Anticancer activity of *Cinnamon tamala* leaf constituents towards human ovarian cancer cells

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Abstract: Bioassay guided fractionation of *Cinnamon tamala* leaf extracts yielded bornyl acetate (1), caryophylene oxide (2), *p*-coumaric acid (3) and vanillic acid (4) using A-2780 human ovarian cancer cell lines. The structures of the isolated compounds were confirmed through spectroscopic techniques (EIMS, ^{1}H and ^{13}C NMR). Compound 1 exhibited highest cytotoxicity with 90.16±1.06% inhibition (IC_{50}=5.30 x 10^{-4} mg/ml) followed by compound 2 (84.40±1.53% inhibition; IC_{50}=8.94 x 10^{-3}mg/ml), while compounds 3 and 4 were inactive in the bioassay.

Keywords: Anticancer, *Cinnamon tamala*, A-2780 and cytotoxicity.

INTRODUCTION

Currently, most cancers such as breast, colorectal, lung, ovarian and prostate cancer are increasing in the incidence. Chemotherapy is a conventional cancer treatment, which is based on the systemic administration of anticancer drugs that travel in the blood circulatory system throughout the body. However, this kind of treatment also has many side effects, such as anemia, nausea, diarrhea, weakening of the immune system, vomiting and hair loss (Spiridon *et al*., 2003). These side effects render it necessary to search more effective and less contra-indicative anticancer drugs. The herbal medicines have been extensively used by all cultures in human history (Dubick, 1986). All over the world sixty percent of the approved anti-cancer medicines (from 1983 to 1994) belong to natural origin (Cragg *et al.,* 1997). C*innamon*, a genus of about 200 species is widely distributed in tropical and sub tropical Himalayas from the Indus to Bhutan. In Pakistan leaves of *C. tamala* are extensively used as spices to improve food flavor. In addition, it also plays an important role in folk medicine (Cavender, 2006). The leaves of *C. tamala* are locally known as Tamala patra or Tez pat. Locally, leaves, which have a pleasant orange smell is used for silkworm cultivation (Kostermans *et al.,* 1978). The essential oil constituents of *Cinnamon* species have been extensively studied (Ahmad *et al.,* 2000; Jayaprakash *et al*., 2002; Jantan *et al.,* 2002; Jantan *et al.,* 2005) but no significant studies related to purification of anticancer compounds from leaves have been reported. Keeping in view traditional and medicinal importance of this plant, we carried out bioassay-guided purification of *C. tamala* leaves using human ovarian cancer cell lines (A-2780) which resulted in the purification of compounds **1-4**. Cytotoxicity of the purified compounds on normal baby hamster kidney cell lines (BHK-21) was also determined.

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MATERIALS AND METHODS

Materials

Alamar blue dye, RPMI, phosphate buffer saline, fetal bovine serum, amphotericin B, ampicillin, streptomycin, gentamycin, polyoxyethylene sorbitan monolaurate (Tween 20) were purchased from MP Biomedicals, PAA Laboratories GmbH, Fluka, Symans Pharmaceuticals and Sigma-Aldrich. All the solvents were purchased from Panreac (Spain). The samples for NMR spectrometry were prepared in $CDCl_3$ containing tetramethylsilane as an internal standard. Silica gel 60 was used for column chromatography, while TLC was performed on precoated silica cards (GF_{254}).

Equipment and apparatus

Mass spectrometry was carried out on a MAT 312 instrument. ^{1}H and ^{13}C NMR spectra were recorded at 400 and 75 MHz respectively using Bruker Avance spectrometer. UV- visible spectrophotometer (Cecil CE-7200), Cytofluor Series 4000 Fluorescence Multi-well plate reader (Per Ceptive Biosystems) were used for spectrophotometry. The other equipments used were orbital shaker-timer control (DIGITEC Instruments), rotary evaporator (Laborota 4000, Heidolph) and incubator.

Collection of plant material

The leaves of *Cinnamon tamala* were purchased from local market and identified at the Department of Botany G. C. University, Lahore, Pakistan, where a specimen voucher (GCU-Herb-Bot-864) was deposited.

Extraction and isolation

The plant material was air dried, pulverized and soaked in methanol for one week. The methanolic extract was dissolved in water and subjected to partition

chromatography successively with n-hexane, chloroform and n-butanol. Column chromatography (CC) of n-hexane extract resulted in compound 1 (system: n-hexane-$CHCl_3$, 1: 1), while compound 2 was purified through preparative tlc (solvent system: n-hexane-$CHCl_3$, 40: 60) of the successive CC fraction obtained in the same system. (136mg, 1.1% yield). The CC fraction of chloroform extract on preparative tlc in the system of MeOH-$CHCl_3$ (94:06) yielded compound 3 and 4 at Rf=0.35 and 0.75 respectively.

Anti-tumor potential

Cell culture

Cell lines A-2780 and BHK-21 (human ovarian carcinoma and baby hamster kidney cell lines respectively) were obtained from Virginia Poly Tech Institute, Virginia State University, USA and Quality Operation Lab (QOL), WTO, UVAS Lahore. Cells were maintained at 37ºC in a 5% CO_2 atmosphere. Both cell lines were grown in RPMI supplemented with 10% fetal bovine serum, vitamins, penicillin (100 IU/ml), gentamycin (10%), amphotericin B (100 units/ml) and essential amino acids

Cytotoxicity assay (alamar blue dye assay)

The human ovarian tumor (A-2780) as well as baby hamster kidney cells, propagated in raux flasks, incubated for 48 hours were seeded as a monolayer culture in 96 well flat bottom plates at the rate of $1.7x10^5$cells/ml in RPMI medium. After 24 hours pre-incubation period 200 ml of RPMI media was changed with fresh media containing final concentrations of samples. These plates containing samples were incubated at 37ºC under a 5% CO_2 atmosphere and after 44 hours incubation the growth medium was gently shaken out by inverting the 96-well micro titer plate. As a final step 1.0% alamar blue dye solution was added to each well and plates were again incubated for remaining 4 hours to complete the two days incubation. The cell viability in each well was recorded as a result of fluorescence using a cytofluor fluorescence plate reader (Clement, 2005).

Growth inhibition and IC_{50} calculation

The wells without cells were considered as dead cell control having 0% cell viability. The viability of untreated cell control was set to 100%. The degree of growth inhibition is expressed as % of the untreated cell control. The IC_{50} value for each sample was calculated from specific equation obtained for each sample as a result of graphical interpolation of percent inhibition below and above 50% from corresponding data of experimental results by applying linear regression analysis (Androtti *et al*., 1995). Degree of growth inhibition was calculated by the following formula (Ulukaya *et al.,* 2008)

Bornyl acetate (1)

EIMS *m/z* (rel. int., %): 196.0 (M^+, 7), 154 (10), 136 (21), 121(16), 108 (14), 95 (100), 80 (15), 69 (12), 55 (14), 43 (76). ^{1}H-NMR (400 MHz, $CDCl_3$): δ 0.76 (3H, s, H-1a), 0.81 (3H, s, H-7a), 0.85 (3H, s, H-7b), 1.18 (1Hm, H-4), 1.61 (2H, t, H-3), 1.69 (2H, t, H-6), 1.87 (2Hm, H-5), 2.00 (3H, s, H-9), 4.81 (1H, t, H-2). ^{13}C-NMR (100 MHz, $CDCl_3$): δ 13.4 (C-1a), 18.7 (C-7a), 19.6 (C-7b), 21.1 (C-9), 26.9 (C-5), 28.0 (C-6), 36.7 (C-3), 44.9 (C-4), 47.7 (C-7) 48.6(C-1), 79.7 (C-2), 171.2 (C-8).

Caryophylene oxide (2)

EIMS *m/z* (rel. int., %): 220 (M^+, 10), 205(3), 187(3), 177(7), 161(9), 149(10), 135 (13), 121 (27), 109 (34), 93 (71), 79 (93), 69 (48), 55 (46), 43 (100). ^{1}H-NMR (400 MHz, $CDCl_3$): δ 0.95 (3H, s, H-12a), 1.09 (3H, s, H-12b), 1.36 (1H, n, H-1), 1.52 (3H, s, 4a), 1.7 (2H, m, H-2), 1.87 (2H, m, H-6), 2.11 (2H, d, H-11), 2.34 (2H, t, H-7), 2.78 (1H, m, H-10), 3.31 (1H, t, H-5), 4.78 (1H d, *J*=4.95, H-9a), 4.97 (1H, d, *J*=4.95, H-9b). ^{13}C-NMR (100 MHz, $CDCl_3$): δ 17.3 (C-4a), 21.8 (C-12a), 27.0 (C-12b), 30.0 (C-2), 34.1 (C-6), 39.1 (C-7) 40.8 (C-12), 48.3 (C-3), 50.1 (C-5), 59.3 (C-10), 62.8 (C-1), 113.1 (C-9), 152.1 (C-8).

p-Coumaric acid (3)

EIMS *m/z* (rel. int., %): 164 (M^+, 100), 14 (16), 136(7), 119 (26), 107 (13), 91(23), 77 (8), 65 (24), 51(11), 45 (6).^{1}H-NMR (400 MHz, $CDCl_3$): δ 6.27 (1H, d, *J*=14.5, H-8), 6.75 (2H, d, *J*=8.5, H-3 and H-5), 6.79 (211, d, *J*=8.5, H-2 and H-8), 7.47 (1H, d, *J*=14.5, H-7).^{13}C-NMR (100 MHz, $CDCl_3$): δ 115.7 (C-8), 116.2 (C-3 and C-5), 125.7 (C-1), 130.5 (C-2 and C-6), 144.6 (C-7), 160.0 (C-4), 168.4 (C-9).

Vanillic acid (4)

EIMS *m/z* (rel. int., %): 168 (M^+, 100), 153 (55), 125 (26), 108 (6), 97 (31), 92 (2), 79 (9), 69 (3), 63 (7), 51 (12), 41(10).^{1}H-NMR (400 MHz, $CDCl_3$): δ 3.81 (3H, s, H-3a), 6.85 (1H, d, *J*=8.5, H-5), 7.40 (1H, s, H-2), 7.47 (1H, d, *J*=8.5, H-6).^{13}C-NMR (400 MHz, $CDCl_3$): δ 55.9 (C-3a), 113.1 (C-2), 115.4 (C-5), 122.0 (C-1), 124 (C-6), 148 (C-3), 151.5 (C-4), 167.6 (C-1a).

RESULTS

Bioassay guided fractionation/purification

The methanolic extracts of *Cinnamon tamala* exhibited significant anticancer activity against A-2780 human ovarian cancer cell lines. Partition chromatography of the crude extract successively with *n*-hexane, $CHCl_3$ and *n*-butanol followed by screening of the extracts in anti cancer assay showed promising results in n-hexane and $CHCl_3$ extracts. Column chromatography of the active extracts yielded compounds 1-2 from n-hexane extract while compounds 3-4 were isolated from $CHCl_3$ extract through CC followed by TLC.

Table 1: % inhibition and IC_{50} data of the isolated compounds from *C.tamala* leaves against human ovarian cancer (A-2780) and normal BHK-21 cells

Code	A-2780 Cells		BHK-21 Cells	
	% Inhibition [a]	IC_{50} (mg/ml) [b]	% Inhibition [a]	IC_{50} (mg/ml) [b]
	Isolates of *C.tamala* extracts			
1	90.16±1.06	$5.30x10^{-4}$	74.28±1.33	$6.50x10^{-3}$
2	84.40±1.53	$8.94x10^{-3}$	86.35±2.39	$7.19x10^{-3}$
3	32.17±0.17	$3.64x10^{-2}$	29.14±1.15	$4.10x10^{-2}$
4	12.27±0.11	$1.51x10^{-1}$	18.07±0.90	$9.50x10^{-2}$

[a]% Inhibition at 20µg /ml concentration of isolated compounds and reference anti tumor agents.
[b]Four half log dilutions i.e. 20, 4.0, 0.8 & 0.16µg/ml of each isolated compound and reference agent were tested to calculate IC_{50} value.
[c]Taxol & vinblastine were used as reference agents against human ovarian cancer cells (A-2780) & BHK-21 cell lines respectively.
*Not significantly different (ANOVA, $p<0.05$) to that reference anti-tumor agent (RA). All data is given as mean ± α-confidence level ($\alpha = 0.05$, n= 3).

Structure elucidation of the isolated compounds 1-4

bornyl acetate (1)

The IR spectrum showed CH stretching at 2966cm^{-1} and carbonyl group at 1737cm^{-1}. EIMS spectrum exhibited molecular ion peak at *m/z* 196 with other characteristic peaks at *m/z* 154, 136, 121, 95 (base peak) and 43. ^{1}HNMR ($CDCl_3$, 400 MHz) spectrum showed four singlets at δ0.76 (H-1a), 0.81 (H-7a), 0.85 (H-7b) and 2.00 (H-9), three triplets due to H-3 (δ1.61), H-6 (δ1.69) and H-2 (δ4.81) and two multiplets at δ 1.87 (H-5) and 1.18 (H-4). ^{13}C NMR ($CDCl_3$, 100 MHz) spectrum exhibited 12 signals comprised of four methyl, three methylene, two methine and three quaternary carbons.

Based upon the spectral values and comparison of the spectral data with the corresponding standard, compound 1 was identified as bornyl acetate (Baldovini *et al*., 2003).

Caryophylene oxide (2)

EIMS showed characteristic peaks at m/z 220 (M^+), followed by the fragment ions at 205, 187, 177, 121, 109, 93, 69, and 43. ^{1}H NMR ($CDCl_3$ 400MHz) spectrum showed one singlet at δ 1.52 (H-4a), three doublets at δ 2.78 (H-10), 4.78 (H-9a) and 4.97 (H-9b) and four multiplets at δ 1.36 (H-1), 1.7 (H-2), 1.87 (H-6) and 2.78 (H-10). ^{13}C NMR ($CDCl_3$, 100 MHz) spectrum displayed 15 signals due to three methyl, six methylene, three methine and three quaternary carbons. The signals, at δ 17.3, 21.8, 27.0, 30.0, 34.1, 39.1, 40.8, 48.3, 50.1, 59.3, 62.8, 113.1, 152.1 were assigned to C-4a, C-12a, C-12b, C-2, C-6, C-7, C-12, C-3, C-5, C-10, C-1, C-9 and C-8′ respectively (Ragasa CY *et al*., 2003).

p-Coumaric acid (3)

The spectral data showed the characteristic features of a simple phenol (Rappoport Z 2003). EIMS showed M^+ (molecular ion) peak at 164 *m/z* with other characteristic peaks at *m/z* 147, 136, 119, 107, 91, 65 and 45. ^{1}H-NMR ($CDCl_3$, 400MH) spectrum showed only four doublets i.e. two doublets due to aromatic protons and two doublets due to H-7 and H-8.^{13}C-NMR spectrum ($CDCl_3$, 100 MHz) displaying seven signals (four for the six methine carbons and three for the three quaternary carbons) indicated the presence of seven different kinds of carbons in the skeleton of the compound 3-(4-hydroxy phenyl)-2-propenoic acid also known with name of *p*-coumaric acid (An SM *et al.* 2008).

Vanillic acid (4)

EIMS spectrum showed M^+ (molecular ion peak) at 168 m/z with other characteristics peaks at m/z 153, 125, 97, 69, and 41. The spectral features were in general agreement with that of a simple phenol (Rappoport Z, 2003). Three aromatic protons at H-5 and H-6 showed singlet (δ 7.40), doublet (δ 6.85) and doublet (δ 7.47) respectively in ^{1}H-NMR ($CDCl_3$, 400 MHz) spectrum while methoxy protons (H-3a) appeared at 83.81 as singlet. Eight signals in ^{13}C-NMR spectrum ($CDCl_3$, 100 MHz) of the compound showed the presence of eight different types of carbon in its skeleton with one CH_3, three CH and four quaternary carbons. Spectral data confirmed the compound as 4-hydroxy-3-methoxy benzoic acid or vanillic acid (Termentzi A *et al*., 2009).

Anticancer activity

Bioassay-directed isolation of *C. tamala* leaves extract yielded four compounds 1-4. Bornyl acetate (1) exhibited 90.16±1.06% inhibition with lowest IC_{50} ($5.30x10^{-4}$ mg/ml), while caryophylene oxide (2) showed moderate results with IC_{50}=8.94 x 10^{-3} (84.40±1.53% inhibition). Compound 3 and 4 were found inactive against the cancer cell lines.

DISCUSSION

The anticancer activity of the extracts and purified compounds was determined on human ovarian cancer lines (A-2780) by the almar blue dye assay and results are shown in table 1. Cytotoxicity on normal cells were also determined using BHK-21 cell lines. A dose-

dependent decrease in the cell viability was observed when the cells were exposed to the growing concentration of the purified compounds. Concentration of the compounds for which each cell line growth was reduced to 50% (IC_{50}) were calculated from curve. As indicated in table 1, bornyl acetate (1) possessing a monoterpene skeleton, showed the most promising results among all other compounds. Anticancer activity of monoterpenes is well reported in the literature.

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REFERENCES

Ahmad A, Choudhary I and Farooq A (2000). Essential oil constituents of the spice *Cinnamomum tamala* (Ham.) Nees & Eberm. *Flavour Fragr J.,* **15**: 388-390.

Andreotti PE, Cree IA, Kurbacher CM, Hartmann DM, Linder D, Harel G, Gleiberman I, Caruso PA, Ricks SH, Untch M, Sartori C and Bruckner HW (1995). Chemosensitivity testing of human tumors using a microplate adenosine triphosphate luminescence assay: Clinical correlation for cisplatin resistance of ovarian carcinoma. *Cancer Res.,* **55**: 5276-5282.

An SM, Lee SI, Choi SW, Moon SW and Boo YC (2008). p-Coumaric acid, a constituent of Sasa quelpaertensis Nakai, inhibits cellular melanogenesis stimulated by alpha-melanocyte stimulating hormone. *British Journal of Dermatology*, **159**: 292-299.

Baldovini N, Tomi F and Casanova J (2003). Enantiomeric differentiation of bornyl acetate by ^{13}C-NMR using a chiral lanthanide shift reagent. *Phytochem Anal.,* **14**: 241-244.

Clement JA (2005) PhD. Dissertation, Virginia Polytechnic Institute & State University, USA.

Cragg GM, Newman DJ and Snader KM (1997). Natural products in drug discovery and development. *J Nat Prod.,* **60**: 52-60.

Cavender (2006). Folk medical uses of plant foods in southern Appalachia, United States. *J. Ethnopharmacol.,* **108**: 74-84.

Dubick MA (1986). Historical perspectives on the use of herbal preparations to promote health. *J Nutr.,* **116**: 1348-1354.

Jayaprakash GK, Rao LJ and Sakaria KK (2002). Chemical composition of volatile oil from *Cinnamomum zeylanicum* buds. *Naturforsch.,* **57**: 990-993.

Jantan I, Ayop N and Hiong AB (2002). Chemical composition of the essential oils of *Cinnamomum cordatum* Kosterm. *Flavour Fragr J.,* **17**: 212-214.

Jantan I, yalvema MF, Bukar BA, Muhammad K, Ayop N and Ahmad AS (2005). Constitution of the Leaf oil of *Cinnamomum pubescents* Kochummen. *J. Essent. Oil Res.,* **17**: 513-515.

Kostermans AJG, Nasir E and Ali SI (1978). Title. *Flora of West Pakistan,* **118**: 1-13.

Ragasa CY, Ganzon J, Hofileña J, Tamboong B and Rideout JA (2003). A new furanoid diterpene from *Caesalpinia pulcherrima*. *Chem. Pharm. Bull.,* **51**: 1208-1210.

Rappoport Z (2003). The chemistry of phenols. Part 2. John Wiley & Sons, Ltd. The Atrium, Southern Gate, Chichester, West Sussex, England.

Spiridon E Kintzios and Maria G Barberaki (2003). Plants that fight cancer. By Taylor & Francis 11 new fatter lane, London, EC4P 4EE P.05.

Termentzi A, Zervou M and Kokkalou E (2009). Isolation and structure elucidation of novel phenolic constituents from *Sorbus domestica* fruits. *Food Chemistry,* **116**: 371-381.

Ulukaya E, Ozdikicioglu F, Oral AY and Demirci M (2008). The MTT assay yields a relatively lower results of growth inhibition than the ATP assay depending on the chemotherapeutic drugs tested. *Toxicology in Vitro,* **22**: 232-239.