was developed from the initial isolation of the compound artemisinin (called qinghaosu, QHS) from the Chinese plant *Artemisia annua* in 1971. The compound, which is a sesquiterpene lactone with a peroxide grouping but lacking the nitrogen-containing ring system commonly found in other antimalarials, has been used to treat thousands of cases of malaria, including those involving chloroquine-resistant strains of the malaria

causing parasite, *Plasmodium falciparum*.[3] The structure for artemisinin is shown in Figure 1.

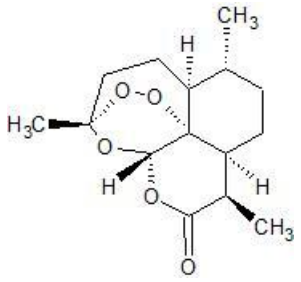
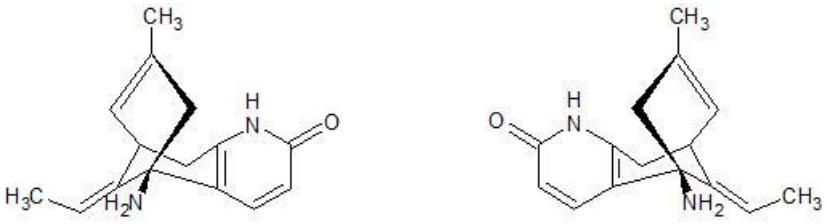


Figure 1. Structure of artemisinin

Huperzine A (HupA) is a sesquiterpene alkaloid compound that was originally isolated from the plant *Huperzia serrata* (a firmoss found in India and southeast Asia) in 1986, and has been used as an effective treatment for Alzheimer's disease (AD). HupA works by inhibiting the activity of acetylcholinesterase (AChE), an enzyme that degrades the neurotransmitter acetylcholine and ultimately leads to dementia. Clinical trials have shown successful inhibition of AChE in elderly patients suffering from AD and overall steady improvement in cognitive abilities.[4,5,6] The two stereoisomers of HupA are shown in Figure 2.



(-)-Huperzine A (+)-Huperzine A

Figure 2. Stereoisomers of huperzine A

While the discovery and isolation of compounds such as artemisinin and huperzine A only touches the surface of the extent of current research being conducted, it illustrates the importance and increased interest in studying the bioactivity of natural products. In fact, a study published in 2008 revealed that over 60% of pharmaceuticals available at that time were either directly or indirectly derived from natural products.[7] Another more recent study revealed that there has been a significant trend in the utilization of natural products for the treatment and cure of diseases over the past 30 years. From 1981 to 2010, 77% of anti-cancer drugs, 80% of anti-viral drugs, and 78% of anti-parasitic drugs were either naturally derived or mimicked naturally occurring compounds.[8]

These studies show promising prospects for the increased amount of new alternatives to medical treatment, yet there are still many diseases worldwide that do not have an effective or efficient treatment or cure. This issue led the World Health Organization (WHO) to publish a report in 2012 that encouraged the

continued research of bioactive natural products in order to alleviate the inability of third world countries in Africa and Asia to receive the often times expensive treatments for fatal diseases.[9]

Disease

One such fatal disease is the illness African Trypanosomiasis, also known as African sleeping sickness. Caused by an infection with the parasite *Trypanosoma brucei*, African sleeping sickness has affected hundreds of thousands of people, with over 10,000 new cases being reported each year (mainly in regions in sub-Saharan Africa). The disease is transmitted via the bite of tsetse flies, and manifests itself in two stages. In the first stage, the parasite remains in peripheral circulation and causes non-fatal symptoms. In the second stage, once the blood-brain barrier is crossed and the parasite enters the central nervous system, more life-threatening symptoms occur, and if left untreated leads to coma and death.[10,11]

There are two distinct versions of the disease, depending on the form of the parasite: East African and West African sleeping sickness. East African sleeping sickness, caused by *T.b. rhodesiense*, affects a few hundred new patients each year and progresses very rapidly, with onset of symptoms such as fever, headache, muscle and joint aches, and enlarged lymph nodes occurring within 1-2 weeks of initial contact. Large sores at the bite site and a rash may also develop.

After a few weeks, the parasite invades the central nervous system, causing mental deterioration and other neurological problems. Eventually, within a few months, the disease leads to coma and death.[12]

West African sleeping sickness, caused by *T.b. gambiense*, progresses more slowly and is much more common, with 7,000-10,000 new cases each year. Initially, mild symptoms such as occasional fever, headaches, muscle and joint aches, and malaise arise, with possible itching of the skin, swollen lymph nodes, and weight loss. Then, after 1-2 years, involvement of the central nervous system is evident, with personality changes, daytime sleepiness and nighttime sleep disturbance, and progressive confusion. Additionally, hormonal imbalances, partial paralysis, and trouble balancing or walking also ensue. The course of the infection rarely lasts longer than 6-7 years, and usually kills within the first 3 years.[13]

Various treatments for African sleeping sickness exist, including pentamidine (the main drug used to treat stage 1 of *T.b. gambiense* infection) suramin, melarsoprol, eflornithine, and nifurtimox. However, there is no cure for the disease, and the treatments are not always widely available or effective.[14] Thus, investigations into alternative treatments and/or cures are highly needed, including prospects of bioactive natural products and TCM.

Stephania longa

The plant *Stephania longa* (fen ji du, 粪箕笃), common name Long Stephania, is a herbaceous vine found in the shrublands and forest margins of the Chinese provinces of Fujian, Guangxi, Guangdong, and Hainan, as well as in Taiwan and southeast Laos.[15] The genus *Stephania* belongs to the family Menispermaceae, and contains 26 species within its genus. The plants of this genus have been used in TCM to treat various ailments, including parasitic diseases, tuberculosis, asthma, hyperglycemia, fever, dysentery, malaria, and cancer. Over 200 alkaloids, including morphines, hasubanas, berberines, hasubanalactams, and aporphines, together with numerous flavonoids, terpenoids, lignans, coumarins, and steroids have been isolated from this genus.[16]

One of the more well studied species is *Stephania tetrandra* (fen fang ji, 粉防己), which has been used in TCM for treating pain, swelling, edema, asthma, high blood pressure, and cancer.[17] Isolations of bioactive agents in *S. tetrandra* have revealed the presence of several compounds, including tetrandine (a smooth muscle relaxant and an anti-carcinogen),[18,19,20] isoquinoline alkaloids (also a smooth muscle relaxant), and flavonoids (anti-inflammatory, anti-bacterial, and anti-carcinogenic properties).[21,22] The structures for these compounds are shown in Figure 3.

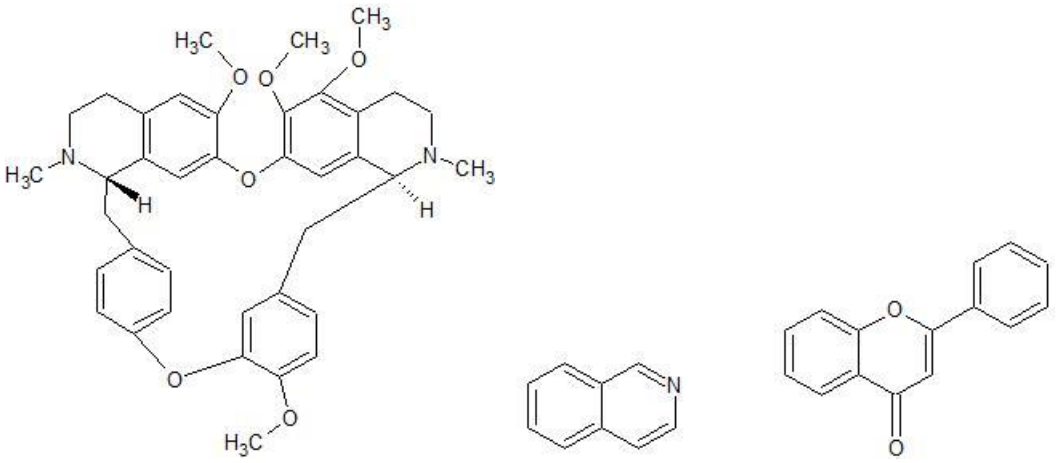


Figure 3. Structure of tetrandine (left), an isoquinoline alkaloid (middle), and a flavonoid (right)

While numerous studies have been published on the bioactivity of several species of the genus *Stephania*, little research has been conducted on the compounds of *S. longa*. Only three compound isolation studies have been published on *S. longa*, all done by the same researchers and all focusing on alkaloids found in the plant. The first, published in 2005, isolated twenty-two hasubanan type alkaloids (thirteen new and nine known) including stephalonines A-I, norprostephabyssine, isoprostephabyssine, isolonganone, and isostephaboline.[23] Figure 4 shows the basic structure of a hasubanan-type alkaloid. Figures 5 and 6 show the structures for some of the alkaloids isolated from the study.

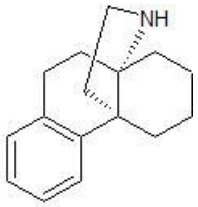


Figure 4. Structure of a hasubanan-type alkaloid

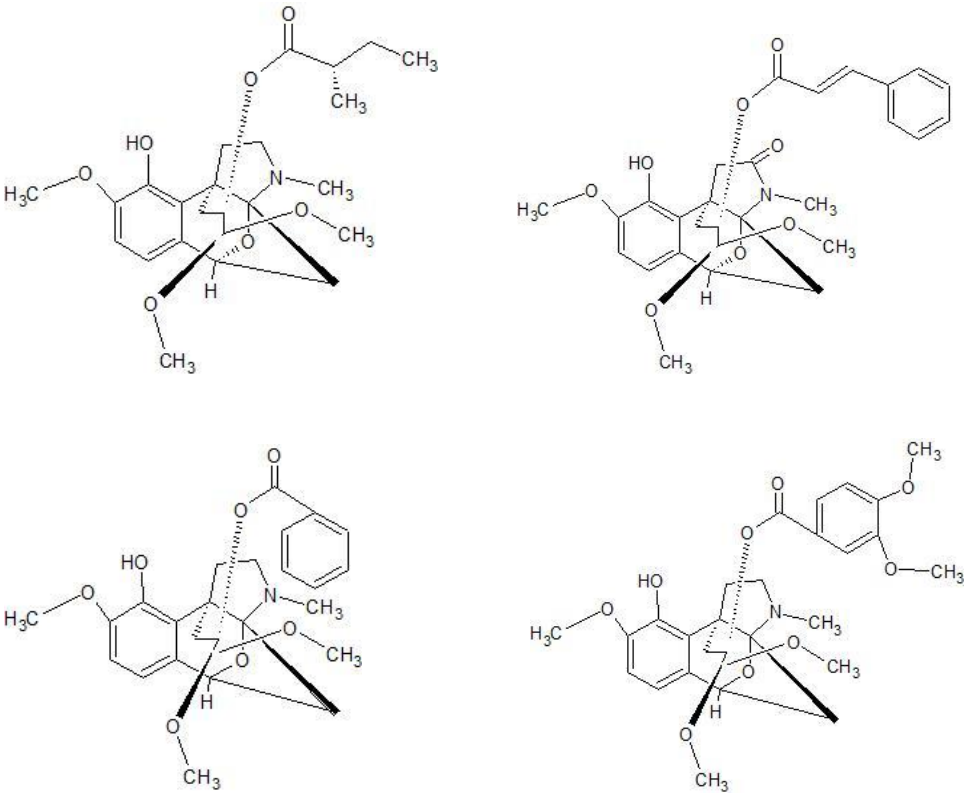


Figure 5. Structure of stephalonine A (top left), E (top right), F (bottom left), and G (bottom right) isolated from *S. longa*

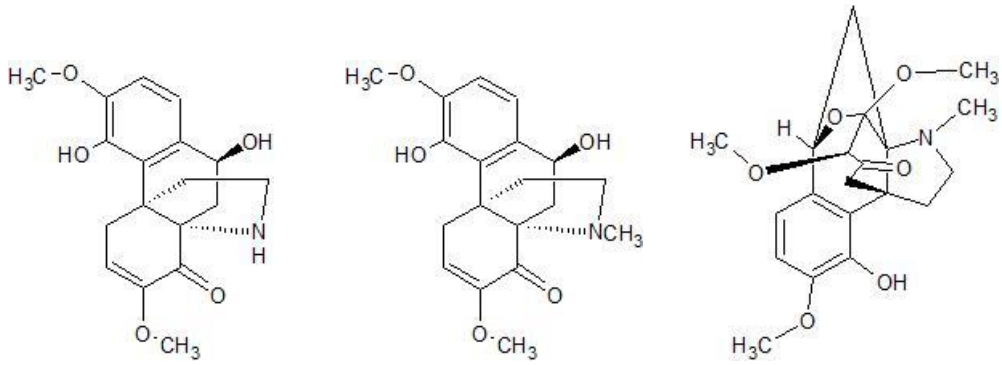


Figure 6. Structure of norprostephabyssine (left), isoprostephabyssine (middle), and isolonganone (right) isolated from *S. longa*

The second study, published in 2006, isolated two new nitro-substituted hasubanan-type alkaloids (stephalonines J and K),[24] and the third study published the same year isolated three new stephaoxocane-type alkaloids, stephalonganines A-C.[25] The stephaoxocane-type alkaloids isolated in the third study are shown in Figure 7.

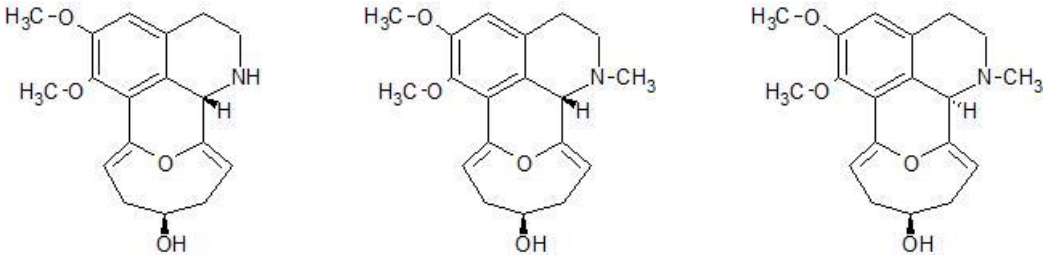


Figure 7. Structure of stephalonganine A (left), B (middle), and C (right) isolated from *S. longa*

The three compound identification studies done on *S. longa* were conducted using various spectroscopy methods, including infrared spectroscopy (IR), mass spectroscopy (MS), and nuclear magnetic resonance spectroscopy (NMR). However, the bioactivities of the isolated compounds were not tested through a bioassay guided fractionation that would measure the bioactivity of the specific agents. Instead, the compounds were simply isolated and elucidated. Further investigation into the specific bioactivity of compounds found in *S. longa* is needed in order to better understand the medicinal properties of the plant.

Research Focus

The objective of this study was to isolate and identify bioactive compounds in *Stephania longa*, a plant commonly used in Traditional Chinese Medicine. Focusing on the anti-parasitic (specifically, anti-trypanosomal) properties of the plant, a bioassay guided fractionation was conducted in order to isolate and identify compounds that may be utilized to treat diseases caused by the parasite *Trypanosoma brucei*, including African sleeping sickness. This plant was chosen as the focus of this research because of the bioactivity measured in the crude extracts, as well as the overall lack of previous research done and thus potential isolation of new bioactive compounds.

CHAPTER II: MATERIALS AND METHODS

The initial crude extract of the plant *Stephania longa* was received from the Guangzi Botanical Garden of Medicinal Plants (GBGMP) located in Nanning, China as part of their joint effort with The Tennessee Center for Botanical Medicine Research (TCBMR) at Middle Tennessee State University (MTSU) in Murfreesboro, TN to isolate and identify bioactive compounds in plants commonly used in TCM. Over 140 extracts of plants used in TCM were sent to MTSU, where initial screening for bioactivity in anti-viral, anti-tumor, and anti-trypanosome systems, along with the further chemical purification of the active compounds was conducted.

The standard protocol for natural products isolation developed in our laboratory was used for this process. It is important to note that this is a bioassay guided fractionation that incorporates measuring the bioactivity of the individual fractions in between each step of the purification process. Continued purification was conducted only for the fractions demonstrating significant levels of bioactivity. Figure 8 illustrates the basic outline for this isolation procedure, as well as the naming pattern of the fractions for each step.

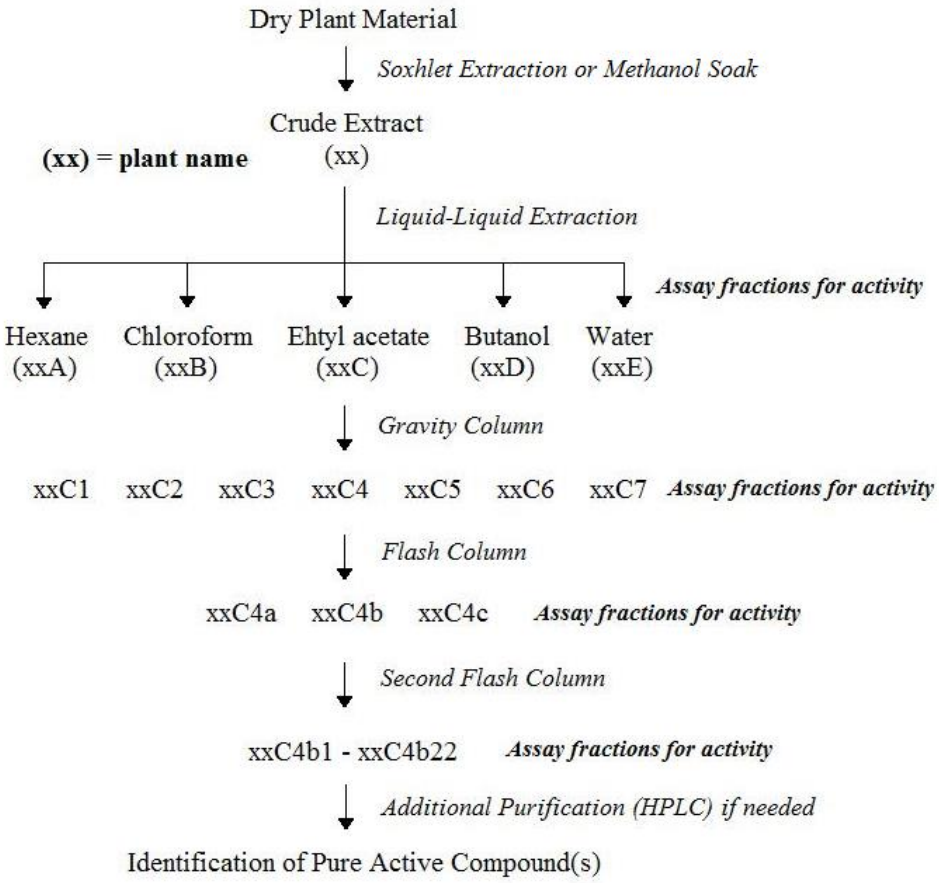


Figure 8. Outline of the isolation procedure and naming scheme

Stephania longa Extractions

The crude extract of *S. longa* sent from China was 73.25 g of the ethanol fraction that was obtained from the soxhlet extraction of the dry plant material. Initial bioscreening showed anti-trypanosome activity in this crude extract. A total of three extractions were conducted from the crude extract, using 1.0 g, 5.0 g, and 5.0 g respectively.

Instruments, Materials, and Reagents

Thin layer chromatography (TLC) was performed on glass plates coated with silica gel and UV active backing purchased from Fisher Scientific, Pittsburgh, PA. The TLC plates were analyzed with a short wavelength (254 nm) UV light and subsequently stained with phosphomolybdic acid (reagent grade, Aldrich, Milwaukee, WI) prepared as a 10% solution in ethanol. Gravity column chromatography was performed with silica gel, 63-200 micron 70-230 mesh ASTM (reagent grade, Fisher Scientific, Pittsburgh, PA). Flash column chromatography was performed with silica gel, 60 Å 230-400 mesh ASTM, reagent grade, Fisher Scientific, (Pittsburgh, PA).

Methanol, acetone, ethyl acetate, ethanol, and hexanes were purchased from Fisher Scientific, (Pittsburgh, PA). Chloroform was purchased reagent grade from Acros Organic, (New Jersey, USA). Magnesium sulfate was purchased from Fisher Scientific, (Pittsburgh, PA). Evaporation of solvents was achieved using a Buchi rotary evaporator (Model RII, Buchi, Switzerland). Materials used for the trypanosome bioassays included 96 well microtiter plates (Corning, Corning, NY), pentamidine (Sigma-Aldrich, St. Louis, MO), Presto Blue indicator (Invitrogen, Frederick, MD), 4% paraformaldehyde solution (Santa Cruz Biotechnology, Dallas, TX), and a SpectraMax M5 plate reader (Molecular Devices, Sunnyvale, CA).

***Stephania longa* Extraction 1**

The first extraction started by dissolving 1.0 g of the ethanol extract (labeled Sl) into 100 mL of ethanol and stirring for a few hours until completely dissolved. Next, 50 mL of water was added to make the solution aqueous, and a liquid-liquid extraction was conducted using roughly 500 mL each of hexane, chloroform, ethyl acetate, and butanol. Each fraction was put through two levels of filtration and dried with magnesium sulfate, and the solvent was evaporated on a rotary evaporator. Yields for the fractions were as follows (note: bioactive fractions are in bold type):

1. **hexane (SlA) - 137.5 mg (13.8%)**
2. chloroform (SlB) - 319.3 mg (31.9%)
3. ethyl acetate (SlC) - 315.6 mg (31.6%)
4. butanol (SlD) - 98.3 mg (9.8%)

Each fraction was then sent for trypanosome assays, and activity was recorded in the SlA and SlB fractions. Thin liquid chromatography (TLC) plates were prepared in 1:1 ethyl acetate/hexane, 1:1 acetone/dichloromethane, 1:1 chloroform/hexane, and pure hexane for each fraction. The plates were then checked under ultraviolet light and stained with phosphomolybdic acid (PMA) and anisaldehyde.

Additional purification was done for the SlA (137.5 mg) and SlB (319.3 mg) fractions. Gravity column chromatography of both SlA and SlB was conducted using 100 mL each of pure hexane, 15% ethyl acetate/hexane, 30% ethyl acetate/hexane, 45% ethyl acetate/hexane, 60% ethyl acetate/hexane, 75% ethyl acetate/hexane, and pure ethyl acetate. The solvents were then evaporated on a rotary evaporator. Yields for the fractions were as follows:

SlA gravity column

1. pure hexane (SlA1) - 6.5 mg (4.7%)
2. 15% ethyl acetate/hexane (SlA2) - 6.4 mg (4.7%)
3. 30% ethyl acetate/hexane (SlA3) - 50.5 mg (36.7%)
4. 45% ethyl acetate/hexane (SlA4) - 31.6 mg (22.9%)
5. 60% ethyl acetate/hexane (SlA5) - 8.2 mg (5.9%)
6. **75% ethyl acetate/hexane (SlA6) - 6.5 mg (4.7%)**
7. **pure ethyl acetate (SlA7) - 6.9 mg (5.0%)**

SlB gravity column

1. pure hexane (SlB1) - 8.1 mg (2.5%)
2. 15% ethyl acetate/hexane (SlB2) - 1.2 mg (0.4%)
3. 30% ethyl acetate/hexane (SlB3) - 7.1 mg (2.2%)
4. 45% ethyl acetate/hexane (SlB4) - 9.4 mg (2.9%)

5. 60% ethyl acetate/hexane (SlB5) - 10.0 mg (3.1%)
6. 75% ethyl acetate/hexane (SlB6) - 12.9 mg (4.0%)
7. pure ethyl acetate (SlB7) - 24.1 mg (7.5%)

Each fraction was then sent for trypanosome assays, and the strongest activity was in fractions SlA6 and SlA7, but there was not enough material to continue. TLC plates for SlA1-7 were run in 1:1 chloroform/hexane and 1:2 ethyl acetate/hexane, and TLC plates for SlB1-7 were run in 1:1 ethyl acetate/hexane.

***Stephania longa* Extraction 2**

The second extraction started with dissolving 5.0 g of the ethanol extract into 100 mL of ethanol and 200 mL of methanol. Then 100 mL of water was added and the same liquid-liquid partitioning done in extraction 1 was conducted, except only the hexane and chloroform fractions were collected. Yields for the fractions were as follows:

1. **hexane (SlA) - 942.0 mg (18.8%)**
2. chloroform (SlB) - 2,781.4 mg (56.0%)

At this point, it was decided that only the purification of the new SlA (942.0 mg) fraction was necessary, as the low activity of the previous SlB fraction was not worth pursuing.

The same gravity column chromatography as done in extraction 1 was conducted, and the yields for the fractions were as follows:

1. pure hexane (SlA1) - 38.2 mg (4.1%)
2. 15% ethyl acetate/hexane (SlA2) - 56.9 mg (6.1%)
3. 30% ethyl acetate/hexane (SlA3) - 298.7 mg (31.7%)
4. 45% ethyl acetate/hexane (SlA4) - 197.3 mg (20.9%)
5. 60% ethyl acetate/hexane (SlA5) - 51.6 mg (5.5%)
6. **75% ethyl acetate/hexane (SlA6) - 337.4 mg (35.8%)**
7. **pure ethyl acetate (SlA7) - 72.8 mg (7.7%)**

Fractions SlA7 from extraction 1 (6.9 mg) and SlA7 from extraction 2 (72.8 mg) were combined (79.7 mg), and TLC plates were run in pure hexane, 1:2 ethyl acetate/hexane, 4:1 ethyl acetate/hexane, and pure ethyl acetate with a drop of methanol. Fractions SlA6 from extraction 1 (6.5 mg) and SlA6 from extraction 2 (337.4 mg) were also combined (343.9), but were not run on TLC plates. Next, flash column chromatography was conducted for both the SlA7 and SlA6 fraction using 1:10 ethyl acetate/hexane, pure ethyl acetate, and 1:1 ethyl acetate/methanol

(30 mL of each was used for SlA7, and 100 mL of each was used for SlA6). The yields for the fractions were as follows:

SlA7 flash column

a. 1:10 ethyl acetate/hexane (SlA7a) - 3.1 mg (3.9%)

b. **pure ethyl acetate (SlA7b) - 27.9 mg (35.0%)**

c. 1:1 ethyl acetate/methanol (SlA7c) - 32.2 mg (40.4%)

SlA6 flash column

a. 1:10 ethyl acetate/hexane (SlA6a) - 27.8 mg (8.1%)

b. **pure ethyl acetate (SlA6b) - 65.6 mg (19.1%)**

c. 1:1 ethyl acetate/methanol (SlA6c) - 60.1 mg (17.5%)

Due to similar activity and possible spillover of compounds between SLA6 and SLA7, fractions SlA7a-c were combined with fractions SlA6a-c and named SlA7a-c. Each fraction was then sent for trypanosome assays, and activity was shown in the SlA7b fraction. TLC plates were also run for SlA7b in pure ethyl acetate, 1:20 methanol/dichloromethane, and 1:1 acetone/dichloromethane. A 2nd flash column was then conducted for the SlA7b fraction in order to separate out the different compounds. Four rounds of chromatography were done using 100 mL each of 1:20 methanol/dichloromethane, 1:10 methanol/dichloromethane,

1:5 methanol/dichloromethane, and 2:1 methanol/dichloromethane. Each round produced 4-6 fractions collected in test tubes, and TLC plates were run for every other fraction in 1:20 methanol/dichloromethane. Thus, only the fractions containing the compounds of the starting material were evaporated on a rotary evaporator. Round 1 produced fractions 1-4, round 2 produced fractions 5-10, round 3 produced fractions 11-16, and round 4 produced fractions 17-22. Based on the TLC plate results, fractions 2, 3, and 18-22 were collected and evaporated. The yields for the fractions were as follows:

1. fraction 2 (SlA7b1) - 12.9 mg (13.8%)
2. fraction 3 **(SlA7b2) - 9.2 mg (9.8%)**
3. fractions 18-22 **(SlA7b3) - 32.7 mg (34.9%)**

Each fraction was then sent for trypanosome assays, with results showing activity in both fractions SlA7b2 and SlA7b3. A crude ^{1}H-NMR was also taken for each fraction, but did not reveal any usable data.

***Stephania longa* Extraction 3**

The third extraction started with dissolving 5.0 g of the ethanol extract into 300 mL of ethanol. Then 100 mL of water was added and liquid-liquid partitioning was conducted for only the hexane (SlA) fraction, yielding 740.3 mg

(14.8%). Then the same gravity column done in both previous extractions was run, yielding 78.0 mg (10.5%) of fraction SlA7. Next, the same flash column done in both previous extractions was run, yielding 53.3 mg (68.3%) of the fraction SlA7b.

All trypanosome bioassays were conducted by Jeannie Stubblefield (Biology Department, Middle Tennessee State University) as follows: Log phase trypanosomes were adjusted in fresh media to deliver $1x10^4$ cells per well in 90 uL into clear 96 well microtiter plates. Extract samples were dissolved in DMSO and diluted in fresh media such that the addition of 10 uL would produce final well concentrations ranging from 3.125 ug/mL – 50 ug/mL. Maximum well concentrations of DMSO did not exceed 0.5%. Control wells were treated with pentamidine, DMSO, or media only. Each condition was plated in triplicate. Treated samples were incubated at 37°C and 5% CO_2 for 48 hours, at which time 11 uL of the resazurin-based indicator, Presto Blue, was added to each well. Plates were incubated for an additional 24 hours under the same conditions. At 72 hours, 60 uL of a 4% paraformaldehyde solution was added to each well and fluorescent intensity was measured on a SpectraMax M5 plate reader using excitation and emission wavelengths of 560 and 590 nm, respectively. Inhibition was calculated based on the difference between fluorescent intensity levels of treated and untreated wells. Samples showing >50% inhibition were considered to be bioactive.

CHAPTER III: RESULTS AND DISCUSSION

Various time constraints and extenuating circumstances prevented the further purification and identification of the active compounds in the extract of *Stephania longa*. Only the soxhlet extraction, liquid-liquid extraction, gravity column, and two flash columns could be conducted. Thus, no data on the identification of active compounds could be acquired, and only the data for the TLC plates and biological assays against *Trypanosoma brucei* that were conducted throughout the purification process are included.

Thin Liquid Chromatography (TLC) Results

The best separation of compounds for the hexane fraction in extraction 1 was seen in the TLC plate run in 1:1 chloroform/hexane, which is shown in Figure 9. No retention factor (Rf) values were taken for this plate due to the inability to measure individual compounds. (*note: figures of TLC plates used throughout this paper are not drawn to scale, and are simply graphical representations).

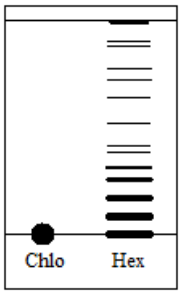


Figure 9. TLC plate for SlA and SlB in 1:1 chloroform/hexane

The TLC plates for the fractions obtained after the gravity column in extraction 1 had better separation than the liquid-liquid extraction fractions. The TLC plate for the fractions SlA1-7 run in 1:1 chloroform/hexane is represented in Figure 10.

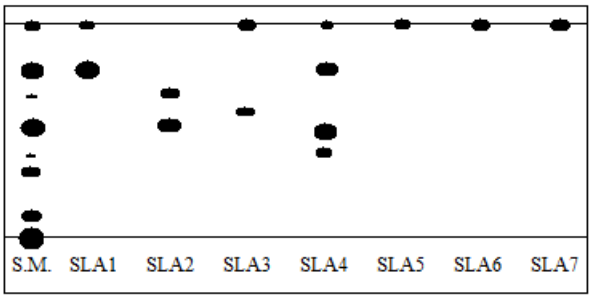


Figure 10. TLC plate for SlA1-7 in 1:1 chloroform/hexane

The Rf values for the compounds in the starting material (S.M.) were as follows, starting from the lowest to the highest: 0.00, 0.06, 0.29, 0.43, 0.51, 0.71,

0.91, 1.00 (total of 8 compounds). For SlA1: 0.91, 1.00 (2). For SlA2: 0.51, 0.70 (2). For SlA3: 0.66, 1.00 (2). For SlA4: 0.39, 0.41, 0.90, 1.00 (4). The Rf value of the compound in fractions SlA5-7 was 1.00.

The TLC plate for the fractions SlB1-7 run in 1:1 ethyl acetate/hexane is shown in Figure 11.

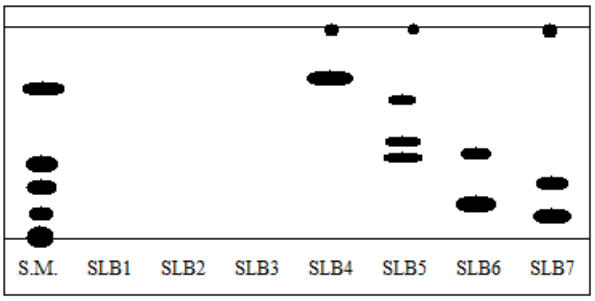


Figure 11. TLC plate for SlB1-7 in 1:1 ethyl acetate/hexane

The Rf values for the compounds in the starting material (S.M) were as follows, starting from the lowest to the highest: 0.00, 0.09, 0.21, 0.30, 0.67 (5). For SlB4: 0.69, 1.00 (2). For SlB5: 0.30, 0.33, 0.64, 1.00 (4). For SlB6: 0.16, 0.35 (2). For SlB7: 0.09, 0.21, 1.00 (3).

The TLC plates for fraction SlA7 from extraction 2 are shown in Figure 12. No Rf values were taken, as this was done to find the right solution to give the best separation (4:1 ethyl acetate/hexane).

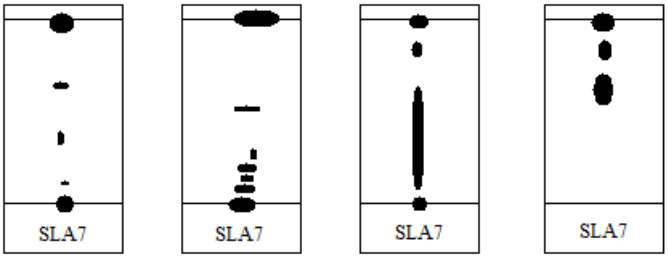


Figure 12. TLC plates for SlA7 run in (from left to right) pure hexane, 1:2 ethyl acetate/hexane, 4:1 ethyl acetate/hexane, and pure ethyl acetate with a drop of methanol

The TLC plate for fraction SlA7b obtained through the 1st flash column from extraction 2 showed the best separation of compounds in 1:20 methanol/dichloromethane, indicating the presence of 5 separate compounds in the fraction, as seen in Figure 13.

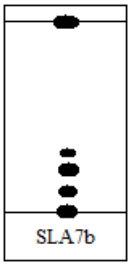


Figure 13. TLC plate for SlA7b in 1:20 methanol/dichloromethane

The Rf values for the compounds were as follows, starting from the lowest to the highest: compound 1: 0.00, compound 2:0.07, compound 3: 0.17, compound 4: 0.24, compound 5: 1.00.

The TLC plates for the fractions obtained through the 2nd flash column from extraction 2 run in 1:20 methanol/dichloromethane are shown in Figure 14.

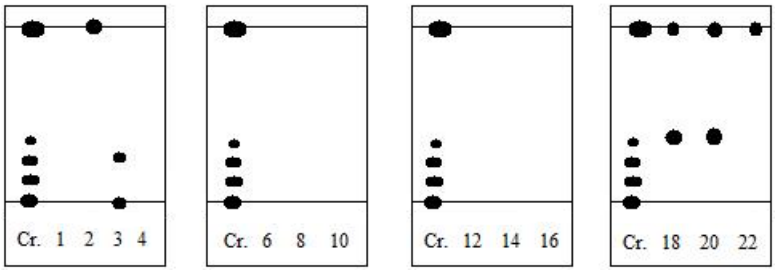


Figure 14. TLC plates for fractions 1-4, and every other fraction from 6-22

The Rf values for the crude are the same as the SlA7b plate: compound 1: 0.00, compound 2: 0.07, compound 3: 0.17, compound 4: 0.24, compound 5: 1.00. For fraction 2: 1.00 (1). For fraction 3: 0.00, 0.17 (2). For fraction 18: 0.24, 1.00 (2). For fraction 20: 0.24, 1.00 (2). For fraction 22: 1.00 (1).

Assay Results of Liquid-Liquid Extraction Fractions

The assay of the fractions obtained from the liquid-liquid extraction of the ethanol extract of *S. longa* showed activity in both the hexane (SlA) and chloroform (SlB) fractions, with inhibition of *T. brucei* occurring at 12.5 ug/mL dose and higher, and 25 ug/mL dose and higher, respectively. Lower doses of SlA and SlB did not show any inhibition activity, while the ethyl acetate (SlC)

and butanol (SlD) showed no activity for all dose points. The data for this assay are shown in Table 1.

Table 1. Bioassay results for fractions SlA-D of *S. longa*

Fractions	Dose Points (ug/mL)				
	50	25	12.5	6.25	3.125
Hexane (SlA)	**Activity**	**Activity**	**Activity**	No activity	No activity
Chloroform (SlB)	**Activity**	**Activity**	No activity	No activity	No activity
Ethyl acetate (SlC)	No activity	No activity	No activity	No activity	No activity
Butanol (SlD)	No activity	No activity	No activity	No activity	No activity

Assay Results of Gravity Column Chromatography Fractions

The assay of the fourteen fractions obtained from the gravity column chromatography of SlA and SlB showed inhibition activity of *T. brucei* in fractions SlA2 (99.19% at 50 ug/mL, 89.38% at 25 ug/mL), SlA3 (96.64% at 50 ug/mL, 50.12% at 25 ug/mL), SlA4 (44.46% at 50 ug/mL), SlA5 (96.92% at 50 ug/mL), SlA6 (99.25 at 50 ug/mL, 98.21 at 25 ug/mL, 28.30% at 12.5 ug/mL), SlA7 (99.06% at 50 ug/mL, 99.23% at 25 ug/mL, 99.23% at 12.5 ug/mL, 98.97%

at 6.25 ug/mL, 98.09% at 3.125 ug/mL), SlB3 (49.11% at 50 ug/mL), and SlB7 (99.03% at 50 ug/mL, 93.80% at 25 ug/mL, 53.10% at 12.5 ug/mL).

The two fractions that were worth pursuing were SlA6 and SlA7, with SlA7 showing over 98% inhibition even down to the lowest dose point of 3.125 ug/mL. Lower dose points of SlA2-7, SlB3, and SlB6-7, along with all the dose points of SlA1, SlB1-2, and SlB4-5 showed insignificant percentages of inhibition. The data for this assay are shown in Table 2.

Table 2. Percent inhibition of *T. brucei* at 5 dose points for fractions SlA1-7 and SlB1-7 of *S. longa*

Fractions	Dose Points (ug/mL)				
	50	25	12.5	6.25	3.125
SlA1	(2.12)	(5.37)	(5.41)	(4.37)	(3.40)
SlA2	**99.19**	**89.38**	(4.64)	(6.25)	(5.04)
SlA3	**96.64**	**50.12**	(5.87)	(5.84)	(4.84)
SlA4	(44.46)	(4.78)	(5.02)	(3.30)	(2.16)
SlA5	**96.92**	(27.32)	(5.47)	(3.74)	(2.89)
SlA6	**99.25**	**98.21**	(28.39)	(2.66)	(1.89)
SlA7	**99.06**	**99.23**	**99.23**	**98.97**	**98.09**
SlB1	(15.04)	(15.38)	(14.79)	(13.63)	(12.24)
SlB2	(11.50)	(14.86)	(13.80)	(12.82)	(11.62)
SlB3	(49.11)	(3.40)	(3.42)	(2.23)	(1.45)
SlB4	(8.09)	(7.87)	(6.39)	(4.78)	(3.57)
SlB5	(6.15)	(6.94)	(5.78)	(3.97)	(2.47)
SlB6	**38.68**	(0.87)	(3.53)	(3.06)	(2.03)
SlB7	**99.03**	**93.80**	**53.10**	(5.19)	(2.74)

Assay Results of 1st Flash Column Chromatography Fractions

The assay of the three fractions obtained from the 1st flash column chromatography of SlA7 showed inhibition activity of *T. brucei* in the fraction SlA7b (100.56% at 50 ug/mL, 100.29% at 25 ug/mL, 95.47% at 12.5 ug/mL). Lower dose points of SlA7b, along with all dose points of SlA7a and SlA7c, showed insignificant percentages of inhibition. The data for this assay are shown in Table 3.

Table 3. Percent inhibition of *T. brucei* at 5 dose points for fractions SlA7a-c of *S. longa*

Fractions	Does Points (ug/mL)				
	50	25	12.5	6.25	3.125
SlA7a	(3.30)	(2.45)	(1.63)	(0.61)	(0.49)
SlA7b	**100.56**	**100.29**	**95.47**	(0.91)	(0.86)
SlA7c	(5.91)	(5.03)	(4.58)	(3.56)	(2.41)

Assay Results of 2nd Flash Column Chromatography Fractions

The assay of the three fractions obtained from the 2nd flash column chromatography of SlA7b showed inhibition activity of *T. brucei* in the fractions SlA7b2 (94.27% at 50 ug/mL, 92.68% at 25 ug/mL) and SlA7b3 (90.61% at 50 ug/mL). Lower dose points of SlA7b2 and SlA7b3, along with all dose points of

SlA7b1, showed insignificant percentages of inhibition. The data for this assay are shown in Table 4.

Table 4. Percent inhibition of *T. brucei* at 5 dose points for fractions SlA7b1-2 of *S. longa*

Fractions	Does Points (ug/mL)				
	50	25	12.5	6.25	3.125
SlA7b1	(1.66)	(1.73)	(0.47)	(0.16)	(0.21)
SlA7b2	**94.27**	**92.68**	(9.11)	(1.49)	(1.47)
SlA7b3	**90.61**	(2.44)	(1.41)	(0.62)	(0.47)

Discussion of TLC Plates and Assay Results

The TLC plate run in 1:1 chloroform/hexane for fractions SlA1-7 from the gravity column (Figure 10) showed 8 compounds present in the starting material, but only 1 compound in fraction SlA7, in which there was activity. This was because a more polar solution was needed in order to bring down and separate the compounds in SlA7 that were at the solvent line. This is why the TLC plate done after the 1st flash column (run in 1:20 methanol/dichloromethane) showed the 5 separate compounds in SlA7b (Figure 13). The 5 compounds were then separated through the 2nd flash column, with TLC plates showing compounds 1 and 3 present in fraction 3, compound 4 present in fractions 18-20, and compound 5 present in fractions 2, 18-20, and 22. Activity from the assay

revealed that the activity was in SlA7b2 (fraction 3) and SlA7b3 (fractions 18-22), indicating that compounds 1, 3, 4, and 5 had the activity.

Assay results for the fractions obtained from the liquid-liquid extraction (SlA-D) showed activity in only SlA and SlB, with more activity in SlA (inhibition down to 12.5 ug/mL). This is why only SlA was considered for further purification later in the research. The assay for fractions SlA1-7 showed the strongest activity in SlA6 and SlA7, with SlA7 inhibiting over 98% of trypanosome activity down to the smallest does point (3.125 ug/mL). However, SlA6 also showed relatively strong activity (over 98% at 25 ug/mL). This is why the two fractions were later combined. Although the fraction SlB7 also showed some activity, it was less than SlB6-7 and therefore disregarded.

The activity for SlA7b showed over 100% inhibition for dose points 50 and 25 ug/mL. This is due to the standard error for the assays, which allows for the margin of a few percentages (+ and -). Also, compared to the activity of SlA7, activity for SlA7b dropped down a bit, showing no activity at all below 12.5 ug/mL. Assay results for SlA7b2 and SlA7b3 showed even less activity, with only 94% at 50 ug/mL, and insignificant inhibition below 25 ug/mL. This steady decrease in activity for each level of purification may be attributed to possible synergistic activities of the compounds present in the fractions. Compounds working together may inhibit trypanosome activity better than they do alone.

Since purification steps separate out the compounds, a decrease in activity may be expected.

In summary, a total of three extractions were done on *S. longa*, proceeding through four purification steps (liquid-liquid extraction, gravity column, and two flash columns) with bioassays conducted between each step and activity isolated in four compounds. Figure 15 represents the schematic for this particular bioassay guided fractionation.

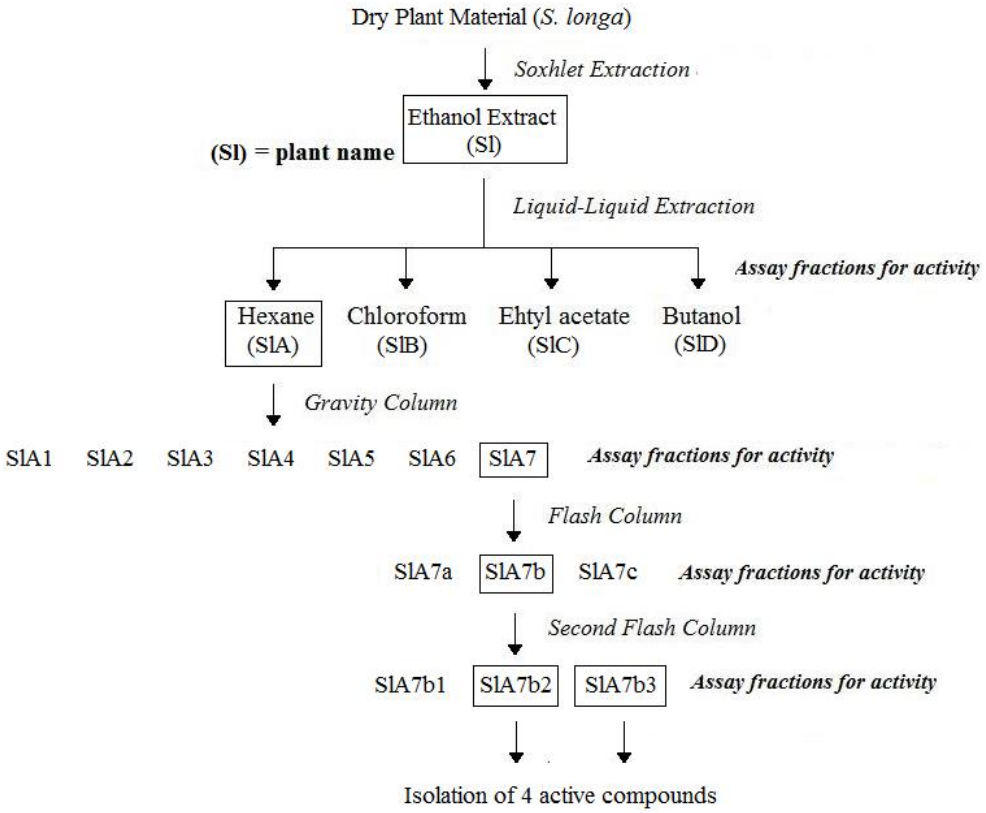


Figure 15. Specific isolation procedure and naming scheme for the bioassay guided fractionation of *S. longa*

Conclusion

After multiple purification steps of the crude ethanol extract of the plant *Stephania longa* and the continuous measuring of inhibition against *Trypanosoma brucei*, a possible four compounds may be responsible for the bioactivity. Further purification steps, including HPLC, and additional bioassays would be needed in order to separate out the compounds into pure compounds and isolate the bioactivity. Additionally, spectroscopy methods including IR, MS, ^{1}H-NMR, ^{13}C-NMR, COSY, $DEPT_{90}$, and $DEPT_{135}$ would be needed to identify the pure compounds. Continued research on *Stephania longa* and its bioactivity against *Trypanosoma brucei* is encouraged, along with the investigation of the bioactivity of other plants used in TCM.

REFERENCES

1. Miller, J.S. The Global Importance of Plants as Sources of Medicines and the Future Potential of Chinese Plants. In *Drug Discovery and Traditional Chinese Medicine: Science, Regulatory and Globalization*; Kluwer Academic Publishers: Netherlands, 2001; pp. 33-42.

2. Yang, S.P.; Yue, J.M. Discovery of structurally diverse and bioactive compounds from plant resources in China. *Acta Pharmacol. Sin*. **2012**, *33*, 1147-1158.

3. Klayman, D.L. Qinghaosu (artemisinin): an antimalarial drug from China. *Science*. **1985**, 228, 1049-1055.

4. Liu, J.S.; Zhu, Y.L.; Yu, C.M.; Zhou, Y.Z.; Han, Y.Y.; Wu, F.W.; Qi, B.F. The structure of huperzine A and B, two new alkaloids exhibiting marked anticholinesterase activity. *Can. J. Chem*. **1986**, 64, 837-839.

5. Ferriera, A.; Rodrigues, M.; Fortuna, A.; Falca, A.; Alves, G. Huperzine A from *Huperzia serrata*: a review of its sources, chemistry, pharmacology and toxicology. *Phytochem. Rev.* **2014**.

6. Liang, X.T.; Fang, W.S.; Zhu, D.Y.; Tan, C.H.; Li, Y.M. Chapter 4. The Overview of Studies on Huperzine A: A Natural Drug for the Treatment of Alzheimer's Disease. In *Medicinal Chemistry of Bioactive Natural Products*; John Wiley & Sons, Inc.: Hoboken, NJ, 2005.

7. Newman, D.J. Natural Products as Leads to Potential drugs: An Old Process or the New Hope for Drug Discovery? *J. Med. Chem*., **2008**, 51, 2589-2599.

8. Newman, D.J.; Cragg, G.M. Natural products as sources of new drugs over the 30 years from 1981 to 2010. *J. Nat. Prod.* **2012,** *75*, 311-335.

9. World Health Organization, The WHO Essential Medicines and Health Products Information Portal. http://apps.who.int/medicinedocs/en/d/Js4917e/3.html (accessed April 2015).

10. Center for Disease Control and Prevention, Parasites - African Trypanosomiasis (also known as Sleeping Sickness): Disease. http://www.cdc.gov/parasites/sleepingsickness/disease.html (accessed April 2015).

11. Center for Disease Control and Prevention, Parasites - African Trypanosomiasis (also known as Sleeping Sickness): Biology. http://www.cdc.gov/parasites/sleepingsickness/disease.html (accessed April 2015).

12. Center for Disease Control and Prevention, Parasites - African Trypanosomiasis (also known as Sleeping Sickness): East African Trypanosomiasis. http://www.cdc.gov/parasites/sleepingsickness/ disease.html (accessed April 2015).

13. Center for Disease Control and Prevention, Parasites - African Trypanosomiasis (also known as Sleeping Sickness): West African Trypanosomiasis. http://www.cdc.gov/parasites/sleepingsickness/ disease.html (accessed April 2015).

14. Center for Disease Control and Prevention, Parasites - African Trypanosomiasis (also known as Sleeping Sickness): Treatment. http://www.cdc.gov/parasites/sleepingsickness/disease.html (accessed April 2015).

15. Wu, Z.; Raven, P.H.; Deyuan, H. Stephania longa. *Flora of China*. **2008**, 7, 16-20.

16. Semwal, D.K.; Badoni, R.; Semwal, R.; Kothiyal, S.K.; Singha, G.J.P.; Rawat, U. The genus Stephania (Menispermaciae): Chemical and pharmacological perspectives. *J. Ethnopharmacol*. **2010**, 132, 369-383.

17. Zhou, J.; Xie, G.; Yan, X. *Encyclopedia of Traditional Chinese Medicines - Molecular Structures, Pharmacological Activities, Natural Sources and Applications*; Springer: New York, NY, 2011.

18. Xu, W.; Debeb, B.G.; Lacerda, L.; Li, J.; Woodward, W.A. Tetrandrine, a Compound Common in Chinese Traditional Medicine, Preferentially Kills Breast Cancer Tumor Initiating Cells (TICs) in Vitro. *Cancers*. **2011**, 3, 2274.

19. Ng, L.T.; Chiang, L.C.; Lin, Y.T.; Lin, C.C. Antiproliferative and apoptotic effects of tetrandrine on different human hepatoma cell lines. *The Americ. J. Chin. Med.* **2006**, 34, 125-135.

20. Chen, Y.J. Potential role of tetrandrine in cancer therapy. *Acta Pharmacol. Sin.* **2002**, 23, 1102-1106.

21. Choi, H.S.; Kim, H.S.; Min, K.R.; Kim, Y.; Lim, H.K.; Chang, Y.K.; Chung, M.W. Anti-inflammatory effects of fangchinoline and tetrandrine. *J. Ethnopharmacol*. **2000**, 69, 173-179.

22. Ma, W.; Nomura, M.; Takahashi-Nishioka, T.; Kobayashi, S. Combined effects of fangchinoline from Stephania tetrandra Radix and formononetin and calycosin from Astragalus membranaceus Radix on hyperglycemia and hypoinsulinemia in streptozotocin-diabetic mice. *Biol. & Pharm. Bull.* **2007**, 30, 2079-2083.

23. Zhang, H.; Yue, J.M. Hasubanan type alkaloids from Stephania longa. *J. Nat. Prod.* **2005**, 68, 1201-1207.

24. Zhang, H.; Wang, F.D.; Yue, J.M. Two Unusual Nitro-substituted Hasubanan-type Alkaloids from Stephania longa. *Chinese J. Chem.* **2006**, 24, 781-784.

25. Zhang, H.; Yue, J.M. New Stephaoxocane Alkaloids from *Stephania longa*. *Helvetica Chimica Acta*. **2006**, 89, 1105-1109.