BIOACTIVITY SCREENING OF PLANTS WITH ANTI-CANCER POTENTIALS FROM DOMINICAN ETHNOBOTANY

Chelsea E. Azarcon and Manuel A. Aregullin
Cornell University, Ithaca, NY

ABSTRACT

Four plants common to the medicinal tradition of the Dominican Republic were prepared as organic extracts and were assayed for cytotoxic, anti-proliferative, and antimicrobial activity. Plants were selected based on anecdotal descriptions of their use in treatment of cancer and assays were selected to provide a preliminary assessment of these potentials. Isopropanol and methanol extracts of *Annona muricata, Eruca sativa,* and *Lantana camara* displayed cytotoxic activity in a time and dose dependent manner. Anti-proliferative properties were observed in the isopropanol and methanol extracts of *Annona muricata, Lantana camara*, *Eruca sativa,* and *Ptychosperma elegans* (a plant unrepresented in published biomedical assays to date). Additionally, *Lantana camara* displayed strong antibacterial and anti-fungal activity.

INTRODUCTION

The Dominican Republic is a region in the Caribbean that contains a rich ethnobotanical tradition. As a developing country, it's citizens still utilize plants for medicinal purposes. However, the medicinal properties of these plants are of interest to modern medicine as well. Many important drugs, including chemotheraputics like vincristine, etoposide, and others have been developed from chemical compounds isolated in plants (Houghton, 1995). Possessing extensive biodiversity, the Dominican Republic is an ideal site for discovering plants that possess biologically active chemistries relevant to pharmacology.

The plants selected for testing were *Annona muricata, Lantana camara*, *Eruca sativa,* and *Ptychosperma elegans*. *A. muricata* is used as an anticancer agent in Dominican ethnobotany and has been confirmed by biomedical studies to possess a variety of bioactivities (Gajalakshmi *et al*., 2012). *L. camara* is also used in Dominican ethnobotany for the treatment of flu and fever and is cited as cytotoxic in several biomedical studies (Sanjeeb *et al*., 2012). Although *E. sativa* is also utilized in Dominican ethnobotany, only a small number of studies regarding its biological activity exist. *E. sativa* belongs to a plant family (Brassicaceae) that exhibits biocativity; *E. sativa* was thus hypothesized to possess biologically active chemistry. *P. elegans* is unrepresented in Dominican ethnobotany or biomedical research; it was selected as a candidate for anti cancer screening based on anecdotal recommendation(Laux, personal communication).

OBJECTIVES

The objective of this research was to examine the biological activity of four plants used in traditional Dominican medicine; confirm ethnobotanical and biomedical claims regarding the anti cancer activity of these plants; examine plants with no or little biomedical representation to confirm hypotheses based on chemistry. Antibiotic activity of these plants was also examined.

MATERIALS AND METHODS

Four plant specimens were collected from the grounds of the Fundacion de Ecologica, Punta Cana. The plant species collected include *Annona muricata, Lantana camara, Eruca sativa,* and *Ptychosperma elegans*. Plant materials were laid flat on a laboratory bench and allowed to air dry, for a period of days, until no moisture remained. Select plant parts were (fruit and seeds of *A. muricata,* leaves of *L. camara* and *E. sativa,* fruit of *P. elegans*) ground in a blender to yield fine particulate. One gram of particulate from each species was transferred to a scintillation vial and 7.5 ml of an organic solvent were added and allowed to stand overnight before use. Two extracts were prepared for each plant species, one with isopropyl alcohol [(CH3)2CHOH] and one with methanol (CH3OH). After 24 hours of extraction the extracts were ready for bioassays.

Brine Shrimp Assay (cytotoxicity):

This assay was used to determine the lethality of the plant extracts to brine shrimp (Artemia salina) nauplii as a proxy for cytotoxicity since it has been shown to have a strong correlation with results from antitumor activity against human cancer cell lines (Anderson et al., 1991). A 24 well plate (6x4) with a capacity of 3 mL per well was used for the Brine Shrimp Assays. The plate was set up for testing as follows: a 1 mL aliquot of brine shrimp from a hatchery were transferred to the first well in column #1 using a pipette followed by the addition of 2 mL of brine from a glass cup. 0.5 mL from well #1 was transferred into each of the remaining 5 wells in the first row (#2-#6). 2 mL of brine were then added to each well to make a total of 2.5mL in each well. This was repeated for each row of wells. Each row was designated for each extract. 1 drop of each extract was added to the first well of its designated row; 2, 3 and 4 drops of each extract were added to the rest of the rows of wells. After all wells in the plate were set up for testing, the test extracts were then added to the wells in three concentrations; 1 drop (25 μL), 2 drops (50 μL) and 3 drops (75 μL) and 4 drops (100 μl). Four control wells were also prepared with isopropyl alcohol and four with methanol at each of the four concentrations. Wells were observed at 12h and 24h under a dissecting microscope and the number of brine shrimp still swimming were counted to determine percent mortality relative to the controls.

Sea Urchin Assay (anti-proliferative):

To determine the anti-proliferative (antimitotic) effects of the plants, sea urchins were gathered from the Punta Cana Resort and Hotel pier in front of the Diving Center and placed on ice to prevent premature spawning. Spawning was induced in sea urchins by injecting approximately 1 mL of potassium chloride (KCl, 0.1 M) through the soft tissue of the oral surface into the body cavity. Approximately 5 mL of eggs were collected inside of a beaker and were fertilized with

around 200 microL of sperm. One drop of fertilized egg solution was placed into each well. One mL aliquots of seawater were delivered to wells of a 6x4 well plate. Extracts were administered at concentrations of 1 and 4 drops. Parallel administration of methanol and isopropanol was conducted and served as controls. Anti-mitotic activity of extracts was determined using a compound microscope after 24 hrs. and observing the stage of cell division and motility of the zygotes.

Disc Diffusion Assay (antibiotic):

To determine the antibacterial and antifungal effect of the plant extracts, Bauer- Kirby disk diffusion method was used. The solid media used for the antimicrobial bioassays was prepared with 23 g of Nutrient Agar [cat# 213000) manufactured by Difco BD, in 1 L of water. For each plant extract, six filter disks were submerged into the crude extract. Two control groups were set up with the media used for dilutions (Isopro- pyl Alcohol and Methanol). After drying the filter disks the first time, they were dipped into the plant extract vial a second time to increase the concentration of plant extract. Saturated filter discs were left out at room temperature for 30 minutes to facilitate solvent evaporation. Five different microorganisms were used to measure the efficacy of the crude plant extract. *Pseudomans aeruginosa, Staphylococcus aureus, Listeria monocytogenes,* and *Escherichia. coli,* were used for determination of antibacterial activity and *Saccharomyces cerevisiae* for determination of antifungal activity. These were grown on Nutrient Agar plates and the saturated filter discs were placed on top after inoculation. The plates were incubated at 37°C for 24 hours and then observed for inhibition.

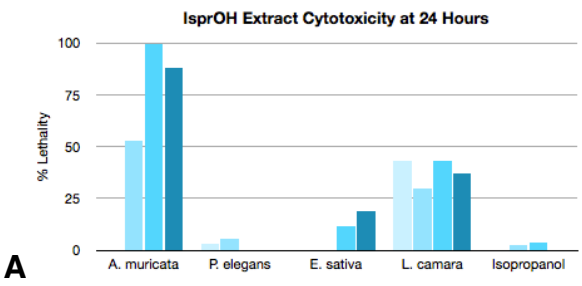
RESULTS

Brine Shrimp Assay (cytotoxicity):

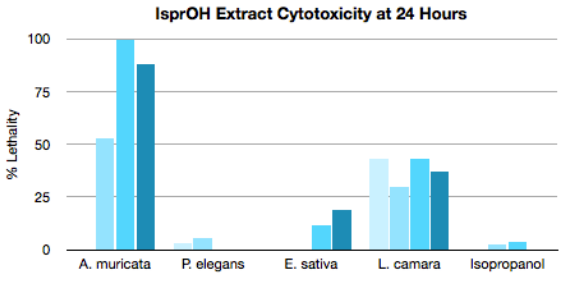
Extracts of *A. muricata*, *E. Sativa*, and *L. camara* exhibited cytotoxic activity (Figure 1). Cytotoxicity of *A. muricata* and *L. camara* was evident in both isopropanol and methanol extracts but enhanced in methanol. The cytotoxicity of *A. muricata* was time dependent and exhibited greater effect at higher concentrations. Methanol extracts of *E. sativa* exhibited cytotoxicity following the same time and concentration patterns as *A. muricata*. *L. camara* extracts also exhibited time dependent cytotoxicity but only methanol extracts displayed concentration dependency. Each of these extracts displayed high percentages of cytotoxicity at 24hrs.

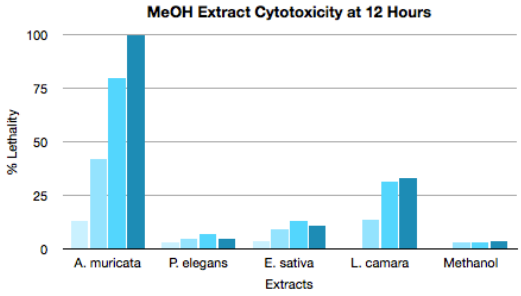
Sea Urchin Assay (anti-proliferative):

The extracts of *A. muricata, P. elegans, E. sativa*, and *L. camara* displayed ability to arrest cell division, with extracts of *P. elegans* and *L. camara* exhibiting the strongest potentials (Table 1). Extracts of both solvents exhibited inhibitory activity but were enhanced in methanol. Isopropanol extracts of *A. muricata*, and *P. elegans* and methanol extract of *E. Sativa* exhibited dose dependency.



A





B

Disc Diffusion Assay (antibiotic):

L. camara exhibited antimicrobial activity (Table 2). Methanol extracts displayed strong inhibition against all microorganisms. Isopropanol extracts exhibited strong inhibition of of *E. coli* and *Staph. aurens*, moderate inhibition of *List.* monocytogenes, and mild inhibtion of *Pseudomonas aetuginosa* and *S. cercvisiae* (fungus). No other extracts exhibited significant inhibition of microbial strains.

A

% of Divided Cells in Methanol

Drops of Extract	A. muricata	P. elegans	E. Sativa	L. camara	Methanol
1d	8.80%	0%	4.70%	0%	22%
4d	0%	0%	2.40%	0%	16.90%

B

% of Divided Cells in Isopropanol

Drops of Extract	A. muricata	P. elegans	E. Sativa	L. camara	Isopropanol
1d	13.80%	14.70%	9.70%	7.20%	55.70%
4d	1.40%	3.30%	0%	2.60%	26%

Table 1

% Cell division in Methanol (A) and Isopropanol (B) extracts

L. Camara Extract Activity

Microbes treated with Solvent	Inhibition	Microbes treated with Solvent	Inhibition
E. Coli (MeOH)	+++	*List. monocytogenes* (MeOH)	+++
E. Coli (IsprOH)	+++	*List. monocytogenes (IsprOH)*	++
Pseud. aetuginosa (MeOH)	+++	*Staph. aurens* (MeOH)	+++
Pseud. aetuginosa (IsprOH)	+	*Staph. aurens* (IsprOH)	+++
S. cercvisiae (MeOH)	+++	Control MeOH	0
S. cercvisiae (IsprOH)	+	Control IsprOH	0

Table 2

Inhibition of microbes by treated with control solvents and L. camara extracts

0= no inhibition + = mild inhibition ++ = moderate inhibition +++ = strong inhibition

DISCUSSION AND CONCLUSION

The objective of the brine shrimp assay was to determine the cytotoxicity of prepared extracts on brine shrimps. *Annona muricata* exhibited significant results in both solvent systems, but was enhanced in methanol. As time and concentration increased, cytotoxic activity of the extracts also increased. In wells containing 2,3, and 4 drops of *A. muricata* methanol extract, 100% brine shrimp mortality was observed at 24 hrs. Methanol extracts of *Eruca sativa* also exhibited significant time and concentration dependent cytotoxic activity. In the well containing one drop of *E. sativa* methanol extract, 87% brine shrimp lethality was observed at 24 hrs., while wells containing 2-4 drops of the same extract exhibited 100% brine shrimp lethality at 24 hours. Like *E. sativa*, *Lantana camara* exhibited significant time and concentration dependent cytotoxicity in methanol. At 24 hrs., wells containing 2 and 3 drops of the extract exhibited 71% brine shrimp lethality and the well containing 4 drops exhibited 83% brine shrimp lethality. The cytotoxicity of the *A. muricata* and *L. camara* extracts has been confirmed by several bioassays and has been accredited to acetogenin and lantadene chemistry respectively (Liaw *et al,* 2002; Sharma et al., 2008). This study's confirmation of the strong cytotoxcicty of *A. muricata* and *L. camara* suggests that additional research regarding the non-target toxicity of these chemistries and their activity in in vivo models would be beneficial. *E. sativa* presents extensive opportunity for further research as it is studied primarily in ecological and commercial applications. In this assay, *E. sativa* extracts displayed strong cytotoxic activity but, in an in vivo assay assessing antitumorigenicity of *E. sativa*, no significant toxicity was observed; significant activity and low toxicity is an intriguing combination that merits further investigation of the plant's bioactivity. (Khoobchandani *et al.,* 2011). The polarity specific results of this assay present a foundation for the isolation of *E. sativa*'s cytotoxic constituents.

The sea urchin assay provided another method of isolating plants with potential "anticancer" chemistries, by observing the ability of those extracts to arrest cell division in sea urchin embryos. All extracts displayed ability to arrest cell division by 85% or greater in isopropanol and 90% or greater in methanol. However, extracts of *Ptychosperma elegans* and *Lantana camara* exhibited the strongest potentials. The methanol extract of *P. elegans* and *L. camara* arrested cell division by 100% at both concentrations (1 and 4 drops). The isopropanol extract of *L. camara* also displayed strong inhibition of cell division by 94% at the low concentration and by 97% at the high concentration. Due to large sample size and the presence of precipitate in some wells, values are averages rather than a precise count. They have been reported as representing inhibition of cell division. However, at time of data collection, embryos had already begun to differentiate making it difficult to determine whether observations represent inhibition of cell division, differentiation, or both. The findings regarding *E. sativa* are of particular interest as there are few studies regarding the bioactivity of this plant. Futhermore, as we were unable to identify any biomedical studies regarding *P. elegans*, this may be the first time that its bioactivity has been reported. The anitmitotic activities of *A. muricata*, *L. camara*, and *E.*

sativa extracts are also particularly intriguing in consideration of their observed cytotoxicity; multiple bioactivities may suggest multiple anticancer mechanisms.

The object of the disc diffusion assay was to asses the antibiotic and antifungal potentials of the prepared plant extracts. Development of resistance to antibiotics is an increasing problem in the microbial world; thus the identification and isolation of efficacious antimicrobial chemistries is an important pursuit (Davies, 2006). This exploration found that *Lantana camara* plant extracts exhibit significant inhibition of gram positive, gram negative, and fungal microbes. Strong inhibition of all microorganisms was apparent upon treatment with methanol and isopropanol extracts of *L. camara*; *Pseudomans aeruginosa* (G-) and *Saccharomyces cerevisiae* were the exception exhibiting only mild inhibition upon treatment with isopropanol extract of *L. camara*. Antibacterial activity of *L. camara* has been previously reported (Tesch *et al.,* 2011). Further research should focus on isolating the active principle of these antimicrobial extracts.

This study has provided evidence that *Annona muricata, Lantana camara, Eruca sativa,* and *Ptychosperam elegans* are promising anticancer compounds. The former three exhibited strong cytotoxic and anti proliferative potentials and the latter exhibited strong anti proliferative activity. *Lantana camara* demonstrated multiple bioactivities and appears to be an effective antimicrobial agent. The findings regarding *Eruca sativa* and *Ptychosperma elegans* are particularly interesting as little or no biomedical research regarding these plants exists in publication.

REFERENCES

Anderson, J. E., Goetz, C. M., McLaughlin, J. L. and Suffness, M. "A Blind Comparison of Simple Bench-top Bioassays and Human Tumour Cell Cytotoxicities as Antitumor Prescreens." Phytochemical Analysis. July, 1991 Vol. 2, No. 3: 107–111.

Davies, Julian. "Where Have All the Antibiotics Gone?" Canadian Journal of Infectious Diseases and Medical Microbiology." Sep-Oct.,2006. Vol. 17, No. 5:287-290.

Gajalaksmi, S., Vijayalakshmi, S., and Rajeswari,V. "Phytochemical and Pharmacological Properties of *Annona Muricata*: A Review." International Journal of Pharmacy and Pharmaceutical Sciences. 2012, Vol. 4, No. 2: 3-6.

Houghton, P. J: 1995.The Role of Plants in Traditional Medi- cine and Current Therapy. The Journal of Alternative and Complementary Medicine. June 1995, Vol. 1, No. 2: 131-143.

Khoobchandani M, N Ganesh, S Gabbanini, L Valgimigli, and MM Srivastava. "Phytochemical potential of Eruca sativa for inhibition of melanoma tumor growth." Fitoterapia. June, 2011. Vol. 82, No.4(2011):647-53.

Laux, Maria. (June, 2013). Correspondence to C. Azarcon [Conversation]. Cornell University MHIRT Program, NIMHD. Punta Cana, Dominican Republic.

Liaw CC, FR Chang, CY Lin, CJ Chou, HF Chiou, MJ Wu, and YC Wu, "New cytotoxic monotetrahydrofuran annonaceous acetogenins from Annona muricata."Journal of Natural

Products. April 2002. Vol. 65, No. 4:470-5. Abstract retrieved from Pubmed.gov (PMID: 11975482).

Sanjeeb, K., Guarav, K., Loganathan, K., and Kohati, V.B.R. "A Review on Medicinal Properties of *Lantana camara* Linn." Research Journal of Pharmacy and Technology. June 2012, Vol. 5, No. 6:711-715.

Sharma M, PD Sharma, and MP Bansal. "Lantadenes and their esters as potential anitutmor agents." Journal of Natural Products. July, 2008. Vol.71., No. 7:1222-7. Abstract retrieved from Pubmed.gov (PMID:18553923).

Tesch NR, F Mora, L Rojas, T Diaz, J Velasco, C Yanez, N Rios, J Carmona, S Pasquale. "Chemical composition and antibacterial activity of the essential oil of Lantana camara var. moritziana." Natural Products Communication. July, 2011.Vol. 6, No. 7:1031-4. Abstract retrieved from Pubmed.gov (PMID: 21834251).