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ARTICLES

Full Length Research Paper

Impact of certain *Solanum* species's natural products as potent cytotoxic and anti-Inflammatory agents

Muhammad A. Alsherbiny[1*], Shahira M. Ezzat[1], Fatma S. Elsakhawy[1], Gehan M. Kamel[2] and Mostafa A. Abdel-Kawy[1]

[1] Department of Pharmacognosy, Faculty of Pharmacy, Cairo University, Kasr El-AiniStreet, Postal code 11562, Cairo, Egypt.
[2] Department of Pharmacology, Faculty of Veterinary Medicine, Cairo University, Giza square, postal code 12211, Egypt.

The present study was conducted to evaluate both the cytotoxic and anti-inflammatory activities of ethanol extracts (T), and both *n*-butanol (B) and total glyco-alkaloid fractions (TGA) of *Solanum seaforthianum* Andr. (SS) and *Solanum macrocarpon* L. (SM) growing in Egypt. Cytotoxic activity was measured using sulforhodamine B (SRB) assay on prostate cancer cell line (PC-3), breast cancer cell line (MCF7), liver cancer cell line (HepG2) and human fibroblast cell line (HFB4) while anti-inflammatory activity was measured using formalin induced paw edema method. The highest cytotoxic potentiality was indicated for those of TGA fraction of *S. seaforthianum* Andr. on PC-3 cell line (IC_{50} = 0.28µg/ml ± 0.01) followed by its activity on MCF-7 cell line (IC_{50} = 2.84 µg/ml±0.20). On the other hand, the potency of TGA fractions of both species showed higher potency followed by *n*-butanol fractions where ethanol extracts showed lowest potency which is emphasizing the cytotoxic potentiality of the glyco-alkaloids. Based on the IC50s indicated for the different extracts and fractions on normal fibroblast cell line, considerable safety was indicated against prostate carcinoma rather than breast or hepatic carcinoma. TGA fraction of *S. macrocarpon* L. and of *S. seaforthianum* Andr. showed the highest anti-inflammatory activity with efficacy of 159 and 156%, respectively as compared to standard indomethacin. That's why the TGA fraction of *S. seaforthianum* Andr. was subjected for isolation of individual alkaloids using different chromatographic techniques and identified using ^{1}H and $^{13}CNMR$ spectroscopy beside Co-chromatography with authentic samples as solamargine (A_1), solasonine (A_2) and solasodine (A_3) which are firstly isolated from *S. seaforthianum* Andr. growing in Egypt.

Key words: *Solanum seaforthianum*, *Solanum macrocarpon*, glyco-alkaloid, anti-inflammatory, cytotoxicity, SRB.

INTRODUCTION

The economically transitioning countries showed increased incidence and mortality rates for most cancers unlike United States and many other western countries (Jemal et al., 2010). Liver cancer incidence for example

in Egyptians was more than 3 times that in US SEER and about 5 to 7 times that in the other Middle East countries consortium populations (Freedman et al., 2006). On the other hand, breast cancer is the most common lethal malignancy especially in the urban areas of the developing countries than rural ones (Dey et al., 2010). In more developed countries, prostate cancer was the most common type of cancer diagnosed among men and was second most common cancer diagnosed among men worldwide (Ferlay et al., 2004; Baade et al., 2009).

Many *Solanum* species are used by humans, and were important sources of food, spice and medicine (Zaidi et al., 1992). The Cytotoxic activities of 20 steroidal glycosides from different *Solanum* species were examined on various cell lines illustrated major structure activity relationship (Nakamura et al., 1996). It was suggested that the cytotoxic activity depends on the kind of oligosaccharide and aglycone moieties of the tested steroidal compounds (Ikeda et al., 2003).

Glycoalkaloids has exhibited apoptotic activity and chemo-preventative effects against known carcinogens and anti-inflammatory activity (Milner et al., 2011). It was suggested that steroidal alkaloids content of *Solanum lycocarpum* St. Hil. fruits account for the anti-inflammatory effect of the crude ethanol extract (Vieira et al., 2003). Also, the aqueous extract of *Sesamum alatum* Moench exhibited anti-inflammatory activity (Lin et al., 1995).

The aim of this study was to evaluate the cytotoxic and anti-inflammatory activities of the different extracts and fractions of *Solanum seaforthianum* Andr. and *Solanum macrocarpon* L isolation of the secondary metabolites from the biologically active fractions.

MATERIALS AND METHODS

Plant materials

Aerial parts of *S. seaforthianum* Andr. and *S. macrocarpon* L. used in this study were collected March, 2012 from the Experimental Station for Aromatic, Medicinal and Toxic plants, Faculty of Pharmacy Cairo University, Giza, Egypt. The plants were kindly authenticated by Dr. Mohamed El-Gebaly, botany specialist, National research center (Dokki, Giza, Egypt). Voucher specimens (23082014 I and II respectively) were kept at the Herbarium of the Department of Pharmacognosy, Faculty of Pharmacy, Cairo University.

Extracts and fractions preparation

Air-dried powdered samples (1000 g, each) of the aerial parts of *S. seaforthianum* Andr. and *S. macrocarpon* L. were, separately, macerated in ethanol (70%) till exhaustion. The extracts were

evaporated to dryness under vacuum. The individual ethanol extracts (104 and 123 g yield for *S. seaforthianum* Andr. and *S. macrocarpon* L. respectively) were successively fractionated, using *n*-hexane (37.7 and 35 g yield), chloroform (2 and 3 g yield), ethyl acetate (2 and 4 g yield) and *n*-butanol saturated with water (35 and 43 g yield). While the total glyco-alkaloid fraction preparation adapted from Bushway et al. (1985) where air-dried powdered samples (1000 g, each) of the aerial parts of both species which were macerated with methanol (3x), and the extract filtered; the solvent was eliminated at a reduced pressure. The resulting dry extracts were dissolved in 500 ml of 5% acetic acid and washed several times with *n*-hexane. Then it was extracted with $CHCl_3$ (v/v). Then filter and adjust supernatant to 10.5 to 11.0 pH with NH_4OH, kept in 70°C water bath for 10 min, cooled and centrifuged. The residue is air dried in desiccator containing anhydrous calcium chloride. Then acid-base purification repeated where 34 and 39 g respectively. The ethanol extracts and both *n*-butanol and TGA fractions of both species evaluated for anti-inflammatory and cytotoxic potentiality.

Experimental models

Carcinoma cell lines

Hepatocellular (HepG2), breast (MCF-7) and prostate (PC-3) carcinoma human cell lines were kindly provided from the Pharmacology and Toxicology Department of Faculty of Pharmacy, Ain-shams University (Cairo, Egypt). Where fibroblast cell lines (HFB4) were provided from Pharmacology Department, National cancer institute (Cairo, Egypt).

Animals

Swiss albino mice (25 to 30 g), used for determination of LD_{50}, and adult male albino rats of Sprague Dawley strain (120 to150 g), utilized for assessment of the anti-inflammatory activity, were obtained from the animal house colony at the National Research Center (Dokki, Giza, Egypt).

Acute toxicity studies (Determination of median lethal doses, LD_{50} of the tested extracts)

The LD_{50} of each tested ethanol extracts and the fractions (*n*-butanol and TGA) were determined following both intraperitonial and oral administration according to Kärber (1931), and the animals were observed for any toxic symptoms for 24 h after administration. Preliminary experiments were carried out to determine the minimal dose that kills all animals LD_{100} and the maximal dose that fails to kill any animal. Several doses at equal logarithmic intervals were selected in between these two doses; each dose was injected in a group of six animals by subcutaneous injection. The mice were observed for 24 h and symptoms of toxicity and mortality rates in each group were recorded.

Cytotoxicity assessment using SRB assay

Cytotoxicity was determined using Sulforhodamine B Assay (SRB)

as described by Skehan et al. (1990). Exponentially growing cells were collected using 0.25% Trypsin-EDTA and seeded in 96-well plates at 1000 to 2000 cells/well in RPMI-1640 supplemented medium. After 24 h, cells were incubated for 72 h with 0, 0.1, 1, 10, 100 and 1000 µg/ml concentrations of the tested compounds in dimethyl sulfoxide (DMSO). Following 72 h treatment, the cells were fixed with 10% trichloroacetic acid for 1 h at 4°C. Wells were stained for 10 min at room temperature with 0.4% SRB dissolved in 1% acetic acid. The plates were air dried for 24 h, and the dye was solubilized with Tris-HCl for 5 min on a shaker at 1600 rpm. The optical density of each well was measured spectrophotometrically at 564 nm with an ELISA microplate reader (ChroMate-4300, FL, USA). The IC_{50} values were calculated according to the equation for Boltzman sigmoidal concentration–response curve using the nonlinear regression fitting models after 3 repetitions. (Graph Pad, Prism Version 5).

Cell culture

PC3 human prostate cancer cell line, MCF-7 human breast cancer cell line, HepG2 human hepatocellular carcinoma cell line and HFB4 human fibroblast cell line were grown in RPMI-1640 medium, supplemented with 10% heat inactivated FBS, 50 units/ml of penicillin and 50 mg/ml of streptomycin and maintained at 37° in a humidified atmosphere containing 5% CO_2. The cells were maintained as "monolayer culture" by serial sub culturing.

Assessment of anti-inflammatory activity by formalin induced paw edema method

Wister albino rats of either sex weighing 150 to 200 g were divided into 8 groups of 5 animals each. They were treated via oral route as follow: the 1st group was given 1% tween 80 and kept as control. The 2nd group administered indomethacin (10 mg/kg body weight) as standard drug according to Young et al. (2005), the tested ethanol extracts and both n-butanol and TGA fractions in the form of 1% tween 80 suspensions were given at a dose of 100 mg/kg body weight to last six groups. After 1 h, 0.1 ml of 2% formaldehyde was injected into the footpad of the left hind paw of each rat for induction of paw edema according to Dharmasiri (2003). The initial paw thickness was measured for each animal using Vernier caliber before induction of edema. The increase in this thickness was determined after 30 min, 1, 2 and 3 h after formaldehyde injection. The anti-inflammatory activity was expressed as inhibition percent in paw thickness in treated groups comparing with the control one using the formula proposed by Adedapo (2008). Where the inhibition percentage of different extracts and fractions divided by the indomethacin inhibition percentage to attain the efficacy were compared to indomethacin. The study protocol was reviewed and approved by the institutional review board PC 8385(REC-FOPCU; Research Ethics Committee- Faculty of pharmacy, Cairo University) in Egypt.

Isolation of the constituents

A weighed amount (4g) of the total glyco-alkaloid fraction of the aerial part of S. seaforthianum Andr. was subjected to fractionation by vacuum liquid chromatography (VLC) on a 50 g Lichoprep Silica gel RP-18 column (25 × 7 cm). Elution was performed starting with methanol 30% in water and the polarity gradually decreased by 5% stepwise addition of methanol. Fractions were collected and monitored by TLC (precoated silica gel plates, Chloroform: Methanol: Ammonia (60:40:1), p-anisaldehyde/H_2SO_4). Fractions

with similar chromatographic pattern were pooled, evaporated under reduced pressure, weighed and saved in a desiccator. Collective fractions I (50% methanol in water) and II (95-100% methanol in water) were subjected to rechromatography as follows:

Fraction I: (1.7 g) showing two major spots (R_f value 0.6 and 0.5 in Chloroform: Methanol: Ammonia (60:40:1)), bluish green and turquoise, respectively with p-anisaldehyde) was subjected to rechromatography on a Licoprep RP-18 silica column (20 cm×1 cm). Elution was started using 40% methanol in water and the polarity of the eluent further decreased with methanol. Fractions, (10 ml, each) were collected and monitored by TLC (Chloroform: Methanol: Ammonia (60:40:1)) and RP-18 TLC (Methanol: Water: Ammonia (90:10:1)). Fractions (6-25) eluted with up to 60% methanol in water yielded concentration under vacuum 33 mg of white microcrystalline powder (compound A_1). And fractions (28-55) eluted with up to 75% methanol in water yielded concentration under vacuum 18 mg of white microcrystalline powder (compound A_2).

Fraction II: (0.6 g) apparently comprised one major constituent (R_f value 0.89, Chloroform: Methanol:Ammonia (60:40:1), Blue with p-anisaldehyde/H_2SO_4), which was purified by rechromatography on a silica gel column using $CHCl_3$: MeOH 95:5 v/vas eluent afforded 35 mg of white microcrystalline powder (compound A3). Identification of the isolated compounds: NMR spectra were recorded using Joel SA NMR-spectrophotometer (Japan) ^{1}H-NMR, 500 MHz, ^{13}C, 125 MHz spectra were recorded in suitable deuterated solvents ($CDCl_3$ or DMSO) using TMS as internal standard and chemical shift values expressed in δ ppm. (National Research Center, Dokki, Giza). Spectra of the isolated compounds were compared with those published by Shabana et al. (2013) and confirmed by Co-TLC with authentic alkaloids kindly provided by prof. Dr. Marawan M. Shabana Pharmacognosy Department, Faculty of Pharmacy, Cairo University.

Statistical analysis

Analysis of variance (ANOVA) followed by Turkey's test was performed using Statistical Package for Social Science (SPSS) version 20 to measure statistical significance. Measurements were carried out in triplicate. Data were presented as mean ±S.E.M. (n=5) for anti-inflammatory activity where significant levels from zero time at $P<0.05$ were tested and accepted as compared to the control group. Where cytotoxic potentiality data were presented as mean ± standard deviation (SD), the values were considered to be significantly different when P Values is less than 0.01.

RESULTS

The results of acute toxicity studies showed no deaths in animal groups which received ethanol extract and n-butanol fraction up to 1000 mg/Kg of body weight. LD_1 of S. seaforthianum Andr. was 1500 mg/Kg b. wt., while at the same dose S. macrocarpon L. did not cause any deaths. Also, TGA fraction of both species causes no deaths up to the dose of 500 mg/Kg b. wt.

The results of the cytotoxic activity of doxorubicin, different extracts and fractions on HepG2, MCF7 and PC3 cell lines were shown in Figure 1. Anti-inflammatory activity of ethanol extracts and both n-butanol and TGA

Table 1. Anti-inflammatory efficacy of ethanol extracts, both *n*-butanol and TGA fractions of *S. seaforthianum* Andr. and *S. macrocarpon* L.as compared to the standard indomethacin.

Treatment	Efficacy			
	30 min	1 h	2 h	3 h
SMT	1.30	1.25	1.01	0.76
SMB	1.25	1.21	1	0.69
SM TGA	1.59	1.58	1.33	1.04
SST	0.78	0.76	0.64	0.50
SSB	0.99	1.11	0.96	0.69
SS TGA	1.56	1.52	1.30	1.04

SMT: Total alcohol extract of *S. macrocarpon* L.; SMB: *n*-butanol fraction of *S. macrocarpon* L.; SM TGA: Total glyco-alkaloid fraction of *S. macrocarpon* L.; SST: Total alcohol extract of *S. seaforthianum* Andr.;SSB: *n*-butanol fraction of *S. seaforthianum* Andr.; SS TGA: Total glyco-alkaloid fraction of *S. seaforthianum* Andr.

Table 2. ^1H NMR chemical shifts (δ ppm) for compound A_3(CDCl$_3$, 500 MHz, *J* in HZ) and A_1-A_2(DMSO, 500 MHz, *J* in Hz).

Proton No.	A_1	A_2	A_3
1	1.70, 1.00 m	1.73, 1.0 m	.72, 0.98
2	2.07,1.85	2.12, 1.87	2.10, 1.85
3	3.92 m	3.99 m	3.71 m
4	2.77 m	2.71 m	2.74 m
6	5.28br.s	5.3 br.s	5.56 r.s
7	1.50, 1.50	1.90, 1.51	1.35, 1.35
8	1.51	1.54	1.50
9	0.90	0.90	0.89
11	1.45, 1.45	1.45, 1.45	1.45, 1.45
12	1.73, 1.12	1.70, 1.10	1.73, 1.13
14	1.07, 2.07	1.10, 2.10	1.08, 2.09
16	4.38	4.51 m	4.50
17	1.85	1.83	1.87
18	0.72	0.75	0.81
19	0.91	0.97	1.18
20	1.98 m,1.09 d, (*J*=7)	2.01 m, 1.17 d, (*J*=7)	1.91m, 1.09 d, (*J*=7)
21	0.82, (*J*=7.6)	0.85, (*J*=6.8)	0.92 d
22	-	-	-
23	1.73, 1.73	1.70, 1.70	1.73, 1.73
24	1.63, 1.63	1.65, 1.65	1.65, 1.65
25	1.50, 2.77	1.48, 2.83	1.48, 2.77
26	2.77	2.83	2.82
27	0.74 d (*J*=6.7)	0.76 d (*J*=8.4)	0.68 d (*J*=6)
29	-	-	-
1`	4.91 d (*J*=12)	4.92 d (*J*=8)	-
1``	5.04br.s	6.39br.s	-
1```	4.98br.s	5.04br.s	-

fractions of both species shown in Figure 2 represent the decrease in paw thickness. While efficacy compared to indomethacin standard is shown in Table 1.

Three compounds were isolated from the TGA fraction of *S. seaforthianum* Andr. The ^{1}H NMR and ^{13}C NMR

chemical shifts of the isolated compounds and their assignments are shown in Tables 2 and 3. The compounds were identified as solamargine (A_1), solasonine (A_2) and solasodine (A_3). The structures of the isolated compounds are shown in Figure 3.

Table 3. ^{3}C NMR chemical shifts (δ in ppm) for compound A_3(CDCl$_3$, 125 MHz) and A_1-A_2 (DMSO, 125 MHz).

Carbon No.	A_1	A_2	A_3
1	37.5	37.0	37.4
2	30.2	29.6	32.0
3	78.2	79.53	73.5
4	39.0	38.3	42.5
5	140.8	140.0	140.0
6	121.8	121.0	120.9
7	32.4	31.8	32.0
8	31.7	31.2	31.5
9	50.4	49.8	50.4
10	37.2	36.6	36.7
11	21.2	20.6	20.9
12	40.1	39.6	39.9
13	40.6	40.1	40.4
14	56.7	56.1	56.5
15	32.6	32.0	31.6
16	76.6	78.5	78.7
17	63.6	62.9	63.2
18	16.5	16.0	15.9
19	19.4	18.9	18.9
20	41.6	41.2	41.4
2	15.7	15.2	14.7
22	97.9	97.48	97.2
23	34.7	34.0	34.2
24	31.0	30.4	30.4
25	31.6	30.8	31.0
26	47.6	47.3	47.5
27	19.8	19.2	18.9
1`	98.6	101.0	-b
2`	77.8	75.6	-
3`	78.0	84.6	-
4`	78.7	69.3	-
5`	76.9	74.3	-
6`	61.3	61.9	-
1``	100.8	102.6	-
2``	72.5	71.7	-
3``	72.7	72.0	-
4``	73.9	73.4	-
5``	69.5	68.9	-
6``	18.6	18.0	-
1```	101	103.4	-
2```	72.8	74.2	-
3```	72.5	77.6	-
4```	74.1	70.9	-
5```	70.4	77.3	-
6```	18.5	61.5	-

DISSCUSSION

The results of acute toxicity studies as deduced through determination of LD_1 of the tested ethanol extract and both *n*-butanol and TGA fractions showed that all could be considered safe, and within the range of the orally administered doses.

The *in-vitro* testing of the cytotoxic potential shown in (Figure 1) the ethanol extracts, TGA and *n*-butanol fractions of the aerial parts of both species on three different human carcinoma cell lines revealed the highest potency for those of TGA fraction of *S. seaforthianum* Andr. on prostate carcinoma cell line PC-3(IC_{50} = 0.28µg/ml ± 0.01) followed by its activity on breast carcinoma cell line MCF-7 (IC_{50} = 2.84 µg/ml±0.20). On the other hand, the potency of TGA fractions of both species shows higher potency followed by *n*-butanol fractions where ethanol extracts show lowest potency which is emphasizing the cytotoxic potential of the glyco-alkaloids. From the IC50s listed for the different extracts and fractions on normal fibroblast cell line, the safety was indicated for prostate carcinoma rather than hepatic or breast carcinoma.

The IC50s on normal cell line (HFB4) indicated that all extracts and fractions except TGA fractions of both species had possible selectivity against cancer cells rather than normal cells compared to doxorubicin (IC_{50} = 4.0±0.21 µg/ml on HFB4).The high toxicity of TGA fractions on HFB4 could justify the anti-proliferative activity of glycoalkaloids.

All the tested extracts and fractions exhibited anti-inflammatory activity as indicated by the percentage of edema inhibition, where the isolated TGA fraction of *S. macrocarpon* L. and of *S. seaforthianum* Andr. showed the highest anti-inflammatory activity with potency 159 and 156%, respectively as compared to standard indomethacin drug following oral dosing. The potency of TGA SS fraction is followed by SSB fraction and SST extract. While the high potency of TGA SM is followed by SMT extract and SMB fraction, respectively. These results are in agreement with the result obtained for *Solanum torvum* Swartz. by Ndebia et al. (2006).

CONCLUSION

TGA fractions of both *S. seaforthianum* Andr. and *S. macrocarpon* L. growing in Egypt possessed significant anti-inflammatory and cytotoxic activity on PC-3, MCF-7 and HepG2, respectively, and highest safety was indicated for prostate carcinoma when comparing the IC_{50} with that for the normal fibroblast cell lines, and safety considerations should guide the use of these compounds as preventative or therapeutic treatments against carcinomas. As far as the available literature is concerned, this is the first report on isolation of
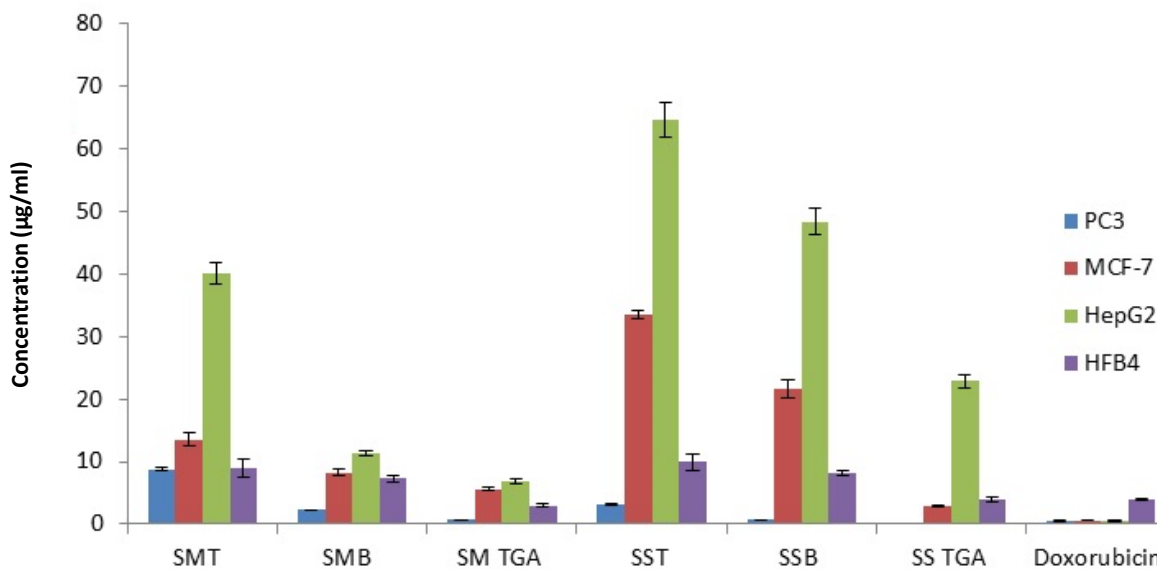
Figure 1. Histogram representing the IC$_{50s}$ results in SRB assay of ethanol extracts and the both *n*-butanol and TGA fractions of *S. Seaforthianum* Andr. and *S. macrocarpon* L. on PC3, MCF-7 HepG2 and HFB-4cell lines. SMT: Total alcohol extract of *S. macrocarpon* L. SMB: *n*-butanol fraction of *S. macrocarpon* L. SMTGA: Total glyco-alkaloid fraction of *S. macrocarpon* L. SST: Total alcohol extract of *S. seaforthianum* Andr. SSB: *n*-butanol fraction of *S. seaforthianum* Andr. SS TGA: Total glyco-alkaloid fraction of *S. seaforthianum* Andr.
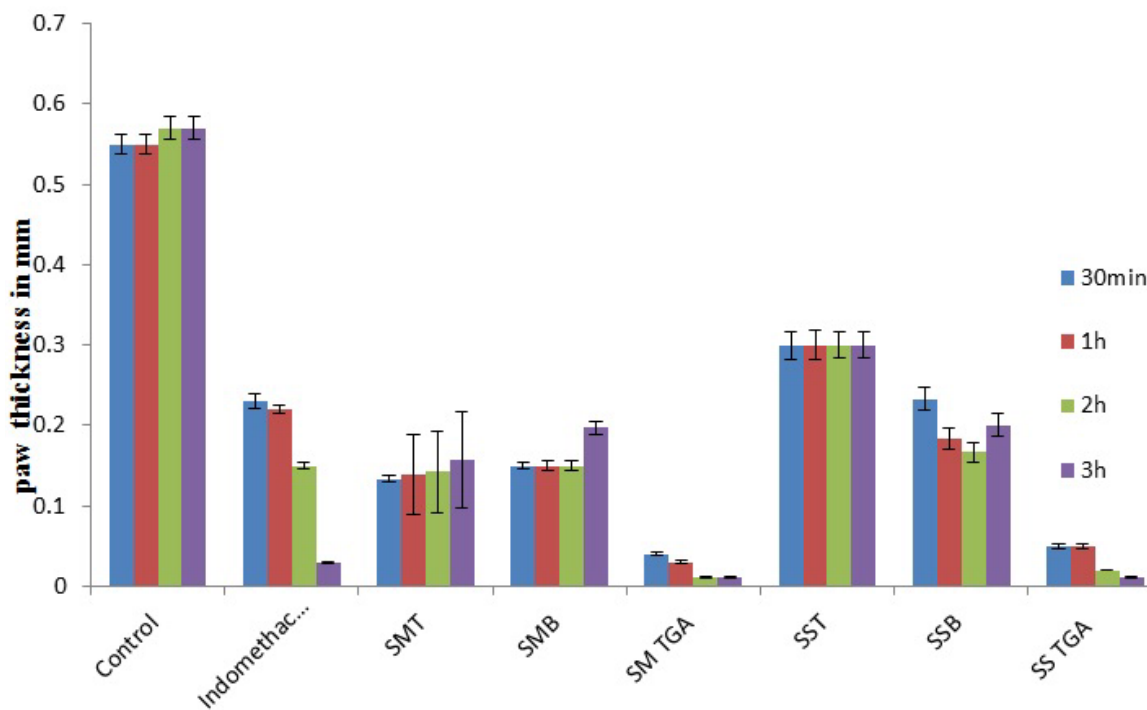
Figure 2. Histogram representing the anti-inflammatory activity *S. Seaforthianum* Andr. and *S. macrocarpon* L. following oral administration in rats. (Mean± S.E., n=5). SMT: Total alcohol extract of *S. macrocarpon* L. SMB: *n*-butanol fraction of *S. macrocarpon* L. SM TGA: Total glyco-alkaloid fraction of *S. macrocarpon* L. SST: Total alcohol extract of *S. seaforthianum* Andr. SSB: *n*-butanol fraction of *S. seaforthianum* Andr. SS TGA: Total glyco-alkaloid fraction of *S. seaforthianum* Andr.

Figure 3. Structures of the isolated compound A_1-A_3

solamargine, solasonine and solasodine from *S. seaforthianum* Andr. growing in Egypt.

Conflicts of interest

The authors have not declare any conflict of interest.

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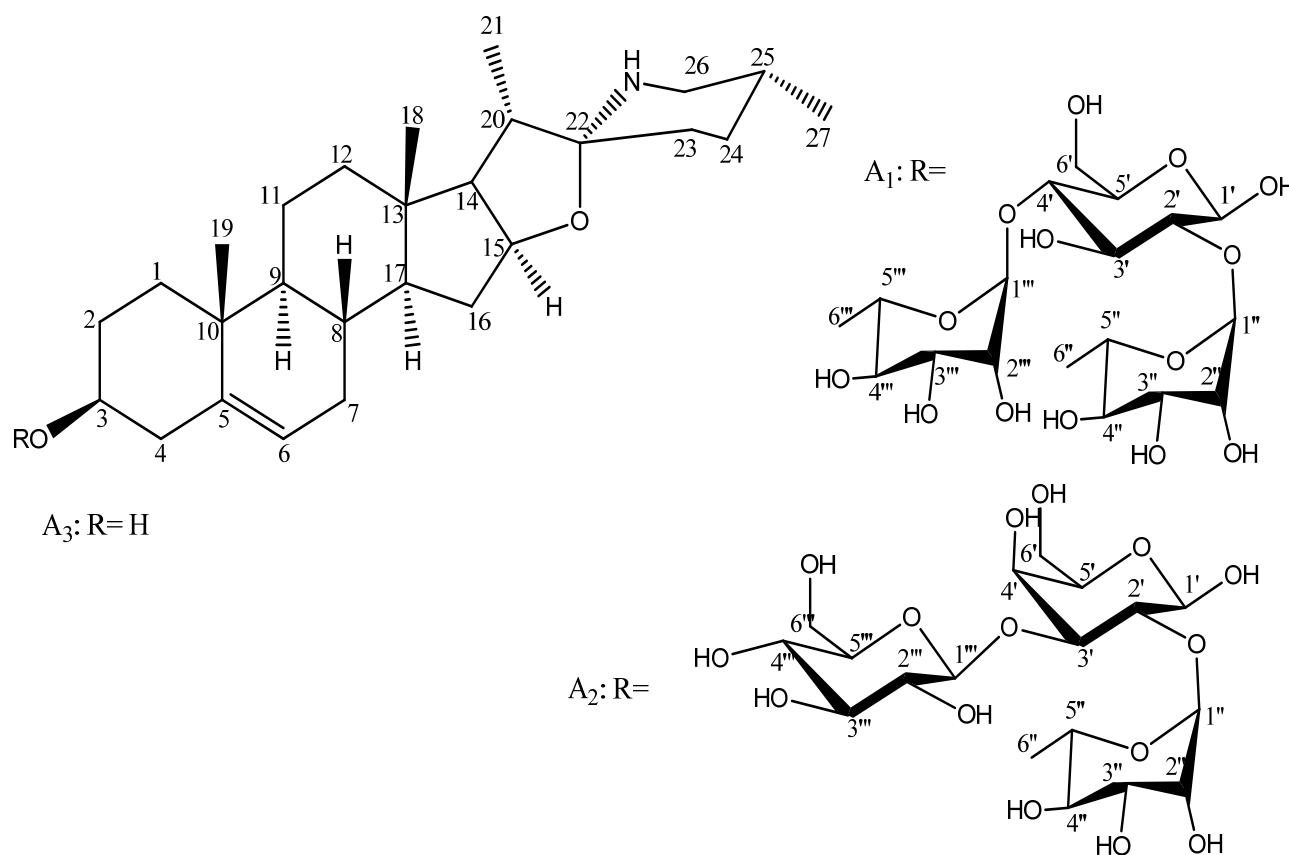Baade PD, Youlden DR, Krnjacki LJ (2009). International epidemiology of prostate cancer: geographical distribution and secular trends. Mol. Nutr. Food Res. 53(2):171-184.

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Full Length Research Paper

Effects of organic fertilizer in the capsaicinoids of red pepper (*Capsicum baccatum* L.)

Andréia Assamy Guinoza[1], Jackeline Guinoza Siraichi[2], Zilda Cristiani Gazim[3], Diógenes Aparício Garcia Cortez[1] and Lucia Elaine Ranieri Cortez[1]*

[1]Programa de Pós-graduação em Promoção da Saúde, Centro Universitário de Maringá, UniCESUMAR, Av. Guerdner nº 1610, Jd. Aclimação, Maringá, PR, Brasil.
[2]Programa de Pós-graduação em Ciências Farmacêuticas, Universidade Estadual de Maringá, Avenida Colombo, 5790, 87020-900 Maringá, Paraná, Brasil.
[3]Programa de Pós-graduação em Biotecnologia Aplicada à Agricultura, Unipar, Praça Mascarenhas de Morães, 4282, 87502-210 Maringá, Paraná, Brasil.

This study aimed to quantify the contents of capsaicinoids (capsaicin, dihydrocapsaicin and nordihydrocapsaicin) in the *Capsicum baccatum* L. species grown in soil with different concentrations of organic fertilizer (0, 1, 2 and 4%). The quantification of capsaicinoids was made by reverse-phase chromatography using the high-performance liquid technique (HPLC) with ultraviolet (UV) detection. The peppers were harvested from November 2009 to March 2010. The capsaicin content of the fruits of *C. baccatum* L. was higher for plants grown with organic fertilizer. The highest dihydrocapsaicin contents (10.49 ± 0.38 mg/100 g dry fruits) were obtained from the first harvest of pepper cultivated in soil at 2% organic fertilizer. The content of nordihydrocapsaicin did not change ($p \geq 0.05$) with different concentrations of organic fertilizer. The contents of capsaicinoids in pepper fruits varied with the different organic fertilizer concentrations used to grow the crop. Plants grown at 2% organic fertilizer presented the highest capsaicin content, with values of 44.70 ± 3.6 and 50.42 ± 4.80 mg/100 g dry fruits, in the first and second harvest, respectively.

Key words: Red pepper, capsaicinoids, capsaicin, organic fertilizer, high performance liquid chromatography (HPLC).

INTRODUCTION

In Brazil, *Capsicum* pepper crop is of major prominence in the economic, social and nutritional areas (Moreira et al., 2006; Sudré et al., 2010). Popularly known as red pepper, *Capsicum baccatum* L. is economically the most demanded pepper species of all cultivated in Brazil. The crop is present especially in the States of Minas Gerais,

São Paulo, Goiás, Ceará and Rio Grande do Sul (Reischeneider and Ribeiro, 2004). Pungency is the key feature of pepper fruits. It is scaled according to the contents of capsaicinoids that are alkaloids accumulated only in the fruits of the *Capsicum* genus (Ishikawa et al., 1998; Carvalho and Bianchetti, 2004).

The production of capsaicinoids in red pepper depends on the Pun 1 gene (formerly known as C), which is dominant for pungency. However, the amount, quality and diversity of capsaicinoids in fruits depend on the soil, climate and growing conditions (Dias et al., 2008; Kappel et al., 2008; Domenico et al., 2012). The water regime, position of the fruits on the plant as well as the farming practices (that is, fertilization) are some important factors affecting the production and accumulation of capsaicins in peeper fruits (Carvalho et al., 2009).

Therefore, knowing the growing conditions of *Capsicum* plants that suit the production of alkaloids is very important since these alkaloids are valued both in the food and in the pharmaceutical industries (Domenico et al., 2012). So, this study aim to quantify the capsinoids content (capsaicin, dihydrocapsaicin and noridihydrocapsaicin) by the HPLC method in the fruits of red pepper (*C. baccatum* L.) grown at different concentrations of organic fertilizer. Scientifically, it implies on the identification of effects of the organic fertilizer on the concentration and diversity of capsaicinoids produced by pepper plants. In practical terms, the increased production of active ingredients may add value to this pepper species for being used as seasoning or medicinal plant and significantly contributing to the field of health and agribusiness.

MATERIALS AND METHODS

The cultivation of *C. baccatum* L. var. *baccatum*

The experiment was conducted in the Medicinal Plant Garden of the University of Maringá (UEM, Maringa-PR, Brazil) from July 2009 to March, 2010. Maringá is located at 23° 25' 31" S and 51° 56' 19" W, 500 to 600 m height. The soil type is Oxisol (Embrapa, 2006).

The experiment was designed in a 2×4 factorial completely randomized to two harvest periods (November 2009 and March 2010) and four levels of organic fertilizer (0, 1, 2 and 4%), with three repetitions. Irrigation was carried out by a drip system with 0.20 m drip spacing (Melo and Nagai, 1998). The seeds of *C. baccatum* were placed to germinate into 128-cell polystyrene trays, 3 seeds per cell, in July 2009. Seedlings were grown in the nursery. Pepper beds were prepared five days before planting with increasing concentrations of plant organic fertilizer (0, 1, 2 and 4% of the soil volume). The organic fertilizer in all mentioned concentrations was incorporated at 0 to 20 cm depth. The transplanting of seedlings to the beds (4.5 m^2, 0.20 m height and 0.50×0.50 m each) was made in August 2009 (Embrapa, 2004). Soil samples were collected at 20 cm deep in the Garden of Medicinal Plants of the UNI-Cesumar Maringa-PR. The samples were homogenized and sent for analysis to the Soil Fertile Laboratory (CRQ-03 751 and CRF-PR 015963/0) in Maringá-PR (Table 1). The fruits of *C. baccatum* L. were harvested in the early maturation stage. The first harvest was made in November 30, 2009 (three months after seedling transplanting) and the second in

February 28, 2010; both in the morning (between 8:00 and 9:00 am).

Plant

The voucher specimens of *C. baccatum* L. var. *baccatum* have been deposited in the BHCB Herbarium at the Department of Botany, Federal University of Minas Gerais (UFMG, BHCB 139438).

Plant extracts

Fruits with seeds were washed under running water, chopped into small pieces, dried at 45°C and then milled. The fruit powder (10 g) was added to ethyl alcohol (96%) at 1:10 and subjected to hydrodynamic maceration (Brazilian Pharmacopeia, 2010). The organic solvent was removed using a rotary evaporator at 45°C and then lyophilized to yield 635 mg of the crude extract, which was stocked in a freezer at -15°C.

HPLC method

The capsaicinoids were analyzed using 20 mg of the lyophilized sample of *C. baccatum* L., which was subjected to extraction with 1 ml acetonitrile in an ultrasound bath (Unique, Ultracleaner 1400 A) for 10 min, twice consecutively. The extracts were combined and centrifuged at 13,000 rpm for 5 min at room temperature and then pellets were suspended in 1 ml of acetonitrile and filtered through a membrane filter cellulose acetate 0.45 µm pore size. Samples were then stored at -20°C until analysis. The contents of capsaicinoids in the extracts of red pepper fruits grown with different concentrations of organic fertilizer (0, 1, 2 and 4%) were quantified by the HPLC, according to the technical standards of the Association of Official Analytical Chemists (AOAC, 2000). The HPLC data handling was made by the Gilson system controller software equipped with the Rheodyne manual injector for 20 µl injection volume (pump model 307) and UV detector (model 151) at 280 nm. The mobile phase was acetonitrile/water/acetic acid (39.9/60/0.1 v/v) and Microsorb-MV column (4.6 mm × 250 mm, I.D., 5 µm) with a flow rate of 0.8 ml/min at 25°C. The calibration curves were obtained with standard solutions from 20 to 200 µg ml^{-1} capsaicin (Sigma Aldrich), 5 to 200 µg ml^{-1} dihydrocapsaicin (Sigma Aldrich) or nordihydrocapsaicin (Crescent Chemical Company). All analyzes were performed in triplicate. Statistical analysis was performed by SISVAR (a computer statistical ANOVA system) and complemented by the Tukey test using the 5% probability level.

RESULTS

This study quantified the amount (%) of capsaicin, dihydrocapsaicin, and nordihydrocapsaicin of the *C. baccatum* L. species that were cultivated in North Parana State, Southern Brazil. The Reversed-Phase Chromatography was the HPLC technique applied to quantify the contents of capsaicinoids in dry fruits of *C. baccatum* L. that were obtained from different concentrations of organic fertilizer. The capsaicin contents of the fruits harvested in November (First Harvest) were 12.00 ± 0.54, 19.40±0.80*, 44.70 ± 3.6* and 40.39 ± 0.77 mg/100 g dry fruits for 0, 1, 2 and 4% organic fertilizer, respectively (Table 1). The capsaicin

Table 1. Chemical analysis of the soil and organic fertilizer.

Soil analysis							
cmol$_c$ dm^{-3}					mg dm^{-3}	g dm^{-3}	pH
H$^+$Al^{3+}	Al^{3+}	Ca^{2+}	Mg^{2+}	K$^+$	P	C	CaCl$_2$
4.61	0.20	2.44	1.27	0.09	1.66	8.96	4.98

Organic fertilizer										
Humidity (%)		%								pH
65°C	110°C	C	MO	N total	CaO	MgO	K$_2$O	P$_2$O$_5$	Rel. C/N	H$_2$O
4.61	6.80	16.90	30.76	1.10	1.4	0.47	0.70	0.01	15:1	7.23

content of the fruits harvested in March (Second Harvest) were higher, reaching 26.15 ± 4.17, 37.73 ± 3.55, 50.42 ± 4.80* and 44.32 ± 1.22 mg/100 g dry fruits for 0, 1, 2 and 4% organic fertilizer, respectively (Table 2). The plants grown at 2% organic fertilizer presented the highest capsaicin contents, with values of 50.42 ± 4.80 mg/100 g dry fruits from the second harvest. The retention times for fruit extracts obtained from 2% organic fertilizer are 29 min for capsaicin, 26 min for nordihydrocapsaicin, and 46 min for dihydrocapsaicin (Figure 1). In this work, treatments up to 2% organic fertilizer favored the accumulation of capsaicin in the *C. baccatum* fruits. When compared to treatments with 4% organic fertilizer, the capsaicin contents declined to 40.39 ± 0.77 and 44.32 ± 1.22 mg/100 g dry fruits in the first and second harvest, respectively.

DISCUSSION

The pungency of red peppers is a distinguished quality parameter. About 20 capsaicinoids have already been identified in red peppers. The capsaicin, dihydrocapsaicin, and nordihydrocapsaicin are responsible for the pungent taste. The capsaicin and dihydrocapsaicin together represent 90% of the capsaicinoids (Nwokem et al., 2010). The *C. baccatum* L. spp. is very similar to the *Capsicum annuum* L. species that is one of *Capsicum* spp. most commercialized worldwide. Contents of capsaicin, dihydrocapsaicin, and nordihydrocapsaicin were determined by the HPLC technique in the dry fruits of *C. baccatum* L. grown at different concentrations of organic fertilizer. Capsaicin was the major compound of the capsaicinoids. Giuffrida et al. (2013) also found capsaicin as the major compound of all capsaicinoids obtained from 12 different varieties of *Capsicum* by the HPLC technique. The highest capsaicin contents were found in soils that received 2% organic fertilizer: 44.70 ± 3.6 and 50.42 ± 4.80 mg/100 g dry fruits in the first and second harvest, respectively.

These results agree with the findings of Pino et al. (2007) for the *Capsicum chinense* species with total capsaicinoids of 41.8 to 65.9 mg/100 g dry fruit.

Materska and Perucka (2005) quantified the capsaicin contents in different varieties of *C. annuum* L. by reversed-phase chromatography using the HPLC technique. The authors obtained 30 to 50 mg/100 g dry fruits, similarly to the values found in this present work for the *C. baccatum* L. spp. grown at concentration of 2% organic fertilizer. *C. baccatum* L. is similar to the pepper species most commonly used in the world and might therefore serve as a substitute in the manufacture of sauces and use by the food industry. Higher capsaicin contents were found in the second harvest of fruits grown with 2% organic fertilizer, in the months from January to March, which present high temperature and high rainfall. Pepper crops require high temperatures (25 to 30°C) for their development (Mercado et al., 1997).

Therefore, the high temperature observed in March in the second harvest perhaps increased the plant metabolism (Yaldiz et al., 2010) and consequently the contents of capsaicin in fruits. In addition, the hot and rainy weather generally observed in the months of January and March (second harvest) suits the development of pests and pathogens (Torquato, 2010) and these can induce the plant to produce capsaicinoids (Carvalho et al., 2009). Rodrigues et al. (2013) reported the effect of an organic fertilizer (concentrations of 0, 1, 2 and 4%) on the *C. baccatum* biomass development. The authors observed a decline in the pepper biomass performance for 4% organic fertilizer, suggesting a lower content of capsaicin. In pepper, the capsaicin production results in pungency and this is expressed by a dominant gene (Gene C). However, the accumulated capsaicin is as a result of the crop, soil and climate conditions (Dias et al., 2008; Kappel, 2008; Domenico et al., 2012).

In this work, the increased concentrations of organic fertilizer favored the accumulation of capsaicin in the *C. baccatum* fruits. It can be explained by the factors stimulating the primary metabolism and plant growth. Fertilization favors the availability of nutrients, especially N in soil, and can induce the production of secondary metabolites such as alkaloids, in this case, capsaicin (Costa et al., 2000). Other authors also verified the benefits of organic fertilization in crops, such as increased biomass production, improved productivity and

Table 2. Capsaicinoids contents (mg/100 g dry fruits) of *Capsicum baccatum* obtained to 4 different soil fertility concentrations and 2 harvest periods (November 2009 and March 2010).

Capsaicinoids	November 2009 (Soil fertility levels)				March 2010 (Soil fertility levels)			
	0%	1%	2%	4%	0%	1%	2%	4%
Nordihydrocapsaicin	2.46 ± 0.006	3.49 ± 0.10	3.52 ± 0.09	5.08 ± 0.29	2.64 ± 0.44	3.49 ± 0.015	4.74 ± 0,17	7.56 ± 0.17
Capsaicin	12.00 ± 0.54	19.40 ± 0.80*	44.70 ± 3.6*	40.39 ± 0.77	26.15 ± 4.17	37.73 ± 3.55*	50.42 ± 4.80*	44.32 ± 1.22
Dihydrocapsaicin	3.13 ± 0.14	3.70 ± 3.25	10.49 ± 0.38*	14.69 ± 0.18	3.29 ± 2.89	7.21 ± 0.34	7.70 ± 6.65	10.54 ± 0.66

The values correspond to the mean and standard deviation of triplicate extraction and quantification by HPLC. *The statistical significance ($p \leq 0.05$) was calculated between the harvest periods and among the fertility concentrations (ANOVA).
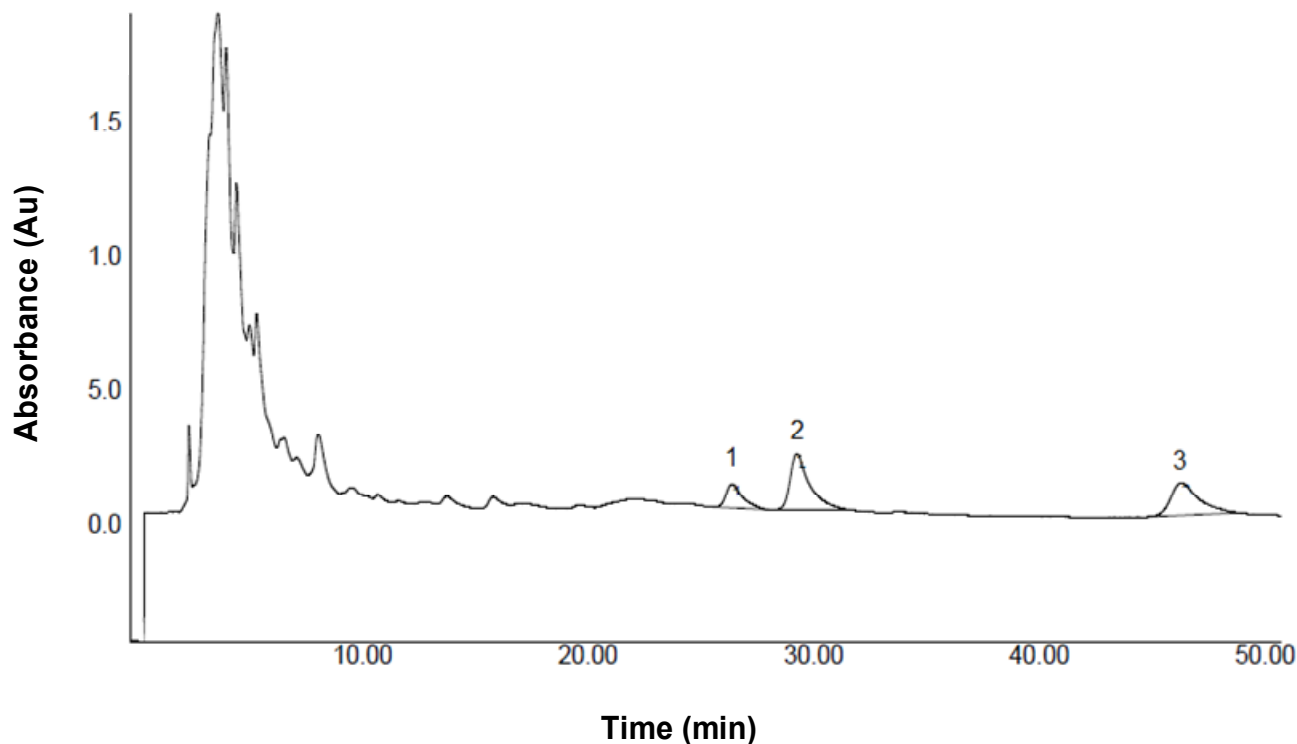
Figure 1. HPLC chromatogram of the extract of pepper fruits of *Capsicum bacattum* L obtained in the second harvest (March, 2010) and grown to 2% organic fertilizer: (1) Nordihydrocapsaicin, (2) Capsaicin and (3) Dihydrocapsaicin. Column: Microsorb-MV (4.6 mm × 250 mm, I.D., 5 μm). The mobile phase was an acetonitrile/water/acetic acid (39.9/60/0.1 v/v). Other parameters: flow rate, 0.8 ml/min; wavelength, 280 nm; column temperature, 25°C.

higher yield of active ingredients (Demeyer and Dejaegere, 1992; Becker et al., 2000; Chaves et al., 2006; Gómez-López and Del Amor, 2013).

Conclusion

The capsaicinoids content of red pepper fruits (*C. baccatum*) may vary according to different organic fertilizer concentrations used to grow the crop. The organic fertilizer at 2% concentration promoted the highest increase in the capsaicin content in pepper fruits, regardless of the harvest period.

Conflicts of interest

The authors have not declare any conflict of interest.

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Full Length Research Paper

Role of sacred groves in the conservation and management of medicinal plants

Manoj Kumar Behera*, Tapas Ranjan Pradhan and Jangyeswar Sahoo

College of Forestry, Odisha University of Agriculture and Technology, Bhubaneswar, India.

Sacred groves play a vital role in context of sustainable use and conservation of medicinal plants. The involvement of local communities offers several advantages in the management of traditionally known medicinal wealth of forests. Considering the importance of sacred groves in the conservation of medicinal plants, a study was carried out in Phulbani forest division of Odisha to record the status, distribution and use of medicinal plants in different sacred grove areas of this division. The study recorded about 40 medicinal plants (including trees, shrubs, herbs and climbers) across different sacred groves and their use for human welfare. The local people were consulted to know about the use of different medicinal plants and the existing management strategy. The study suggested the promotion of medicinal plant conservation through effective capacity building activities for the sacred grove committee members and local people to realize the goals of sustainability.

Key words: Sacred groves, Phulbani, Odisha, Kondha tribe, medicinal plants.

INTRODUCTION

India, a mega diverse country with only 2.4% of the world's land area, harbours 7 to 8% of all recorded species, including over 45,000 species of plants and 91,000 species of animals. It is also one of the 12 primary centres of origin of cultivated plants and domesticated animals (MoEF, 2014). In regard to medicinal plant biodiversity, there are some estimated 6560 species of medicinal plants found in different parts of India (MoEF, 2014). The uniqueness in India's medicinal plant diversity lies with the interlinkage or traditional association between community and nature. However, the culturally linked and traditionally well managed biodiversity of India

is under severe threat due to various anthropogenic causes (Yadav et al., 2010). The situation seems to be more critical in near future as India has been predicted to surpass China to become the most populous country in the world by 2050. Thus the issue of biodiversity conservation and environmental sustainability has undergone a substantial transformation across the country. The importance of sustainable community involvement programs for effective conservation and management of biological wealth has been well recognized in India (Gadgil and Vartak, 1974). The traditionally managed and socio-culturally linked small

patches of forest tress located inside or near to the forest area better known as sacred groves (Bhakat and Sen, 2008), scientifically and culturally considered as treasure house of plants (Basu, 2000) and folk medicines are gaining more attentions with policy makers, researchers, academicians and environmentalists are exploring the critical role of sacred groves in the management and conservation of a variety of medicinal, rare and endemic plants.

Scared groves are traditionally managed by the local communities. Sacred groves remain untouched and well-guarded by the local people due to their traditional and religious attachments with the area and their belief in the local deities (Khumbongmayum et al., 2005). Since time immemorial, the local communities mostly tribals have been using different medicinal plants mostly found in these sacred groves to cure different diseases. The medicinal properties of different plants are well known to the local inhabitants and it gets transferred from generation to generation (Semwal et al., 2010). Sacred groves have been reported and well documented from across different states of India. The sacred groves in India are known by different names (Khan et al., 2008; Bhakat and Sen, 2008) at different places such as 'Devray' in Maharastra, 'Devarkand and Siddarvanam' in Karnataka, 'Oraans, Kenkari, Malvan and Yogmaya' in Rajasthan and 'Saranya' in Bihar.

In tribal regions of Jharkhand and Orissa, sacred groves are popularly known as Jaher. In Odisha, these are mostly confined to the districts like Kandhamal, Koraput, Rayagada, Deogarh, Mayurbhanj and Keonjhar etc. Sacred groves have potential to conserve the medicinal plant biodiversity due to the advantage of community ownership and traditional sustainable management practices. However, the concern is that most of these sacred grove areas are evincing degradation and conservation threats. The degradation of sacred groves has an adverse impact on tribal communities and their traditional knowledge on plants (Basu, 2009). The present study was conducted to document the medicinal plant wealth and their uses by the local communities of different sacred groves under Phulbani forest division of Odisha which is mostly dominated by the culturally rich and traditionally dressed Kondha tribes. Figure 1 provides a view of a sacred grove located in Phulbani forest division of Odisha. The aim was also to investigate the traditional management practices, role and status of sacred groves in regards to medicinal plant biodiversity and its conservation.

MATERIALS AND METHODS

The study area Phulbani is situated in the southern parts of Odisha and comes under the North-Eastern Ghat Agro-climatic zone of the

State and the altitude Ranges from 300 to 1100 m from the mean sea level.The district is mostly inhabited by the Kondh tribes. The study was carried out in 10 identified sacred groves of Phulbani forest division of Odisha. The location details are provided in Figure 2. A survey was carried out in the sacred grove areas of Phulbani forest division to record the medicinal plant biodiversity of the sacred grove areas and also to record the ethnobotanical use of different plants by the local people. Individual sacred grove sites were visited and quadrats were laid down randomly to estimate the medicinal plant biodiversity of these areas. A total of 10 sacred groves belonging to 4 different blocks were studied for this purpose.

Efforts were made to organize meetings with the sacred grove committee members to carry out the study with active participation of the local people. The local vaidyas, village old men and school teachers were consulted to record the medicinal uses of different trees, shrubs and herbs. A total of 250 local inhabitants were interviewed for this study. The information was collected in the pre-designed questionnaires of local language. The plant parts collected were processed for herbarium to ensure proper identification of all the medicinal plants and for the same help of Botany Professors of local college, Specimen Collectors of Regional Plant Resource Centre (RPRC) and Professors of College of Forestry, OUAT, Bhubaneswar were sought. The data so obtained were collated to derive the required information as per the objective of the study.

RESULTS

The present study reported 40 plant species comprising of trees, shrubs, herbs and climbers belong to 30 different families (Table 1). The local people mostly use these medicinal plants to cure a number of diseases such as dysentery, constipation, skin diseases, cardiotonic, cough, fever, diarrhea, indigestion, wound healing, headache, stomach pain, snake bite etc. They use specific part of plants for curing different diseases. The medicinal plants found in the studied area belong to different families like Asteraceae, Combretaceae and Verbenaceae closely followed by Solanacease, Rutaceae and Acanthaceae. The number of medicinal plant species found in 10 different sacred groves studied is presented in Table 2. Although all sacred groves have been explored by the local communities for preparing folk medicine, however, there exists significant variation in species distribution by habit and extent of knowledge with the locals to better understand and utilize the medicinal plants as per their specific uses.

Sacred groves such as Maa Dwarapala, Maa Hansa debi, Maa Pitabali and Baba Kapileswar reportedly have more number of medicinal plant species used by local community. On the other hand, sacred groves such as Ram Mandir located in Raikia, Baba Dhabaleswar Rameswar Pithha in Tikabali and Pachamukhhi Hanuman in Phulbani have less number of medicinal plant species traditionally used by the locals. Among the medicinal plants listed in Table 2, 50% are herbs, 30% are tree species, 15% are shrub and 5% are climbers

Table 1. Use of medicinal plants from sacred grove areas by the local people.

Local name	Scientific name	Family	Parts used	Uses
Agnijala	*Clausena excaveta* Burm.f	Rutaceae	Root	Dysentery
Apamaranga	*Achyranthes aspera* L.	Amaranthaceae	Barks	Constipation and dysentery
Arjuna	*Terminalia arjuna* Wight & Arn	Combretaceae	Bark	Skin diseases and cardio tonic
Asoka	*Saraca asoca* (Roxb.)	Caesalpiniaceae	Bark	Tonic
Aswagandha	*Withenia somnifera* (L.)	Solanaceae	Root	Cough and fever
Aswastha	*Ficus religiosa* (L.)	Moraceae	Bark	Vomiting
Bahada	*Terminalia bellerica* (Gaerth.). Roxb	Combretaceae	Fruit	skin diseases and hair fall
Banakunduri	*Coccinia grandis* (L.)	Cucurbitaceae	Leaf	Jaundice
Basanga	*Adhathoda vasica* L.	Acanthaceae	Leaf	Cold fever and cough
Begunia	*Vitex negundo* L.	Verbenaceae	Leaf	Body pain
Bela	*Aegle mormelos* L.	Rutaceae	Leaf and fruit	Diarrhoea and indigestion
Bhang	*Cannabis sativa* Linn.	Cannabaceae	Leaf	Fever and indigestion
Bhejibaigana	*Solanum xanthocarpum* Schrad & Wendi	Solanaceae	Leaf and fruit	Fever
Bhuinnimba	*Andrographis paniculata* (Burm.f.) Wall	Acanthaceae	whole plant	Skin diseases and malaria
Bisalyakarani	*Tridax procumbens* Linn.	Asteraceae	Leaf	Wounds and burns
Brahmi	*Centella asiatica* (L.)	Apiaceae	Leaf	Headache and memory enhancement
Chara	*Buchanania lanzan* Sperg.	Sapotaceae	fruits	Indigestion
Dimiri	*Ficus recemosa* L.	Moraceae	Fruit	Headache
Dubaghasa	*Cynodon dactylon* (L.)	Poaceae	Leaf	Vomiting
Dudhi	*Euphorbia hirta* L.	Euphorbiaceae	Leaf and stem	Asthma
Hadajoda	*Cissus quadrangularis* L.	Vitaceae	stem	Bone fracture
Harida	*Terminalia chebula* Retz.	Combretaceae	Fruit	digestive problems
Kalamoricha	*Piper nigrum* L.	Piperaceae	Fruit	Cold, cough and indigestion
Kanchan	*Bauhinia veriegata* L.	Fabaceae	Root	snake bite
Khiralai	*Hemidesmus indicus* (L.) R. Br	Asclepiadaceae	Root	Wound
Kuluchi	*Holarrhena pubescens* Wall.ex. DC	Apocynaceae	Root	Headache
Kumbhi	*Careya arborea Roxb.*	Lecythidaceae	Bark	Diarrhoea
Kumti	*Clerodendrum viscosum Vent.*	Verbenaceae	Leaf	Diabetes and dysentery
Lantana	*Lantana camara* L.	Verbenaceae	Leaf	Digestion and wounds
Mayurachulia	*Elephantopus scaber* Linn.	Asteraceae	entire plant	Head ache
Neem	*Azadirachtaindica*a. Juss	Meliaceae	Bark, leaf	Skin diseases and malaria

Table 1 cont'd

Nirmuli	*Cuscuta reflexa Roxb.*	Convolvulaceae	Stem	Fever
Pita alu	*Dioscorea wallichi* Hook.f	Dioscoreaceae	Root	Body pain
Pokasungha	*Ageratum conyzoids* L.	Asteraceae	Leaf	Ulcer
Punarnova	*Boerhavia diffusa* L.	Nyctaginaceae	whole plant	Jaundice
Sal	*Shorea robusta* (Gaertn.f)	Diperocarpaceae	Bark, stem	Dysentery and ear pain
Satabari	*Asparagus recemosus* Wild.	Liliaceae	Root	Stomach pain and indigestion
Satyanasi	*Argemone maxicana* Linn.	Papaveraceae	Seeds and roots	Malaria and skin diseases
Sitaphala	*Annona squamosa* L.	Annonaceae	Fruit	Dysentery
Tulasi	*Ocimum sanctum* L.	Lamiaceae	Leaf	Cold, fever and indigestion

Table 2. Sacred grove wise medicinal plant species distribution in Phulbani Forest division of Odisha.

S/N	Name of sacred grove	FMU/Range	No. of medicinal plant species used by local people by habit				
			Tree	Shrub	Herb	Climber	Total
1	Panchamukhi Hanuman	Phulbani	5	3	8	1	17
2	Maa Pitabali Baba Kapileswar pitha	Phulbani	7	6	13	1	27
3	MaaHansadebi	Sudrukumpa	6	4	15	1	26
4	Maa Dwarapala	Sudrukumpa	5	4	17	0	26
5	Baba Akhandalamani	Phiringia	4	4	12	1	21
6	Baba Akhandalamani	Tikabali	5	3	7	1	16
7	Baba Dhabaleswar Rameswar pitha	Tikabali	4	5	7	0	16
8	Rameswar pitha	G. Udaygiri	5	6	5	2	18
9	Ram Mandir	Raikia	4	5	6	0	15
10	Mandusagiri Veda Bhawan	Raikia	6	3	10	0	19

(Figure 3). In total, these 40 medicinal plants are being used to cure 28 different diseases. Besides the ethno-botanical association, the locals also worship certain tree species such as *Ficusreligiosa* (L.), *Aeglemormelos* (L.)*, Shorearobusta* (Gaertn.f)*, Saracaasoca* (Roxb.)*, Terminalia chebula* Retz. and *Terminalia arjuna* in the sacred grove areas considering the closeness of these species with the local deities.

DISCUSSION

Odisha is well known for tribal communities with 62 tribes residing in more than 40% of the total geographical area of the state. In Kandhamal, 51.96% of the district population belongs to Kandha tribe (Rath, 2011). Sahu et al. (2013) reported that Kandhas have remarkable detailed knowledge of uses of medicinal plants with some major ailments and diseases such as cancer,

Figure 1. A view of a sacred grove in Phulbani Forest Division of Odisha.
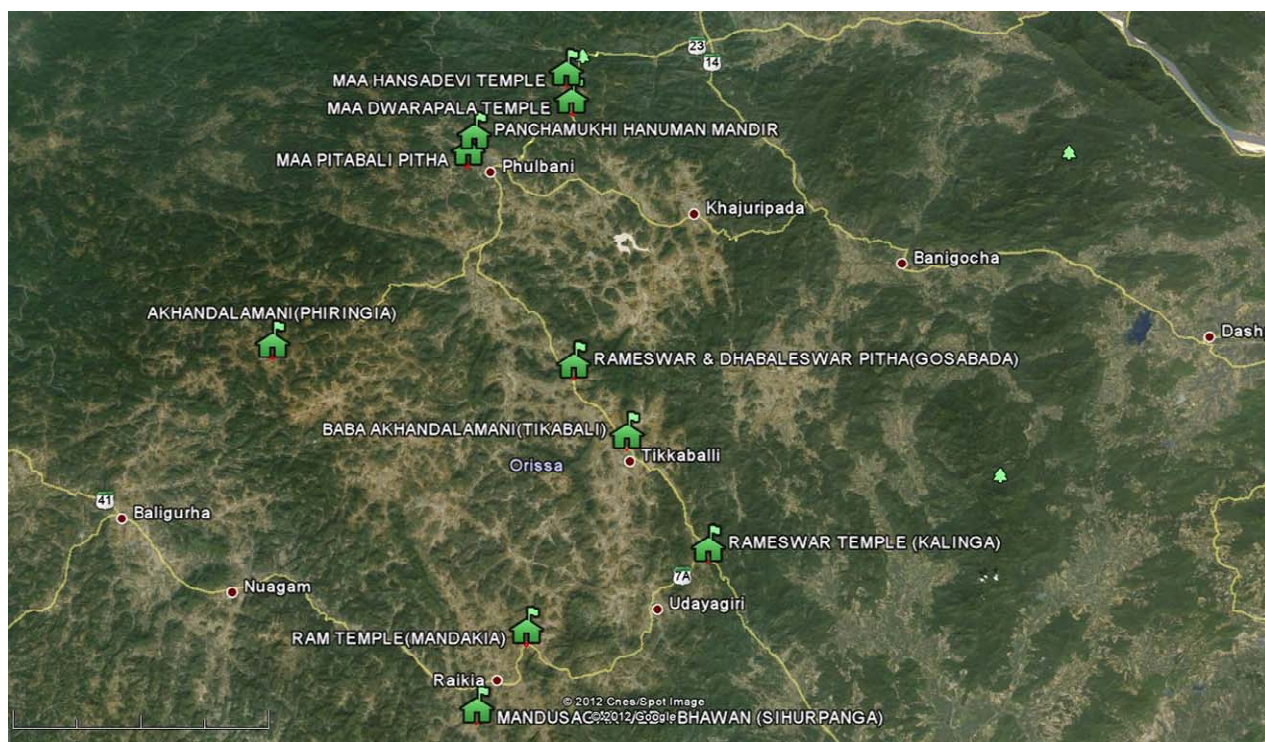

Figure 2. Google earth map of studied sacred groves in Phulbani Forest Divisions of Odisha.

heart diseases, kidney-stones, skin diseases, abortion inducing drugs, respiratory diseases etc. are being effectively treated using traditional knowledge and locally available plant resources by Kondha community. The present study could come out with the same notion about the culture and traditional knowledge of kondha tribe. It
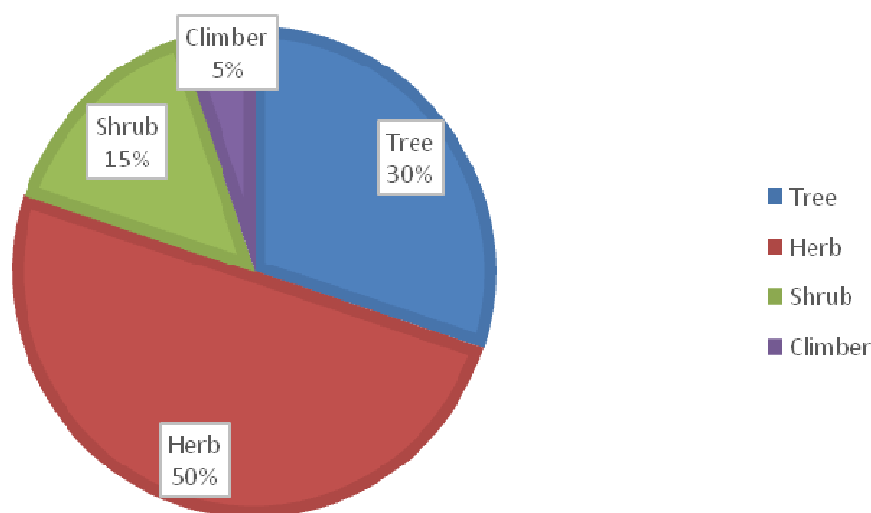
Figure 3. Percentage distribution of different category of medicinal plants in the sacred grove areas.

was found that the local communities with limited knowledge on medicinal uses of different plants depend largely on the sacred grove areas for preparing folk medicines. They are culturally enriched and traditionally trained to explore the uses of different medicinal plants as well as protecting the biodiversity richness and sacredness of these small patches of forests better known as sacred groves.

They use different parts of plants for preparing medicines for different ailments. Generally, leaves of different medicinal plants are used for preparing medicines for a number of diseases. Besides leaves, other plant parts like barks, fruits, stems and roots of different plants are also being used by the local vaidyas or herbal practitioners to treat a myriad of diseases. Medicinal plants such as *Tridaxprocumbens* Linn, *Terminalia bellirica* (Gaerth.). Roxb and *Vitex negundo* have common presence and maximum use by locals in almost all sacred groves studied. This seems to be because of traditional and common uses of these plants by the locals. Among the sacred groves, Maa Pitabali, Maa Dwarapala and Maa Hansa devi sacred groves were found to have maximum number of medicinal plants. Much of this is because of the fact that these are located away from the village (mostly inside the forest) and active participation of the villagers to guard the sacred grove areas. They are certainly aware of the medicinal uses of different plants though not all found in the sacred grove areas. Apart from curing common diseases like cold, cough, fever, diarrhea and indigestion, some critical ailments like cardiotonic and stomach pain are also being treated by the use of herbal medicines. On the other hand, the reasons behind less occurrence and limited

exploration of medicinal plant species found in sacred groves such as Ram Mandir located in Raikia, Baba Dhabaleswar Rameswar Pithha in Tikabali and Pachamukhhi Hanuman in Phulbani are increased or unchecked movement by locals and outsiders in the sacred grove areas due to location along the road as well effect of modern culture which seems destroying to the tribal culture. The high rate of anthropogenic interventions has led to the narrowing of genetic base of medicinal plants in these important biodiversity rich areas.

All the sacred groves studied are managed by a local committee named as Sacred Grove Conservation Committee (SGCC) with both formal and informal arrangements. The collective understandings among them helped in developing strategy for effective management and conservation of these valuable plant species in the sacred grove areas. It was also found that in some cases, the villagers mostly the committee members have shared responsibilities among them for better safeguard of the sacred grove areas. They have also created certain rules which are binding in nature and any violations of these results in imposition of fines. The traditional practice of worshipping soil (Mati puja) and hills and mountains (Giridebata) by the locals better reflects their belief and traditional practices around natural resources. It is well established that the entranced cultural practices along with the ethno-botanical associations of the local people could decide the future sustainability in the management and conservation of medicinal plants while maintaining the ecological balance. It was also found that some of these sacred groves are critically threatened because of heavy soil

erosion influenced by undulating terrains. This is also adversely affecting regeneration of medicinal plants in the sacred grove areas. Above all, as mentioned earlier, the adulteration of tribal culture coupled with degradation of sacred grove areas due to edaphic, biological or anthropogenic causes could derail the path of sustainable use and conservation of medicinal plants, traditional knowledge and practice.

Conclusion

The importance of sacred groves has been well recognized for effective conservation of both biodiversity as well as culture of the tribals. The sacred groves of Phulbani forest division of Odisha are repositories of important medicinal plants and represents ancient cultures of Kondha tribe associated with these forest patches. The existence of sacred groves is contributing immensely towards conservation of traditional knowledge and medicinal plants as both are interlinked. In order to sustain the flow of conservation benefits that emanated mostly from sacred grove areas, Government and Civil society organizations shall proactively guide and support the local communities for effective conservation of biodiversity and tribal culture. Furthermore, there is a need to assess the perceived threats to the sacred groves because of land degradation. This can help in designing effective strategies for better management and conservation of the sacred groves. The conservation efforts of local communities need greater recognition and appreciation. They shall be provided financial incentives for conservation efforts. Most importantly, they need training and demonstration on sustainable harvesting, collection, processing, regeneration and management of medicinal plants in the sacred grove areas. By addressing these critical concerns associated with conservation of sacred groves, we can ensure the sustainable use and conservation of medicinal plants.

Conflicts of interest

The authors declare that they have no conflicts of interest.

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Full Length Research Paper

Effect of *Mansoa alliacea* (Bignonaceae) leaf extract on embryonic and tumorigenic mouse cell lines

Camden M. Towne*, Jan F. Dudt, and Durwood B. Ray

Biology Department, Grove City College, 100 Campus Drive, Grove City, PA 16127, USA.

Mansoa alliacea contains high concentrations of chemicals thought to be associated with the suppression of tumor growth. Additionally, this plant has been reported to possess analgesic, anti-fungal, and anti-bacterial properties, thereby providing other potential benefits for cancer patients. Low doses of a water extract of *M. alliacea* were applied to a cancerous and non-cancerous cell line. Doses between 1.254 to 10.04 mg/ml of extract applied to T3-HA cancer cells inhibited cell growth, but higher doses of 29.92 to 89.6 mg/ml destroyed colonies of the cancer cells. Application of the extract to NIH Swiss mouse cell cultures resulted in the inhibition of growth at higher concentrations, but at a concentration of 10.14 mg/ml, cell growth began to increase after three days. However, cell death was less at lower concentrations than that of T3-HA cancer cells, thereby confirming that lower concentrations of Ajo de Monte will inhibit cancer cell growth as well as initially inhibit non-cancer cells. Thus, *M. alliacea* extract selectively targets T3-HA mouse cancer cells but not NIH Swiss embryonic mouse cells. Future research may consider the use of this plant for human cancer patients.

Key words: *Mansoa alliacea*, Garlic Vine, Ajo de Monte, cancer

INTRODUCTION

In the Amazonian jungles of east Ecuador, native people profess that many plants have healing or other beneficial properties. In Ecuador, indigenous people utilize a woody vine called "Ajo de Monte" or "Garlic Vine" to treat and alleviate a variety of ailments. Ajo de Monte is used as a seasoning in cooking and the leaves are used as a topical anesthetic by placing a leaf in contact with the patient's skin. The analgesic effect penetrates sufficiently to alleviate joint pain. A tea preparation of the leaves may

be used to effect a systemic analgesic response. This systemic effect is regarded by the Waorani people in Ecuador to temporarily assuage musculoskeletal pain from over exertion (personal observation).

The Ajo de Monte woody vine produces net-branching leaves that when mature, average between 10 and 22.5 cm in length. New shoot growth originates in apical meristems from between two mature leaves. When mature, the plants produce purple flowers with a white

*Corresponding author. E-mail: cameden@gmail.com. Tel: 603-387-0631.

center that fade to paler shades. Ajo de Monte is native to Brazil, Costa Rica, Ecuador, French Guyana, Guyana, Peru and Suriname (Taylor, 2006).

Taylor (2006) described Ajo de Monte as *Mansoa alliacea* and placed it in the family Bignoniaceae. Ajo de Monte is otherwise referred to as *Adenocalymma alliaceum* and *Bignonia alliaceae* (Rana et al., 1999; Pandya et al., 2012; Granados-Echegoyen et al., 2014). Herbal applications may use the name Ajos Sacha. Indigenous people of the Amazon have used Ajo de Monte for spiritual rituals and as a cure for medical maladies including "bumps, swellings, rheumatism, arthritis, colds, uterine disorders, inflammation, epilepsy, and infertility (Taylor, 2006). Other systemic problems such as fever, flu, body aches, cramps, fatigue, and pain are also treated (Zoghbi et al., 2009). In addition, the leaves may be applied in direct contact with the skin for analgesic purposes or are prepared instead as an infusion or decoction. Bark is prepared as a tincture or decoction, and the root is used as a tincture or cold maceration (Taylor, 1996). Although these ethnobotanical uses are common among indigenous populations living within the natural range of *M. alliacea*, further study is necessary to confirm the clinical effectiveness of Ajo de Monte.

MATERIALS AND METHODS

Extraction

A leaf extraction was prepared using an adaptation of the procedure described in Rana et al. (1999) to produce a polar molecule extraction which could be applied as a component of cell growth medium. The effects of different concentrations of *M. alliacea* on the T3-HA mouse tumor cell line and normal NIH Swiss mouse cells were examined using a photomicrograhic method to evaluate *in vitro* cell growth. *M. alliacea* sample was obtained as a dry powder extract from Rain Tree Pharmaceuticals, a company avowing their support of sustainable harvesting of materials and rainforest preservation (Taylor, 1996, Rain Tree Pharmaceuticals, Milam County, TX). Fifty grams (50 g) *M. alliacea* powder was mixed with 250 ml water at 25°C and stirred for five min at room temperature. This 1 g/ 5 ml H_2O mixture formed a viscous liquid which was strained with cheesecloth. The effluent liquid was filtered via Buchner funnel suction filtration with #5 Whatman filtration paper. The resulting solution was centrifuged at 12,000 rpm for 20 min to eliminate chloroplasts and other organelles (Damon/IEC Division IEC HN-SII Centrifuge). The supernatant from centrifugation was then eluted through a 0.2 μm millipore filter. These sterile aliquots were frozen at -20°C for later use.

Cell culture

NIH Swiss mouse embryonic cells

Normal primary NIH Swiss mouse embryonic cells and the

tumorigenic mouse T3-HA cell lines were utilized in this experiment. These cells were kindly provided by Durwood B. Ray and their origins, growth properties, morphologies, and tumorigenic capabilities have been described previously. The following description of cell lines and methodology was based on Ray et al. (2015). The NIH Swiss mouse embryonic non-transformed cells were produced as described by Todaro and Green (1963) by establishing primary cultures from eight whole 17 to 19 day old NIH Swiss mouse embryos. These normal mouse cells do not produce tumors when injected into mice and these normal cells exhibited a 22 h doubling time in previous assessment. The NIH Swiss embryonic cells used in this study were comprised of normal immortal cells within passage numbers from 10 to 22.

T3-HA tumor mouse cell line

The highly metastatic T3-HA tumor mouse cell line was obtained during the establishment of a new series of cell lines. They were derived from a new human h-ras oncogene (HRAS) transfection system using T24 human bladder carcinoma DNA to establish cells derived from it that represent various stages in one tumor progression that includes several metastatic cell types. The advantage of this approach is that all transformed cells in the series are derived from a common transfected parent population, namely, the GhrasT-NIH/3T3 cell line generated by transfection of immortalized NIH/3T3 cells with DNA from the T24 human bladder carcinoma. The design to intentionally expose different metastatic cells to *in vivo* micro-environmental effects as they adapt to multiple targets was accomplished by alternating between *in vitro* and *in vivo* growth in the development of this series. The first cell line in the series (T1-A) was cultured from a primary tumor in a NIH/Swiss mouse injected s.c. with the GhrasT-NIH/3T3 cells. The second cell line (T-2A) was cultured from a subsequent secondary local metastasis in a NIH/Swiss mouse injected i.v. with T1-A cells. The third cell line produced (T3-HA)(H=hepatic) used in this current study with *M. alliacea* extract was cultured from a tertiary liver metastatic tumor in a nude NIH/Swiss mouse injected i.v. with T2-A cells. Further cell line developments revealed the maintenance of the HRAS oncogene past the T3-HA stage. The tumorigenic growth and morphological properties of these newly reported T24 bladder carcinoma derived cell lines appear to be unique from those reported by others using a different human EJ-6-2-Bam-6a cell line. These outcomes indicated that the lung and liver are the most common sites for distant metastasis to occur in NIH Swiss nude mice injected i.v. in the tail vein with either T2-A or T3-HA cells. T3-HA cells exhibited a 17.5 h doubling time in previous assessment. The embryonic mouse cells and all the cell lines including the T3-HA cells were expanded and subcultures were frozen in Recovery Cell Culture Freezing Media (catalogue #12648-010, GIBCO) and stored in liquid nitrogen tanks as stocks.

Cell growth conditions

Embryonic mouse cells and T3-HA cells were grown in Dulbecco's modified Eagle's medium (DMEM) (Catalog # 12320-032, GIBCO, Grand Island, NY) with extra added D (+) glucose (Catalogue # G-5400, Sigma Chemical Company, St. Louis, MO) (4.5 g/L), 10% fetal calf serum (Catalogue # SH30070.02, Hyclone Laboratories, Logan, Utah), and 20 units/ml of penicillin, 20 μg/ml streptomycin (Catalogue #15140-122, Sigma Chemical Company). Each culture was incubated at 37°C in 5% CO_2/95% air.
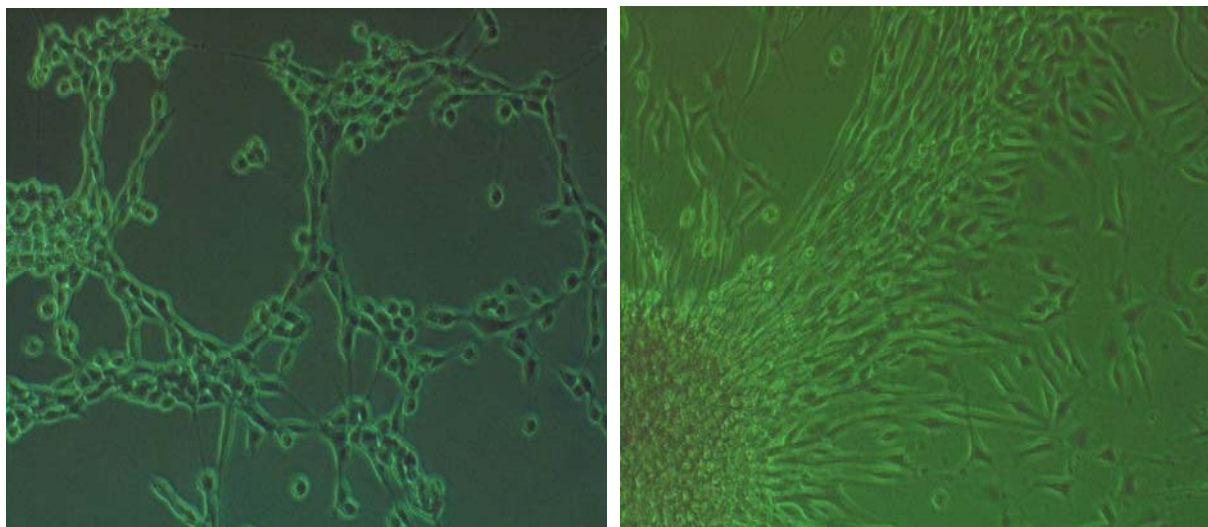
Figure 1. Growth comparison. A comparison of quantity of cultured T3-HA cells from Experiment 1 receiving little treatment over five days; cells from day one shown on left and day five shown on right.

Cell growth determination

A sterile cell culture protocol was utilized for all cell cultures. T3-HA cultured cancer cells or NIH Swiss mouse embryonic cells were plated onto 60 mm gridded tissue culture plates (Corning cat# 430196, Corning, NY). Six predetermined squares were pre-marked with marking pen on each dish prior to use. The squares were scattered in such a way that each 2 × 2 mm square area would be representative of the entire plate. Prior to each experiment, cells were plated and grown for 3 days prior to application of leaf extract. As a control solution, 900 µl H_2O was combined with 10 ml DMEM as described. The rapidly growing tumor cells used in this experiment metabolized the extra added glucose via aerobic glycolysis as their primary energy source and glycolytic intermediates (Warburg, 1956; Wallace et al., 2010). For the test solution, an aliquot of extract was thawed at room temperature, and 900 µl of Ajo de Monte (ADM) extract and 10 ml of media were mixed together within test tubes. Once the confluency of the cancer cells were assessed, the old media was aspirated off, and the plates were washed with sterile saline. Leaf extract was dissolved into cell culture media and pipetted onto each of the plates of cells to make a range of five different concentrations on a logarithmic scale as depicted in Table 1. The control plate of cells received only the growth media and water. Each of the plates received equivalent volumes of media and were swirled gently to distribute the solutions evenly over the plates.

At the same time each day for five days, six pictures were taken of each plate in specific 2 × 2 mm grids on each of the cell plates. Photomicrographs were taken daily when cells reached approximately 15 to 25% confluency (day 0). Photomicrographs were taken with an Olympus DP12 inverted microscope (Olympus America, Inc., Melville, NY) with a digital camera system at 100× in a standardized location within each selected 2 × 2 mm square. These photomicrographs were used to assess quantities of live cells once per day at the same time each day for five days by manual counting. Effectiveness of concentrations of Ajo de Monte extract were determined by observing cell death. Cell death is associated with cell detachment from the bottom of the culture plate. Remaining attached live cells can be easily counted.

Data analysis

The number of live cells was determined by manually counting cells in photomicrographs and subtracting the numbers of detached cells from the number of cells counted at the time the photograph was taken. Unattached cells were presumed to be dead and thereby not included in the cell counts. Counts were made on six 2 × 2 mm sampling areas on each plate and averaged for each day. Results for each day were normalized to fold increase relative to day 0. These procedures were used for the three treatments involving cancer cells and the two experiments using non-cancer cells. Normalized cell counts were produced by dividing the quantity of cells on a given day by the initial quantity of cells in the first photograph. The normalized cell count was used to compare populations of cells between plates. The mean normalized cell counts in all replications were averaged to compare the effects of different concentrations of Ajo de Monte extract between cancer and non-cancerous cell cultures. The differences in cell counts after the applications of Ajo de Monte extract were determined by comparing the average number of cell deaths per plate over five days of treatment as exemplified in Figure 1. The changes in the cell deaths were compared using analysis of variance (ANOVA) with an alpha value of 0.05 as significant for all treatments. Three replications were completed for treatments of T3-HA cancer cells and two replications for each treatment of NIH Swiss non-cancer cells.

A series of three experiments with identical concentrations of leaf extract was conducted with T3-HA mouse embryonic cells. Cell counts were compared before and after the trial and results across the series were averaged for each concentration. Concentration level was determined by grams dry weight leaf powder/ml cell culture media. This series produced growth curves depicted in Figure 2. Two experiments were conducted with NIH Swiss non-
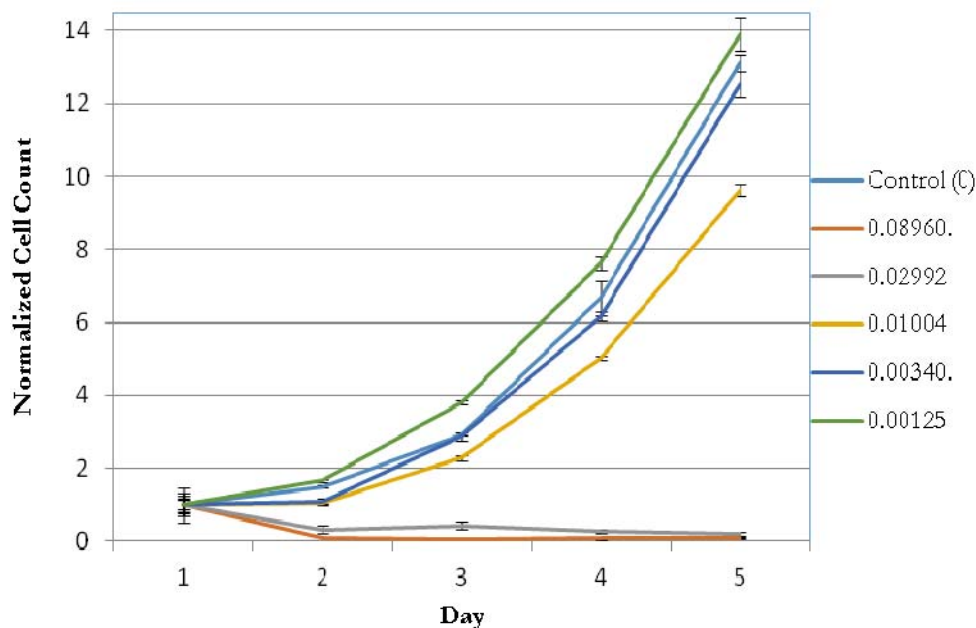
Figure 2. T3-HA Growth, mean normalized cell count of T3-HA cancer cells each day for each treatment condition (0 to 0.0896 g/ml) of *M. alliacea* extract. Normalized cell count produced by dividing the quantity of cells on a given day by the initial quantity of cells. "Control (0)" implies that water was added to the medium with no extract. Error bars represent the standard deviation.

cancerous cells under identical conditions as the experiments conducted with T3-HA cells. These data were averaged in the same way to produce Figure 3. NIH Swiss mouse cells were plated and treated using the same methods described for the T3-HA cancer cell line.

RESULTS

The normalized cell count was used to compare the final counts of cells with the initial cell counts for each experiment (Figures 2 and 3). There was a significant decline in the number of cancer cells at the treatment concentrations, whereas there was not a reduction in cancer cells at the lower concentrations (Figure 2). Moreover, the extract produced statistically different outcomes on the control (NIH Swiss, non-cancerous) populations of cells, as shown in Table 2. Figure 4 provides a visual of this effect. Garlic vine extract resulted in inhibition of the non-cancerous NIH Swiss cell growth without eliminating these populations or significantly reducing their population size. The two highest treatment levels revealed no significant difference in effect between themselves (sig ≤ 0.001). Most notably, at these levels of treatment, the cancerous cell population is significantly decreased but non-cancerous cells only are inhibited in their growth (sig ≤ 0.001).

DISCUSSION

Several researchers have studied other properties of Ajo de Monte, confirming fungicidal, anti-bacterial, cholesterol-lowering benefits, and anti-inflammatory characteristics (Pandya et al., 2012). Rana et al. (1999) reported that the exposure of *Alterniaria brassicae (Berk.) Sacc.* spores to an isolate from Ajo de Monte inhibited germination rates of the spores. Additionally, Khurana and Bhargava (1969) found that Ajo de Monte extract was effective against mild mosaic papaya viruses, whereas it was only slightly effective (20%) in eliminating distortion ring-spot and ring-spot viruses. Additional research is required to determine the efficacy of Ajo de Monte in other applications.

Ajo de Monte is seen as a possible treatment for cancer based on the alleged success in treating cancer patients in the town of Puyo, Ecuador (personal communication with Galo Ortiz, director of the Indigenous People's Technology and Education Center). Neither Ajo de Monte nor extracts or preparations of the plant have been tested for effects on cancer cells or other *in vitro* assays. Additionally, although no documented sources confirm Ajo de Monte's effects on cancer, Davila et al. (2008) reported that Ajo de Monte contains several organosulfur ingredients that also occur in garlic, these organosulfur compounds are correlated with the lower
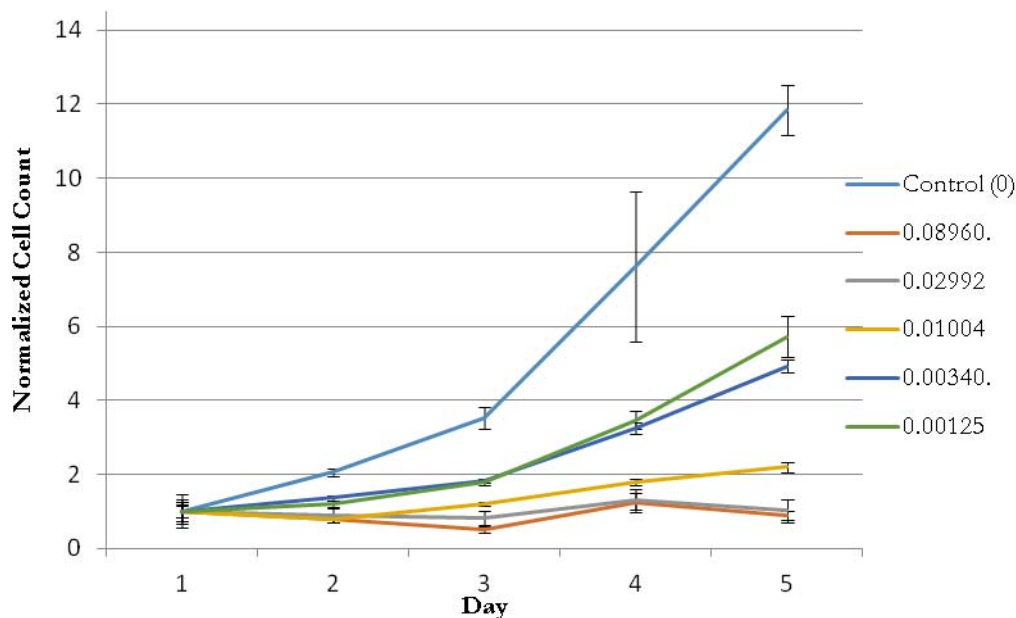
Figure 3. NIH Swiss Growth. Mean normalized cell count of NIH Swiss non-cancerous mouse cells each day in response to various levels of *M. alliacea* treatment (0 to 0.0896 g/ml). Normalized cell count produced by dividing the quantity of cells on a given day by the initial quantity of cells. "Control (0)" implies that water was added to the medium with no extract.
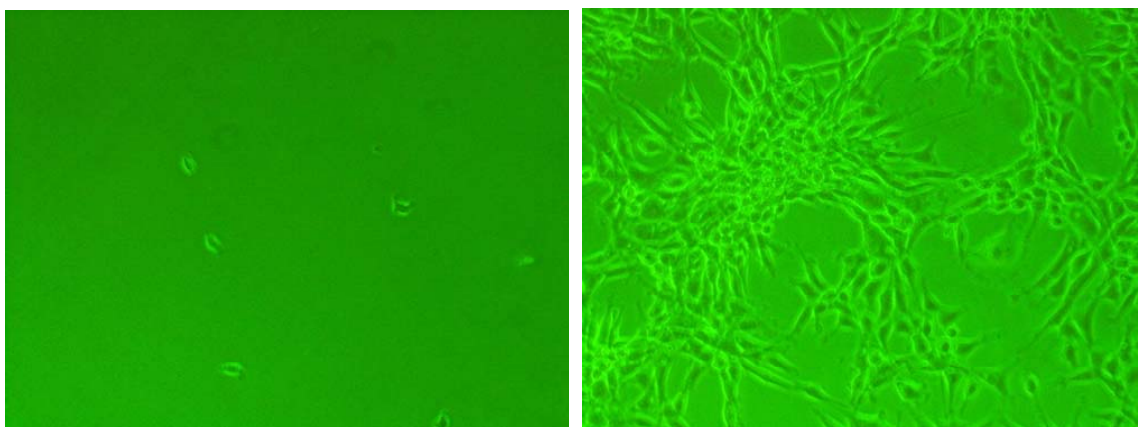

Figure 4. T3-HA Treatment Comparison. A comparison of quantity of cultured T3-HA cells from Experiment 2 on day five, with cells receiving the highest treatment level on the left, and those receiving the lowest treatment on the right.

incidence of cancers in clinical trials (Dorant et al., 1993; Thomson and Ali, 2003; Sakamoto et al., 1997; Wang et al., 2010). Of these, the chemically potent diallyl sulfides comprise 65.9% of the volatile compounds of Ajo de Monte and diallyl trisulfides 29.6% (Granados-Echegoyen et al., 2014). Additionally, these sulfonic compounds in garlic inhibit hepatic cholesterol synthesis, resulting in

lower levels of systemic cholesterol with garlic consumption (Yeh and Liu, 2001). The reported success of these sulfonic compounds in garlic suggests that since both Ajo de Monte and garlic possess organosulfur compounds, it is possible that these plants share a common mechanism of action.

Likewise, the chemical compound allicin in Ajo de

Table 1. Concentrations of extract added to each of five treatment plates with one control.

Plate #	Media and water -900 μl H$_2$O -10 ml cell culture media	Extract and media -900 μl Ajo de Monte extract -10 ml media	Extract concentration (g dry weight leaf powder/ml)
1	5	0	0
2	0	5	0.08960
3	3.33	1.67	0.02992
4	4.44	0.56	0.01004
5	4.81	0.19	0.00340
6	4.93	0.07	0.00125

Table 2. ANOVA (analysis of variance) indicating statistically significant (<.001) difference between control and treatment cell populations.

Parameter	Sum of squares	df	Mean Square	F	Sig
Between groups	4788.498	5	957.700	11.175	0.000
Within groups	8741.068	102	85.697	-	-
Total	13529.567	107	-	-	-

Monte has been proposed to explain its anti-microbial properties. Song and Milner (2001) reported that alliin and the enzyme alliinase combine to produce allicin, a plant-defense chemical with a short half-life which they suggested to be responsible for the anti-microbial and anti-cancer properties in garlic. According to Davila et al. (2008), Ajo de Monte has higher concentrations of alliin in the roots and leaves than garlic. Since allicin also occurs in large quantities in Ajo de Monte, it has been associated with the anti-microbial properties of this species.

Allicin and the related allyl sulfide or diallyl sulfide constituents of Ajo de Monte and garlic have been identified with tumor suppression (Song and Milner, 2001; Zhou and Mirvish, 2005). Although the particular mechanism of action has not been completely defined, studies suggest that allyl sulfides prevent carcinogenesis as well as promote apoptosis of cancer cells. Allyl sulfides can prevent the attachment of the metabolite 7,12-dimethylbenzene(a)anthracene (DMBA) to rat mammary epithelial cell DNA (Song and Milner, 2001). Additionally, Zhou and Mirvish (2005) implied that allyl sulfide compounds prevented guanine methylation by nitrosamine compounds in rats, resulting in fewer neoplastic-generating base mismatches. Moreover, once cancer cells have developed, allyl sulfides can arrest cells in G$_2$ – M phase through Cdc25C signaling (Xiao et al., 2005). Knowles and Milner (2000) suggest that these compounds may play a role in increasing cellular membrane fluidity thereby suppressing integrin glycoprotein llb-llla mediated adhesion, ultimately leading to cell cycle arrest and leading to premature apoptosis (Seki et al., 2012). Alternatively, allyl sulfides and polysulfides can be biologically cleaved to produce reactive oxygen species that may result in an anti-proliferative effect for cancerous cells by promoting apoptosis if they are sufficiently concentrated (Filomeni et al., 2008). Xiao et al. (2003) found that allyl sulfides decrease tubulin polymerization in cancer cells, ultimately leading to the structural collapse of the cell. Furthermore, Yeh and Liu (2001) reported that these organosulfur compounds are water soluble.

Allicin or a related polar organosulfur compound may be the active ingredient for these neoplastic effects. Since extracts of these compounds from similar species are reported to be successful in cancer treatment as well as other ethnobotanical uses, similar effects may also occur from extracts of Ajo de Monte.

Based on these results, Ajo de Monte extract has been shown to both inhibit the growth rate of T3-HA cancer cells at low concentrations and to kill colonies of cancer cells at higher concentrations. Therefore, Ajo de Monte could be used in the treatment of certain cancers due to its targeted killing of cancer cells. Besides killing cancer cells, the analgesic effects of garlic vine could prove to be beneficial for cancer patients. Moreover, the fungicidal and antibacterial properties of the plant may be used for the treatment of secondary infections. Further *in vivo* studies would be required to determine the effectiveness of Ajo de Monte in targeting cancer cells and the appro-

priate dosage required. Due to the high concentration of allicin in Ajo de Monte and other phytochemicals in this species, additional studies should be considered to determine their effectiveness in cancer treatment.

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Conflict of interest

Authors have none to declare.

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