POTENTIAL CANCER-FIGHTING *OCIMUM GRATISSIMUM* (*OG*) LEAF EXTRACTS: INCREASED ANTI-PROLIFERATION ACTIVITY OF PARTIALLY PURIFIED FRACTIONS AND THEIR SPECTRAL FINGERPRINTS

Stephen I. N. Ekunwe, PhD; Melvanique S. Thomas, MS; Xuan Luo, MS; Hengshan Wang, PhD; Yong Chen, MSc; Xiaopu Zhang, MSc; Gregorio B. Begonia, PhD

Introduction: Cancer causes about 13% of all deaths. According to the American Cancer Society, world-wide cancer deaths were 7.6 million in 2007. African Americans and other minorities are disproportionately affected. Effective cancer chemotherapy is scarce. This study is part of an ongoing search for potential cancer-fighting agents in medicinal herbs. In previous *in-vitro* studies, we have shown that the aqueous extracts of the medicinal herb *Ocimum gratissimum* (*Og*) inhibit the proliferation of several cancer cell lines, especially prostate adenocarcinoma (PC-3) cells. Therefore, *Og* leaf extracts may harbor novel cancer-fighting compounds that need to be isolated, purified and characterized.

Methods: Partially purified *Og* fractions were obtained from sequential extraction of *Og* powder with organic solvents of different polarities. The hypothesis that the anti-proliferation activity of the fractions will be significantly greater than that of either aqueous or ethanol extracts was tested by treating PC-3 cells with 1.61 mg/mL of each fraction. Spectral analysis of the fractions was also conducted.

Results: Activity of the fractions was $P_2 > P_{3\text{-}2} > P_{4\text{-}2} > P_{3\text{-}1} > P_{4\text{-}1}$. Fractions P_2, $P_{3\text{-}2}$ and $P_{4\text{-}2}$ were 725, 75 and 2.3 times more active than the aqueous extract, respectively. Spectral analysis revealed peaks for: $P_{3\text{-}2}$ and aqueous extract at 208 nm, P_2 and $P_{4\text{-}2}$ at 210 nm, and $P_{3\text{-}1}$ at 220 nm.

Conclusions: These findings suggest that fractions P_2, $P_{3\text{-}2}$ and $P_{4\text{-}2}$ could be potential sources of *Og's* bioactive component(s) that warrant further purification and characterization. (*Ethn Dis*. 2010;20[Suppl 1]:S1-12–S1-16)

Key Words: *Ocimum gratissimum* (*Og*), Prostate Adenocarcinoma (PC-3) Cells, Anti-proliferation Activity

From the Department of Biology, Jackson State University, Jackson, MS (SINE, MST, GBB); Department of Chemistry, Jackson State University, Jackson, MS (XL); Key Laboratory for the Chemistry and Molecular Engineering of Medicinal Resources, College of Chemistry & Chemical Engineering, Guangxi Normal University, Guilin, P.R. China (HW, YC, XZ).

Address correspondence and reprint requests to Stephen I. N. Ekunwe, PhD; Department of Biology; Jackson State University; 1400 JR Lynch Street, P.O. Box 18540; Jackson, MS 39217; 601-979-3476; 601-979-5853 (fax); stephen.i.ekunwe@jsums.edu

INTRODUCTION

Prostate cancer is the most common cancer and the second leading cause of cancer related deaths in men over the age of 50. One out of nine men over 50 is often diagnosed with prostate cancer in the United States.[1] It affects African American men and other minorities disproportionately. There is neither a cure nor an effective chemotherapy for prostate cancer, even at its early stage when the disease is still localized to the gland. The often-prescribed treatment is either radical prostatectomy or radioactive seed implantation (brachytherapy), whose major adverse side effects are erectile dysfunction and urinary incontinence.[2] Other treatment options include immunotherapy and intensity modulated radiotherapy (IMRT), which delivers radiation to the tumor while sparing the surrounding normal tissues. In IMRT, high intensity beams of radiation are directed at a specific location on the prostate gland rather than at the entire gland, therefore making it more precise.[3] The side effects of these treatments are very harsh. Therefore, better tolerated treatments are desirable. We believe that compounds found in edible medicinal herbs are good prospects for such treatments. Our research efforts are directed to finding novel plant-derived cancer-fighting compounds in edible medicinal herbs from the rain forests of Nigeria. *Ocimum gratissimum (Og)* also called *Eb'amwonkho*, is a member of the genus *Ocimum* L. (*Lamiaceae*). It is used for food and medicine.[4] In Nigeria, extracts of *Og* leaves or of whole plants are popular for the treatment of diarrhea, upset stomach, and hemorrhoids.[5] There is anecdotal evidence that *Og* leaf extract shrinks prolapsed hemorrhoids, stopping the bleeding and itching associated with the condition. Because *Og's* folklore use as anti-hemorrhoidal treatment suggests that *Og* possesses anti-inflammatory and anti-angiogenesis properties, coupled with results from our work with aqueous *Og* leaf extract on bladder, colon, liver and prostate cancer cells, we are optimistic that *Og* leaf extract may harbor cancer-fighting compounds. Hence in this study, we investigated the anti-proliferation activity of partially purified fractions of *Og* leaf extract on prostate cancer (PC-3) cells *in-vitro*, along with qualitative spectral analysis of the fractions, a necessary step for quantitative analysis.

METHODS

Plant Culture

Initial stem cuttings of *O. gratissimum* imported from Benin City, Nigeria (Phyto-sanitary Certificate No. IB 2004/7, Plant Quarantine Service, Federal Department of Agriculture, Nigeria) were propagated and multiplied at the Jackson State University (JSU) greenhouse until maturity (ie, brown coloration of the inflorescence). Stem cuttings (with at least 3 nodes) obtained

from these original plants were subsequently planted in 4L plastic pots containing potting soil. Plants were grown devoid of pesticides and maintained in the same JSU greenhouse at 35/28°C day/night temperatures under a 12-hour photoperiod until maturity. During the growth period, plants were watered as needed based on evaporative demand, and fertilized every two weeks with soluble fertilizer (20-20-20; N-P_2O_5-K_20). The plants were identified by Dr. G. Begonia, a botanist, Department of Biology, Jackson State University, Jackson, MS USA, based on their vegetative (eg, shape and color of leaves, stem) and reproductive (eg, inflorescence) characteristics. The matured leaves were harvested, air-dried in the laboratory, ground into powder and used to prepare crude aqueous and ethanol extracts.

EXTRACT PREPARATION

Aqueous extract of powdered *Og* leaves was prepared by suspending 50 g of powdered *Og* leaves in 500 mL distilled water overnight in orbital shaker at 37°C (98.6°F).[6,7] Extract was centrifuged and supernatant was lyophilized and stored at 4°C.

Crude ethanol extract was prepared as follows: 3 sets of 100 g of powdered *Og* leaves were extracted in 500 mL round bottom flasks fitted with reflux condensers using 450 mL aliquots of 95% ethanol (EtOH) in a 50°C water bath for 2 hours. Extracts from all three flasks were vacuum filtered, combined, diluted to 70% EtOH and incubated at −20°C for 2 hours to precipitate chlorophyll from the extract.[8] The extract was vacuum dried using a rotary evaporator. The extract was then completely re-dissolved in distilled water and acetone. The acetone was volatized using the rotary evaporator at 50°C with occasional rotation of the flask, yielding a dark brown crude ethanol extract.

Fractionation of Crude Ethanol Extract

Using a 1L separatory funnel, the crude ethanol extract was re-extracted with 250 mL chloroform ($CHCl_3$). The $CHCl_3$ fraction, P_1, was collected and dried in a rotary evaporator. P_1 (13.506 g) was stored at −20°C. This procedure was repeated sequentially with ethyl acetate (EtOAc), P_2 (6.5862 grams) and butanol, P_3 (4.9832 g). Fraction P_3, a viscous liquid, was stored at −20°C. The remaining water fraction, P_4 (26.4104 g) was stored at 4°C. P_3 and P_4 were fractionated further in a chromatography column packed with D101 resin (Shanghai Hualing Resin Co., Ltd, Shanghai, China). Both were eluted with water and 95% EtOH respectively.[9,10] P_3 yielded $P_{3\text{-}1}$ (1.559 g) and $P_{3\text{-}2}$ (2.217 g), while P_4 yielded $P_{4\text{-}1}$ (23.208 g) and $P_{4\text{-}2}$ (2.202 g).

Cell Culture

PC-3 cells and McCoy's 5A modified medium supplemented with 10% fetal bovine serum (FBS) and 1% Penicillin/Streptomycin (pen/strep) antibiotic used as the complete growth medium (CGM) were purchased from American Type Culture Collection (Rockville, MD). PC-3 cells were expanded in Primaria™ T-75 tissue culture flasks incubated in a 5% CO_2 humidified incubator at 37°C by standard tissue culture techniques. Confluent cells were trypsinized, counted with a hemocytometer and diluted accordingly for the different experiments.

Anti-proliferation Activity of *Og* Extracts

Anti-proliferation activity of aqueous, ethanol crude extracts and fractions P_2, $P_{3\text{-}1}$, $P_{3\text{-}2}$, $P_{4\text{-}1}$ and $P_{4\text{-}2}$ on PC-3 cells was assessed by [^{3}H]-thymidine incorporation assay.[11,12] Primaria™ 6-well tissue culture plates were seeded with 2 mL/well PC-3 cell suspension at cell density of 5.0×10^4 cells/mL, and incubated in a 5% CO_2 humidified incubator at 37°C. CGM was changed every 48 hours until cell growth was 60–65% confluent at which time cells were treated with aqueous and crude ethanol extracts at the following concentrations: 0.25, 0.50, 1.0, 2.0, 4.0, 8.0, 12.0, and 16.0 mg/mL for 18 hours in a 5% CO_2 humidified incubator at 37°C. Cells were then labeled with 2μCi/mL (20μL) per well of [^{3}H]-thymidine (MP Biomedicals, Inc., Irvine, CA) and incubated in a 5% CO_2 humidified incubator at 37°C for 4–6 hours. Medium was aspirated, cells were first washed 3 times with 2 mL per well cold 1X PBS and then fixed by incubating for 10 minutes at 4°C with 2 mL per well of 10% trichloro-acetic acid (TCA). Fixed cells were washed 3 times with 2 mL per well dH_2O and solubilized by incubating for 30 minutes in a 5% CO_2 humidified incubator at 37°C with 2 mL per well 0.5M sodium hydroxide (NaOH). One milliliter solubilized cells was mixed with 5 mL scintillation cocktail. Radioactivity was counted in a TRI-CARB 2700TR Liquid Scintillation Counter. Untreated cells grown in CGM and CGM+0.8% EtOH served as negative controls for aqueous and crude ethanol extracts respectively. Because of the limited amount of the partially purified fractions, the concentration of each fraction used in the experiment was 1.6 mg/mL. Fractions $P_{1\text{-}2}$ and P_2 were dissolved in a 0.8% EtOH solution, $P_{3\text{-}1}$, $P_{4\text{-}1}$, and $P_{4\text{-}2}$ in distilled water (dH_2O), and $P_{3\text{-}2}$ in a 1% dimethyl sulfoxide (DMSO) solution. Appropriate controls were set up for tests involving these fractions. The following chemicals PBS, EtOH, NaOH, TCA, DMSO were purchased from Sigma-Aldrich, St. Louis, MO.

UV-Spectrophotometry of *Og* Extracts and Partially Purified Fractions

In a Wheaton vial, 2 mg of each sample were dissolved in 2mL EtOH (anhydrous) incubated in a 60 Hz ultrasonic water bath at 60°C for 15 minutes. Each sample was filtered using a 0.45 micron filter. Two milliliters anhy-

drous EtOH in a quartz cuvette was used to blank the Cary 300 Bio UV-vis spectrophotometer. Samples were diluted with anhydrous EtOH as needed for absorbance of each sample to be measured at wavelength of 190–990 nm.

Statistical Analysis

Results represent the mean +/− SD values of experiment performed in triplicate. *P* value <.05 was considered significantly different from negative control according to Dunett test and is indicated by an asterisk [*] on the figures.

RESULTS

We found that aqueous *Og* leaf extract inhibits proliferation of treated prostate adenocarcinoma (PC-3) cells in a concentration dependent manner (Figure 1). At a concentration of 1.6 mg/mL, all partially purified fractions but one, $P_{1\text{-}2}$, inhibit proliferation of treated PC-3 cells (Figure 2). A comparison of anti-proliferation activity of aqueous *Og* leaf extract, crude ethanol *Og* leaf extract and the three most potent partially purified fractions (P_2, $P_{3\text{-}2}$, $P_{4\text{-}2}$) derived from the crude ethanol extract is shown in Figure 3. The results show that the crude ethanol extract is 1.6 times less active than the aqueous extract, while fractions P_2, $P_{3\text{-}2}$, $P_{4\text{-}2}$ are 725, 75, and 2.3 times more active than the aqueous extract, respectively. As an initial step in the isolation, purification and characterization of the bioactive components in *Og* leaf extracts, we carried out a qualitative spectral analysis of *Og* samples. In Figure 4, the qualitative spectral analysis results reveal peaks for: $P_{3\text{-}2}$ and aqueous extract at 208nm, P_2 and $P_{4\text{-}2}$ at 210nm, and $P_{3\text{-}1}$ at 220nm.

DISCUSSION

The therapeutic use of plant products is among the oldest of medical practices. It is a central feature of many

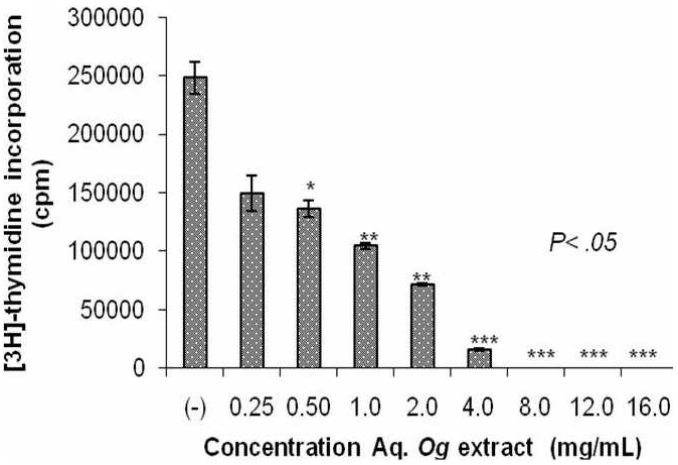

Fig 1. Effect of aqueous *Og* leaf extract on prostate adenocarcinoma (PC-3) cells

This figure represents the mean of triplicate counts ± SD. Compared to the untreated cells (negative control), aqueous *Og* leaf extract significantly inhibits proliferation of treated PC-3 cells, and it does so in a concentration dependent manner

*** difference is significant; ** difference is very significant; ***indicates difference is highly significant**

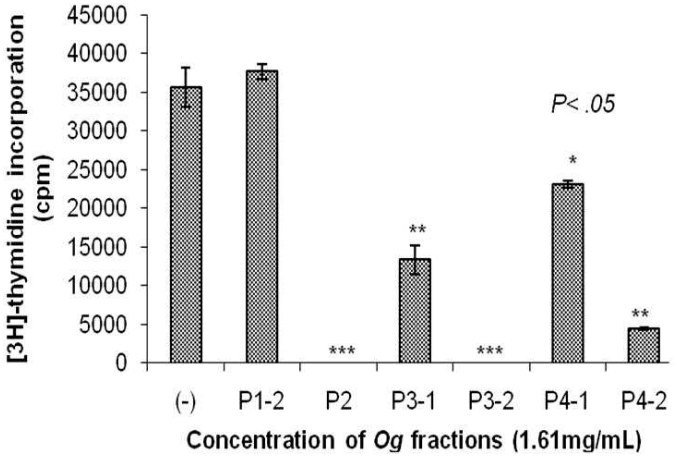

Fig 2. Effect of partially purified fractions of *Og* leaf extract on prostate adenocarcinoma (PC-3) cells

This figure represents the mean of triplicate counts ± SD. The results show that at a concentration of 1.6 mg/mL, all partially purified fractions but one, $P_{1\text{-}2}$, inhibit proliferation of treated PC-3 cells. This supports the hypothesis that partially purified fractions of the extract will show increased anti-proliferation activity as compared to the crude extracts. The negative control is untreated cells grown in CGM+0.8% EtOH

***difference is significant; ** difference is very significant; ***difference is highly significant**

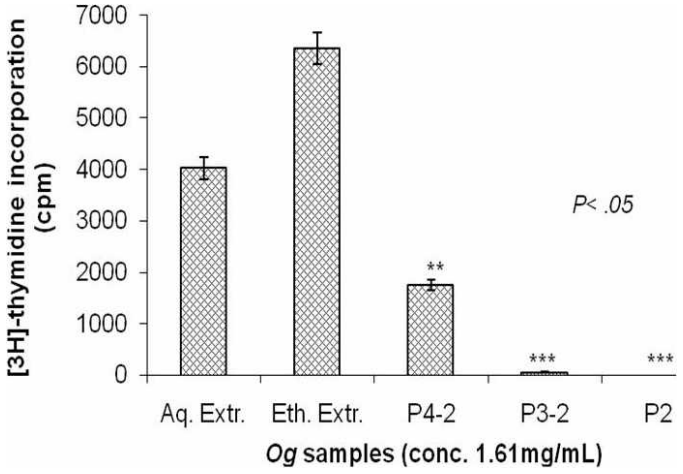

Fig 3. A comparison of anti-proliferation activity of aqueous, crude ethanol *Og* leaf extracts and three most active partially purified fractions P_2, P_{3-2} and P_{4-2}

This figure represents the mean of triplicate counts ± SD. The results show that the crude ethanol *Og* leaf extract is 1.6 times less active than the aqueous extract, while fractions P_2, P_{3-2}, P_{4-2} are 725, 75, and 2.3 times more active than the aqueous extract, respectively

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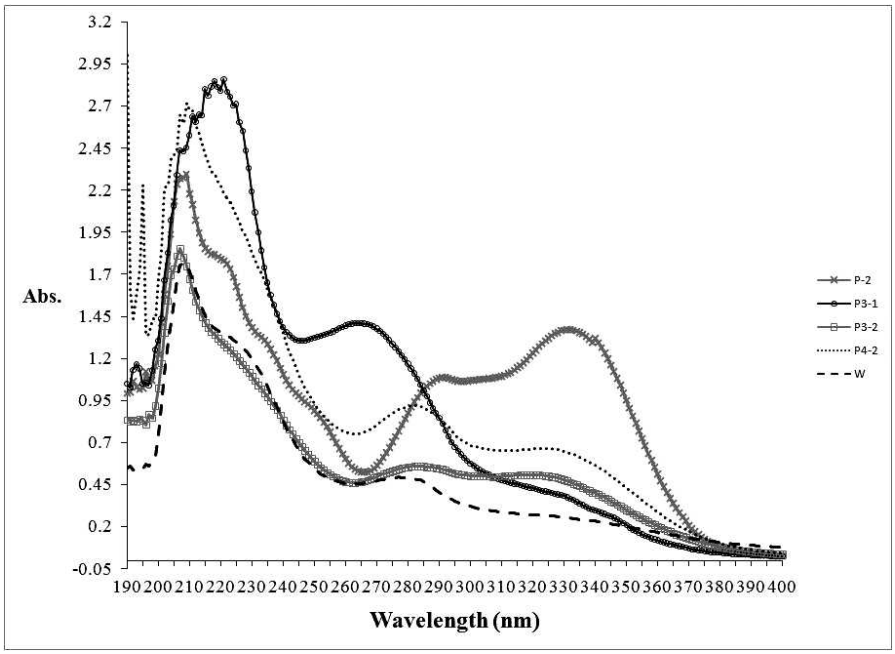

Fig 4. Ultra violet spectral absorption of *Og* leaf extract and its partially purified fractions

This figure is a qualitative spectral analysis of *Og* samples. It reveals peaks for P_{3-2} and aqueous extract at 208 nm, P_2 and P_{4-2} at 210 nm, and P_{3-1} at 220 nm wavelength. This result will be used to guide the quantitative analysis of the samples to determine the relative abundance of the component in the different fractions of *Og* leaf extract

current forms of folk and traditional medicine, for example, traditional Chinese medicine, Native American healing and Nigerian native medicine. Currently, there is increased use of herbal products due to the fact that people believe that these products have therapeutic benefits where conventional medicines have failed.[13] Since about one-fourth of all medications contain an active ingredient derived from a plant, there is an increased effort to isolate bioactive micro-chemicals from medicinal plants for their use in treatment of various ailments.[1] Many diet-derived micro-chemicals have been shown to display significant efficacy in tumor prevention for example: plant polyphenolic compounds, anti-oxidants, vitamins, and ω-3 fatty acids.[14–17] In this study, we investigated the anti-proliferation activity of *Og* leaf extract on prostate cancer (PC-3) cells *in-vitro*, because we believe that *Og* leaf extract may contain novel cancer-fighting compounds. Our results show that aqueous *Og* leaf extract inhibits proliferation of treated PC-3 cells in a concentration dependent manner. Our results also show that partially purified fractions of *Og* leaf extract have a higher anti-proliferation activity. Therefore, *Og* leaf extract could potentially be a source of novel cancer-fighting compounds that, if successfully isolated, purified and identified, could be used in formulating new treatments for prostate cancer. In a recent work on *Og* at Wayne State University, it was shown that aqueous *Og* leaf extract inhibited proliferation, migration, anchorage independent growth, 3D growth and morphogenesis and induction of COX-2 protein in breast cancer cells. *Og* also reduced the size of the tumors and neo-angiogenesis in a MCF10 DCIS.com xenograft model of human DCIS.[18] This shows the potential cancer-fighting abilities of *Og*. Such a report vindicates our continued effort to find the bioactive components in *Og* leaf extract. While our results are exciting and encouraging,

we are a long way from our ultimate goal of identifying the novel cancer-fighting compounds that may be present in *Og* leaf extract.

IMPLICATIONS FOR IMPROVING HEALTH DISPARITIES

Og is an edible medicinal herb. Therefore, if the efficacy of drugs formulated with its isolated bioactive components is successfully established, such medications are likely to have little or no toxicity. They may also be quite affordable.

ACKNOWLEDGMENTS

This research was supported in part by a grant from the NAFEO BEAT CANCER Research Consortium (Grant#: YS2MP9700308011) awarded to SINE. Melvanique S. Thomas, who used part of this work to satisfy the Masters Degree thesis requirements at Jackson State University, was supported with funds from the NIH MBRS-RISE (Grant#: R25 GM067122) awarded to Jackson State University. Part of this work was conducted in the Molecular and Cellular Biology Core Facility supported with funds from the National Institutes of Health Research Centers in Minority Institutions, NIH-RCMI (Grant #1G12RR13459) awarded to Jackson State University.

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