PJN
ISSN 1680-5194

# PAKISTAN JOURNAL OF NUTRITION

ANSInet
308 Lasani Town, Sargodha Road, Faisalabad - Pakistan
Mob: +92 300 3008585, Fax: +92 41 8815544
E-mail: editorpjn@gmail.com

Pakistan Journal of Nutrition 9 (4): 307-313, 2010
ISSN 1680-5194


# Antioxidant, Antimicrobial and Antitopoisomerase Screening of the Stem Bark Extracts of *Ardisia compressa*

Marco Vinicio Ramírez-Mares, Jorge Alberto Sánchez-Burgos and Beatriz Hernández-Carlos
Resources Institute, Universidad Del Mar, Oaxaca, México

**Abstract:** The *in vitro* antioxidant, antimicrobial and antitopoisomerase activities of crude Methanolic (Me) and Hexane (He) extracts of the stem bark of *Ardisia compressa* (AC) were investigated. Free radical-scavenging activity against 2,2-Diphenyl-1-picrylhydrazyl (DPPH) was evaluated, acid ascorbic was used as reference standard. Antimicrobial activity was determined using the agar diffusion method; the bacteria *K. pneumoniae*, *E. coli*, *S. epidermidis* and the fungus *C. albicans*. Controls employed were chloranphenicol (30 µg) and nystatin (100 units). Topoisomerase inhibition was determined by a clone-forming assay, which uses yeast (*S. cerevisiae*) strains as a model. Controls included dimethyl sulfoxide (1.66%); campothecin (50 µg/ml), etoposide (100 µg/ml) and sobuzoxane (150 µg/ml). Ascorbic acid was a substantially more powerful antioxidant than the extracts from the stem bark of AC. The Antiradical Efficiency (AE) of ascorbic acid was 249-fold that for He and 8.3-fold that for Me. Only the growth of *Klebsiella pneumoniae* was inhibited by Me (MIC = 1.875 mg/ml) and He (MIC = 0.9375 mg/ml), while the growth of the other strains were not inhibited. He extract not showed antitopoisomerase activity. Me extract showed antitopoisomerase activity (-15.3%). Open column chromatography of the methanolic extract was conducted and fourteen fractions were collected and tested. Fractions VI (-21.55%), X (-42.49%), XI (-97.68%), XII (-52.34%) and XIII (-35.97%) showed antitopoisomerase I activity and fraction XIV (-79%) antitopoisomerase II (poison) activity. These results suggest that Me extract of the stem bark of *A. compressa* could be promising in its potential usefulness for treatment of cancer and deserves further investigation.

**Key words:** *Ardisia compressa*, bioactivity, natural products, chemoprevention

## INTRODUCTION

The potential for using herbal tea components as anticancer botanicals and functional foods is promising. Tea is one of the most popular beverages consumed worldwide and several reports have attributed to tea chemopreventive and therapeutic properties (Mann *et al*., 2009; Adhami and Mukhtar, 2007; Siddiqui *et al*., 2007). In this regard, a newly investigated tea is ardisia extracted from the leaves of *Ardisia compressa* (AC), a plant of the Myrsinaceae family found in tropical and sub-tropical regions. There are approximately 500 species of Ardisia throughout the world (Kobayashi and De Mejia, 2005) and a few species such as *A. japonica* (Nikolovska-Coleska *et al*., 2004) and *A. compressa* (Ramirez-Mares *et al*., 1999) have been used for cancer treatment in indigenous medicine. While *A. japonica* is used in traditional Chinese medicine for treatment of pancreatic cancer (Nikolovska-Coleska *et al*., 2004), *A. compressa* is used in some Latin America regions, where its leaf-extracts are consumed in the form of herbal tea for the treatment of various liver conditions including liver cancer (Ramirez-Mares *et al*., 1999). However, data from well-controlled clinical studies are lacking and the medicinal claims are only substantiated by testimonial reports. Nevertheless, various alkylphenols have been isolated from ardisia species and some of them have anticancer properties (Chitra *et al*., 1994; Kang *et al*., 2001; Sumino *et al*., 2002). For example ardisin, an alkylphenol found in AC, has been reported to possess antioxidant and anti tumor activities in experimental animals (Gonzalez De Mejia *et al*., 2002). The antioxidant properties of ardisin were discovered when preincubation of hepatocytes with ardisin, in comparison to Epigallocatechin Gallate (EGCG), resulted in improvement in glutathione content combined with significant reductions in glutathione peroxidase activity and malondialdehyde formation (Ramirez-Mares and Gonzalez De Mejia, 2003). Additionally, ardisin also showed potent catalytic inhibition of topoisomerases I and II (Ramirez-Mares *et al*., 2004) and the administration of AC tea completely prevented tumor formation in Wistar rats exposed to diethylnitrosamine and acetylaminofluorene (Gonzalez De Mejia *et al*., 2004). Moreover, AC tea was cytotoxic to HT-29 and Caco-2 cells (Gonzalez De Mejia *et al*., 2006). However, there is no data that supports the claims made about the benefits for health of the *A. compressa* stem bark.

Several assays have been developed to evaluate the ability of a compound to modulate biochemical events

**Corresponding Author:** Marco Vinicio Ramírez-Mares, Instituto de Recursos, Universidad del Mar, Ciudad Universitaria, Puerto Ángel, C.P. 70902, Oaxaca, México

presumed to be mechanistically linked to carcinogenesis (Shureiqi *et al*., 2000). Examples of such assays include: (a) topoisomerase inhibitors, which constitute a class of agents that inhibit carcinogenesis via their antiproliferative or cell-differentiating action and are considered an attractive targeting strategy in both chemotherapy and chemoprevention (Cho *et al*., 2000); (b) antimicrobial activity, allows the identification of novel agents capable of interfere with a specific molecular target, that may avoid the shortcomings of conventional chemotherapy because certain antimicrobials exhibit selective cytotoxicity against a broad spectrum of human cancer cells (Schweizer 2009); (c) antioxidant activity, potent scavengers of Reactive Oxygen Species (ROS) may serve as a possible preventive intervention for free radical-mediated diseases such as cancer (Ralph *et al*., 2010). The aim of this work was to investigate the *in vitro* antioxidant, antitopoisomerase and antimicrobial activities of crude methanolic and hexanic extracts of the stem bark of *A. compressa*, as part of the exploration for new and novel bioactive compounds.

## MATERIALS AND METHODS

**Biological material:** *Saccharomyces cerevisiae* mutant cells JN362a, JN394, JN394 $t_1$, JN394$t_{2-4}$ and JN394$t_{2-5}$; were kindly provided by Dr. John Nitiss of St. Jude Children's Research Hospital, Memphis, Tennessee. *Ardisia compressa* stem bark was collected from the Pacific Coast of Mexico (State of Michoacan). Standard microorganisms were purchased from American Type Culture Collection (ATCC, Manassas, VA): Gram negative bacteria; *Klebsiella pneumoniae (*ATCC 13883), *Escherichia coli (*ATCC 35218), Gram positive bacteria; *Staphylococcus epidermidis (*ATCC 12228) and the fungus *Candida albicans* (ATCC 14053).

**Chemicals:** Peptone bacto, yeast extract, agar bacto, Mueller Hinton (MH) agar, MH broth, trypticase soy agar, trypticase soy broth, sabouraud dextrose agar, sabouraud dextrose broth and dextrose were purchase from Difco (Sparks, MD). Methanol (HPLC grade), methanol (spectrophotometric grade), Hexane (HPLC grade), Camptothecin (CPT), Etoposide (ETP), Sobuzoxane (SBZ), dimethyl sulfoxide (DMSO-Hybri-Max), adenine hemisulfate salt, chloranphenicol, nystatin, ascorbic acid and 2,2-Diphenyl-1-picrylhydrazyl (DPPH) were obtained from Sigma Chemical (St. Louis, MO). $C_{18}$ (Li Chroprep RP-18, 40-63 μm) was from Merck (Darmstadt, Germany).

**Collection of plant material:** Fresh stem bark of *A. compressa* collected on the Pacific coast of Mexico (Michoacan State) was first air-dried without exposure to sunlight; the dry material was chopped into pieces and kept in large plastic bags and stored in a cool and dry place.

**Preparation of extracts:** 475 g of powdered bark of *A. compressa* were soaked in 2000 ml of methanol and hexane for two weeks respectively, at room temperature (25°C). The extracts were filtered and the filtrates were concentrated to dryness using a rotary evaporator at 30°C at reduced pressure, to form Methanolic (Me) and Hexanic (He) extract respectively. The dried and powdered crude extracts (Me = 6.65 g and He = 3.2 g) were kept at -20°C and protected from light and moisture in a glass container sealed with parafilm. The extracts were dissolved in DMSO prior to use in all assays.

**Fractionation of the methanol extract:** Open Column Chromatography (OPC) using a column (300 mm x 20 mm I.D.) packed with $C_{18}$ (reversed-phase) were conducted to isolate the fractions responsible for antitopoisomerase activity of the methanolic extract of the stem bark of AC. A combination of MeOH and $H_2O$, were used to perform the separation of 1 g of Me. The analysis was started with 200 ml of MeOH/$H_2O$: 50%/50%, then 200 ml of MeOH/$H_2O$: 70%/30%, after 200 ml of MeOH/$H_2O$: 90%/10% and finally 200 ml of pure MeOH. Fourteen fractions were collected and passed through a 0.22 μm filter and evaporated until dried using a rotary evaporator and a freeze dryer. The dried solid fractions were protected from light and kept at -20°C. The inhibition of topoisomerase activity was then measured in the fractions using the antitopoisomerase yeast assay.

**Antioxidant activity of the extracts:** The antioxidant activity was evaluated in terms of radical-scavenging ability of Me and He extracts using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical assay (Brand-Williams *et al*., 1995). A methanolic (spectrophotometric grade) solution (50 μl) of the herbal extract (Me or He) at five different concentrations was added to 1.95 ml of DPPH● solution ($7.6 \times 10^{-5}$ M in methanol). The decrease in the absorbance at 515 nm was determined using a uv/vis spectrophotometer (Beckman DU-530) until the reaction reached the steady state in the dark (Siddhuraju and Becker, 2003).

The DPPH● concentration in the reaction medium was calculated from the following calibration curve, determined by lineal regression:

$$A_{515nm} = 0.009\ [DPPH\bullet]_T - 0.007$$

Where $[DPPH\bullet]_T$ was expressed as μM, $r^2 = 0.998$

The percentage of remaining DPPH● (% $DPPH\bullet_{REM}$) was calculated as follows:

$$\%\ DPPH\bullet_{REM} = [DPPH\bullet]_T / [DPPH\bullet]_{T=0}$$

Where $[DPPH\bullet]_T$ was the concentration of DPPH● at the time of steady state and $[DPPH\bullet]_{T=0}$ was the

concentration of DPPH● at zero time. The % $DPPH●_{REM}$ against the standard concentration was plotted to obtain the amount of antioxidant necessary to decrease by 50% the initial DPPH● concentration ($EC_{50}$). The time needed to reach the steady state to $EC_{50}$ concentration ($T_{EC50}$) was determined. Antiradical Efficiency (AE) was also calculated ($AE = 1/EC_{50}\ T_{EC50}$). Ascorbic acid was used as a reference standard. All experimental were carried out in triplicate. The activity of each extract was expressed as percentage of that achieved for the reference standard.

**Antibacterial and antifungal screening:** Antibacterial and antifungal assays were carried out of the Me and He extracts against *Klebsiella pneumoniae (*ATCC 13883), *Escherichia coli (*ATCC 35218), *Staphylococcus epidermidis (*ATCC 12228) and the fungus *Candida albicans* (ATCC 14053). The antibacterial and antifungal activities were determined using the agar diffusion method. The bacterial strains were placed in plates of trypticase soy agar and the fungus in plates of sabouraud dextrose agar. After 24 h incubation at 37°C (bacteria) and 30°C (fungus), four or five colonies were inoculated in 4 ml of Mueller-Hinton broth or sabouraud dextrose broth and incubated for 2 h at 37°C and 35°C, respectively. These inocula were adjusted to the 0.5 MacFarland standard (0.048 M $BaCl_2$ 0.5 ml + 0.18 M $H_2SO_4$ 99.5 ml).

For susceptibility testing, each 150 µl of adjusted bacterial or fungal suspension was spread on the sterile medium (trypticase soy agar or sabouraud dextrose agar) using sterile cotton swabs. The positive controls employed were chloranphenicol (30 µg) in the antibacterial and nystatin (100 units) in the antifungal assays. Application of the samples and controls (25 µl) was done directly in the solid medium. The application point was marked on the lower surface of the Petri dish. The preparations were left to diffuse. Subsequently the plates were incubated at 37°C for 24 h in the case of the bacteria; while the fungus was cultured at 30°C for 48 h. Plates were prepared using the same procedures without extract or antibiotic, but with DMSO (25 µl) were equally set as negative control. After incubation, the growth inhibition rings were quantified by measuring the diameter for the zone of inhibition in millimeters from the lower surface of the plates. All assays were carried out in triplicate.

**Determination of minimal inhibitory concentration (MIC):** The method of Greenwood (1989) was used to determine the MIC of the extracts. 50 µl of extract (Me or He) was mixed with 950 µl of sterile water to get an X concentration. Eight sterile test tubes were arranged in a test tube rack and 1 ml of sterile water was pipetted into each test tube. Thereafter, there was a two fold serial dilution of the extract to obtain 1 ml of each one of the following concentrations: X/2, X/4, X/8, X/16, X/32, X/64, X/128 and X/256. The test organism (1 ml = 5 x $10^5$ CFU/ml) was pipetted into each of the test tubes containing the extract, the final concentrations were: X/4, X/8, X/16, X/32, X/64, X/128, X/256 and X/512. Finally the tubes were incubated at 37°C for 24 h in the case of the bacteria, while the fungus was cultured at 30°C for 48 h. The MIC was recorded as the least concentration of extract that completely inhibited the growth of the test organism.

**Yeast antitopoisomerase assay:** The antitopoisomerase activity was assessed using mutants *Saccharomyces cerevisiae* JN362a, JN394, JN394 $t_1$, JN394$t_{2-4}$ and JN394$t_{2-5}$ strains (Nitiss and Nitiss, 2001). Briefly, yeast cells were grown in YPDA media at 30°C (25°C for JN394$t_{2-4}$ and JN394$t_{2-5}$) for 18 h in a shaking incubator. The logarithmically growing cells were then counted using a hematocytometer and adjusted to a concentration of 2 x $10^6$ cells/ml media. Yeast cells (6 x $10^6$ cells) were incubated at 30 or 25°C for 24 h in the shaking incubator, in the presence of the Me or He that were dissolved in 50 µl DMSO. DMSO (1.66%) was used as negative control, while CPT (50 µg/ml) a topoisomerase I inhibitor, ETP (100 µg/ml) a topoisomerase II poison and SBZ (150 µg/ml) a topoisomerase II inhibitor were the positive controls. Treated cells from each mixture were then duplicate plated to petri dishes containing 1.75% Agar Bacto solidified YPDA as media. Cells were incubated at growth temperature of 30°C or 25°C for 48 h. The antitopoisomerase activity was then measured by comparing the number of counted colonies in each plate to that of the negative control plate (DMSO).

## RESULTS AND DISCUSSION

**Evaluation of the antioxidant activity:** The antioxidant activity of plant extracts which containing various kinds of compounds are due to their abilities to be donors of hydrogen atoms or electrons and to capture free radicals (Wu *et al*., 2010). DPPH, a stable free radical with purple color, changes into a stable yellow compound on reacting with an antioxidant. The extent of the reaction depends on the hydrogen-donating ability of the antioxidant. The concentration of the antioxidant needed to decrease the initial DPPH concentration by 50% ($EC_{50}$) is a parameter widely used to measure antioxidant activity. Another parameter was defined as antiradical efficiency ($AE = 1/EC_{50}\ T_{EC50}$) where $T_{EC50}$ is the time needed to reach the steady state to $EC_{50}$ concentration. The lower the $EC_{50}$ or $T_{EC50}$, the higher is the antioxidant activity (Brand-Williams *et al*., 1995). The Table 1 shows the free radical-scavenging activity of the Me and He extracts from the stem bark of AC. The antioxidant activity of the extracts was expressed as percentage of efficiency by comparing AE of the

Table 1: Estimation of free radical-scavenging activity of the stem bark of *Ardisia compressa* extracts

| Extracts and control | $EC_{50}$ g/kg $_{DPPH}$ | $T_{EC50}$ min | AE x $10^{-3}$ | % of efficiency |
|---|---|---|---|---|
| Me | 473±10 | 2.83±0.2 | 0.747 | 12.1 |
| He | 2882±15 | 14.00±0.3 | 0.0248 | 0.402 |
| Ascorbic acid | 103±2 | 1.57±0.1 | 6.183 | 100 |

Results are means±SD (n = 3)

extracts with AE of ascorbic acid. From the results it was appeared that the methanol extract has the strongest DPPH radical-scavenging activity ($EC_{50}$ = 473. g/$Kg_{DPPH}$) whereas the hexane extract gave the lowest ($EC_{50}$ = 2882 g/$Kg_{DPPH}$). Table 1 also shows that the lower the $EC_{50}$, the shorter was the reaction time ($T_{AC50}$) and the higher the Antiradical Efficiency (AE). Inspection of Table 1 showed that ascorbic acid was a substantially more powerful antioxidant than the extracts from the stem bark of AC. The AE of ascorbic acid was 249-fold that for He and 8.3-fold that for Me. The classification order of AE for the tested antioxidants was: ascorbic acid > Me > He. Due to the diversity and complexity of the natural mixtures of antioxidants compounds in the different plant extracts, it is rather difficult to characterize every compound and assess or compare their antioxidant activities. Antioxidant activity of the extracts may also be attributable to synergistic interactions. Because of these the 12.1% of efficiency of Me compared to ascorbic acid (100%) do not deserve to fractionate the extract to look for the responsible compounds of the antioxidant effect.

**Antimicrobial activity and MIC:** In order to improve the screening of natural products with antimicrobial activity, we applied the extracts directly to the agar medium, because when the paper (cellulose) disc is used, the free hydroxyl groups present on each glucose residues becomes the surface of the disc hydrophilic (Burgess *et al*., 1999). Consequently the polar natural products would be expected to adsorb to the surface of the disc and not diffuse into the agar medium.

The results of antibacterial activity of the extracts against *Klebsiella pneumoniae, Escherichia coli* and *Staphylococcus epidermidis* showed that the methanolic and hexanic extracts only inhibited the growth of *Klebsiella pneumoniae* while the growth of the other strains were not inhibited (Table 2). The hexane extract (inhibition zone 13 mm) was found to be more effective than the methanol extract (12 mm) against *Klebsiella pneumoniae* at the same concentration tested (10 mg/ml). Both extracts showed low antifungal activity with inhibition zones ranging between 1 and 3 mm for *Candida albicans*. Contrary to the results of Nostro *et al.* (2000), the antibacterial activity of the Me and He extracts was more pronounced against Gram-negative bacteria than against Gram-positive bacteria. Gram-negative is expected to be impermeable to lipophilic solutes due to have an outer phospholipidic membrane carrying the structural lipopolysaccharide components. Gram-positive bacteria have an outer peptidoglycane layer that is not an effective permeability barrier, thus these bacteria are more susceptible to antibacterial compounds (Nikaido and Vaara, 1985). The antibacterial activity not only depends of the kind of microorganism and its morphology, but also of other factors such as: (a) diffusion capacity of substances present in the extracts (number, size, polarity and shape of particles) in the agar medium, (b) antimicrobial activity of diffused compounds, (c) density of inoculation, (d) growth and metabolic activity of bacteria in the medium and (e) pH of substrates in plates. This suggests that the antibacterial activity that can be seen in the agar medium, not necessarily is due to the morphological differences between bacteria. These could be the reasons why our results were different than those from Nostro *et al.* (2000). Moreover, in support of our explanation, polar (Me extract) and no-polar (He extract) compounds were effective against *Klebsiella pneumoniae*.

The MIC illustrates (Table 3) a decreasing inhibitory effect of the bark extracts and chloranphenicol as the concentration decreases. This suggests that antimicrobial activity of a compound is a concentration dependent and that the antimicrobial activity is a function of the bioactive ingredient reaching a microorganism. The minimum inhibitory concentration of Me and He against *Klebsiella pneumoniae* were 1.875 and 0.9375 mg/ml respectively (Table 3). These concentrations are remarkable for a crude extract and indicate that when further isolation and purification of the extracts is carried out, increased activity would be obtained or a possible synergism of antimicrobial activity among the components of the extract would be demonstrated. It is important to point out that there is a clear dependence of inhibition zone sizes on antibiotic concentration and the MIC value determined by the broth dilution assay. The hexane extract resulted more effective (inhibition zone 13 mm, MIC = 0.9375 mg/ml) than methanol extract ((inhibition zone 12 mm, MIC = 1.875 mg/ml. The agar diffusion method do not expose the test bacteria to the full volume of extract solution and are less sensitive to the size of the inoculum and in the broth dilution assay, bacterial susceptibility depends on the ratio of extract solution concentration to inoculum size. Nevertheless, there was a good agreement between the results of both methods.

Table 2: Zone of inhibition (mm) produced by extracts of the stem bark of *Ardisia compressa* and standard antimicrobial agents

| Organism | Gram | Me (10 mg/ml) | He (10 mg/ml) | Chloranphenicol (1.2 mg/ml) | Nystatin (30 units) |
|---|---|---|---|---|---|
| *Escherichia coli* | (-) | 0 | 0 | 12±0 | --- |
| *Staphylococcus epidermidis* | (+) | 0 | 0 | 24.8±0.8 | --- |
| *Klebsiella pneumoniae* | (-) | 12±0 | 13±0 | 20±0.5 | --- |
| *Candida albicans* | --- | 2.5±0.5 | 1.5±0.5 | --- | 25±0.7 |

Results are means±SD (n = 3)

Table 3: Minimal inhibitory concentration of the extracts of the stem bark of *Ardisia compressa* and chloranphenicol against *Klebsiella pneumoniae*

| | mg/ml | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Extracts | 3.75 | 1.875 | 0.9375 | 0.46875 | 0.23437 | 0.11718 | 0.05859 | 0.02929 |
| Me | - | - | + | + | + | + | + | + |
| He | - | - | - | + | + | + | + | + |
| | µg/ml | | | | | | | |
| Standard | 36 | 18 | 9 | 4.5 | 2.25 | 1.125 | 0.5625 | 0.28125 |
| Chloranphenicol | - | - | + | + | + | + | + | + |

-: Susceptible; +: Resistance

Table 4: Percent[a] of inhibition of Me and He extracts treatments on the survival of JN394 and JN362a strains

| Extract or control | Concentration (µg/ml) | JN394 | JN362a |
|---|---|---|---|
| Me | 21,500 | -15.3±4.5 | +17.5±6.2 |
| He | 21,000 | - 33.0±5.7 | -16.9±4.3 |
| CPT (TOP 1 poison) | 50 | - 99.0±0.4 | 0 |

DMSO (1.66%) was used as a control and all the results were referred to this value. [a]Values are means±SD of triplicate determinations

Table 5: Percent[a] of inhibition of methanol extract fractions on the survival of JN394 and JN362a strains

| Fraction or control | Concentration (µg/ml) | JN394 | JN362a |
|---|---|---|---|
| I | 190 | -39.10±2.4 | -49.79±3.9 |
| II | 480 | +19.00±5.3 | ND |
| III | 10 | -86.85±4.1 | -52.09±7.2 |
| IV | 60 | -42.05±5.2 | -72.31±5.4 |
| V | 1160 | -88.12±3.4 | -89.10±4.4 |
| VI | 680 | -21.55±6.3 | +180.61±8.9 |
| VII | 1120 | +12.75±3.3 | ND |
| VIII | 45 | +36.24±4.7 | ND |
| IX | 22 | +22.15±5.2 | ND |
| X | 16 | -42.49±3.3 | +104.59±5.8 |
| XI | 30 | -97.68±2.3 | -97.20±2.1 |
| XII | 480 | -52.34±4.4 | +66.84±4.6 |
| XIII | 300 | -35.97±3.9 | +87.76±5.0 |
| XIV | 230 | -12.23±6.5 | +80.58±4.5 |
| CPT (TOP 1 poison) | 50 | -99.0±0.4 | 0 |

DMSO (1.66%) was used as a control and all the results were referred to this value. [a]Values are means±SD of triplicate determinations. ND: Not Determined

**Antitopoisomerase activity:** The concentration of the extracts used in this assay was based on the solubility factor of each solid extract in DMSO. As shown in Table 4, the strain JN394 was hypersensitive to CPT (99.0%), which is a Topo I poison. Me and He showed 15.3 and 33% inhibition, respectively. The strain JN394 is DNA repair-deficient and drug-permeable (carry *ise2* and *rad52* mutations) (Nitiss and Wang, 1988). These mutations increase the sensitivity of these cells to drugs. The yeast JN362a, a DNA repair-proficient strain (Nitiss and Wang, 1988), was no affected by Me (+17.5%) or CPT (0%), but was inhibited by He (-16.9%). These results mean that the methanol extract has compounds with antitopoisomerase activity. In order to find the fraction or fractions responsible for the antitopoisomerase activity, a guide-fractionation was conducted using an open column packed with $C_{18}$ and a combination of MeOH and $H_2O$. Fourteen fractions were collected and tested in the strains JN394 and JN362a (Table 5). Fractions I (-39.10%, 190 µg/ml), III (-86.85%, 10 µg/ml), IV (-42.05%, 60 µg/ml), V (-88.12%, 1160 µg/ml), VI (-21.55%, 680 µg/ml), X(-42.49%, 16 µg/ml), XI (-97.68%, 30 µg/ml), XII (-52.34%, 480 µg/ml), XIII (-35.97%, 300 µg/ml) and XIV (-12.23%, 230 µg/ml), showed inhibition against the JN394 strain. The rest of the fractions (II, VII, VIII and IX) not inhibited the growth of this strain and for this reason they were not tested against the yeast strain JN362a. The yeast JN362a was affected by fractions I (-49.79%), III (-52.09%), IV (-72.31%), V (-89.10%) and XI (-97.20). The fractions VI, X, XII, XIII and XIV no inhibited the growth of JN362a, these are the fractions with antitopoisomerase activity.

The strain JN394$t_{.1}$ is isogenic to JN394 and contains a disrupted top1 gene (Nitiss and Wang, 1988); the absence of the gene resulted in diminished cytotoxicity of antitopoisomerase I drugs. Table 6 shows that CPT failed to reduce the growth of these mutant cells and also the fractions VI, X, XII and XIII were incapable of affect the growth of the cells with the top1 mutation. This means that Topo I is the target of these fractions. In

Table 6: Percent[a] of inhibition of methanol extract fractions with antitopoisomerase activity on the survival of JN394$t_{-1}$, JN394$t_{2-4}$ and JN394$t_{2-5}$ strains

| Fraction or control | Concentration (µg/ml) | JN394$t_{1}$ | JN394$t_{2-5}$ 25°C | JN394$t_{2-4}$ 25°C | JN394$t_{2-4}$ 30°C |
|---|---|---|---|---|---|
| VI | 680 | 0 | ND | ND | ND |
| X | 16 | 0 | ND | ND | ND |
| XII | 480 | 0 | ND | ND | ND |
| XIII | 300 | 0 | ND | ND | ND |
| XIV | 230 | -99.0±0.3 | +145±10.2 | -79±5.2 | -15±3.6 |
| CPT (TOP 1 poison) | 50 | 0 | -99±0.4 | -80±4.6 | -99±0.3 |
| ETP (TOP 2 poison) | 100 | -99±0.4 | +71±6.3 | -99±0.4 | -80±3.9 |
| SBZ (TOP 2 catalytic) | 150 | -99±0.2 | -17±4.4 | +213±7.8 | -99±0.4 |

DMSO (1.66%) was used as a control and all the results were referred to this value.
[a]Values are means±SD of triplicate determinations. ND: Not Determined

contrast, no resistance was observed when top1 cells were treated with fraction XIV (-99%) or the antitopoisomerase II drugs ETP and SBZ (-99%). Sensitivity in the top1 cells was higher than in the parent cells (JN394). This finding provided evidence that Topo I was not the cellular target of fraction XIV.

The strain JN394$t_{2-5}$ carries a top2 allele that is resistant to multiple classes of topoisomerase II poisons at its permissive temperature (25°C) (Jannatipour *et al*., 1993). Table 6 shows that cells with the top2-5 mutation are able to growth in ETP (+71%) or in fraction XIV (+145%). The sensitivity of the top2-5 strain to CPT (-99%) and SBZ (-17%) is also shown in Table 6. The strain has essentially the same sensitivity to CPT as JN394 (*rad52 top2*$^{+}$ cells), indicating that the observed resistance is specific to antitopoisomerase II agents.

Eliminating the possibility that Topo I is the target of fraction XIV, two additional possibilities exist for the physiological mechanism of cytotoxicity. The first is that Topo II is the primary target responsible for killing cells by trapping the enzyme-mediated DNA cleavage. The second is that the cytotoxicity is correlated with the ability to block the overall catalytic activity of the enzyme. The possibilities described above can be scrutinized by utilizing a yeast strain JN394$t_{2-4}$. This yeast strain expresses the temperature-sensitive top2-4 mutant in place of the wild type top2 gene (Nitiss and Wang, 1988). The top2-4 protein shows wild type activity at 25°C, while its activity is reduced to about 5-10% of the wild type at the semi-permissive temperature of 30°C. Therefore, if the fraction XIV functions as a Topo II "poison", a reduction in enzyme activity should greatly diminish the induced cell death. Conversely, if the cytotoxicity is correlated with the ability to impair the catalytic function of the enzyme, cells with decreased levels of Topo II activity should become hypersensitive. ETP was a potent toxic agent toward JN394$t_{2-4}$ yeast cells at 25°C (Table 6). A smaller value was obtained when the cytotoxicity of ETP was examined at 30°C. The sensitivity to CPT at 30°C (99%) was greater than at 25°C (80%) (Table 6). The fraction XIV displayed weak cytotoxicity (-15 %) toward JN394$t_{2-4}$ yeast at 30°C. Top 2-4 cells were hypersensitive (-79%) at the permissive temperature (25°C). The increased toxicity toward cells that contain increased levels of Topo II activity strongly suggests that trapping the enzyme-mediated DNA cleavage is the primary physiological target of fraction XIV.

In summary, the results of the present study indicate that compounds extracted in the methanolic fraction of the stem bark of *Ardisia compressa* possess potential chemopreventive activity, but further work is required in order to isolate the phytochemicals responsible for the antibacterial and antitopoisomerase activities.

## ACKNOWLEDGEMENTS

This study was supported by the National Council for Science and Technology (CONACYT) México, grant 52065.

## REFERENCES

Adhami, V.M. and H. Mukhtar, 2007. Anti-oxidants from green tea and pomegranate for chemoprevention of prostate cancer. Mol. Biotechnol., 37: 52-57.

Brand-Williams, W., M.E. Cuvelier and C. Berset, 1995. Use of a free radical method to evaluate antioxidant activity. Lebensm. Wiss. Technol., 28: 25-30.

Burgess, J.G., E.M. Jordan, M. Bregu, A. Mearns-Spragg and K.G. Boyd, 1999. Microbial antagonism: A neglected avenue of natural products research. J. Biotechnol., 70: 27-32.

Chitra, M., E. Sukumar, V. Suja and C.S. Devi, 1994. Antitumor, antiinflammatory and analgesic property of Embelin, a plant product. Chemotherapy, 40: 109-113.

Cho, K.H., J.M. Pezzuto, J.L. Bolton, V.E. Steele, G.J. Kelloff, S.K. Lee and A. Constantinou, 2000. Selection of cancer chemopreventive agents based on inhibition of topoisomerase II activity. Eur. J. Cancer, 36: 2146-2156.

Gonzalez De Mejia, E., M.V. Ramirez-Mares and M.G. Nair, 2002. Topoisomerase I and II enzyme inhibitory aqueous extract of *Ardisia compressa* and ardisin protect against benomyl oxidation of hepatocytes. J. Agric. Food Chem., 50: 7714-7719.

Gonzalez De Mejia, E., M.V. Ramirez-Mares, E. Arce-Popoca, M. Wallig and S. Villa-Trevino, 2004. Inhibition of liver carcinogenesis in Wistar rats by consumption of an aqueous extract from leaves of *Ardisia compressa*. Food Chem. Toxicol., 42: 509-516.

Gonzalez De Mejia, E., S. Chandra, M.V. Ramirez-Mares and W. Wang, 2006. Catalytic inhibition of human DNA topoisomerase by phenolic compounds in *Ardisia compressa* extracts and their effect on human colon cancer cells. Food Chem. Toxicol., 44: 1191-1203.

Greenwood, D., 1989. Antibiotic Sensitivity Testing. In: Antimicrobial Chemotherapy (Greenwood, D., Ed.), Oxford University press, New York, pp: 91-100.

Jannatipour, M., Y.-X. Liu and J.L. Nitiss, 1993. The *top 2-5* mutant of yeast topoisomerase II encodes an enzyme resistant to etoposide and amsacrine. J. Biol. Chem., 268: 18586-18592.

Kang, Y.H., W.H. Kim, M.K. Park and B.H. Han, 2001. Antimetastatic and antitumor effects of benzoquinonoid AC7-1 from *Ardisia crispa*. Int. J. Cancer, 93: 736-740.

Kobayashi, H. and E. De Mejia, 2005. The genus Ardisia: A novel source of health-promoting compounds and phytopharmaceuticals. J. Ethnopharmacol., 96: 347-354.

Mann, C.D., C.P. Neal, G. Garcea, M.M. Manson, A.R. Dennison and D.P. Berry, 2009. Phytochemicals as potential chemopreventive and chemotherapeutic agents in hepatocarcinogenesis. Eur. J. Cancer Prev., 18: 13-25.

Nikaido, H. and M. Vaara, 1985. Molecular basis of bacterial outer membrane permeability. Microbiol. Rev., 1: 1-32.

Nikolovska-Coleska, Z., L. Xu, Z. Hu, Y. Tomita, P. Li, P. P. Roller, W. Renxiao, X. Fang, R. Guo, M. Zhang, M. E. Lippman, D. Yang and S. Wang, 2004. Discovery of embelin as a cell-permeable, small molecular weight inhibitor of XIAP through structure-based computational screening of traditional herbal medicine three-dimensional structure database. J. Med. Chem., 47: 2430-2440.

Nitiss, J.L. and J.C. Wang, 1988. DNA topoisomerase-targeting antitumor drugs can be studied in yeast. Proc. Natl. Acad. Sci. USA, 85: 7501-7505.

Nitiss, J.L. and K.C. Nitiss, 2001. Yeast systems for demonstrating the targets of antitopoisomerase II agents. Methods Mol. Biol., 95: 315-327.

Nostro, A., M.P. Germano, V. D´Angelo, A. Marino and M. A. Cannatelli, 2000. Extraction methods and bioautography for evaluation of medicinal plant antimicrobial activity. Lett. Appl. Microbiol., 30: 379-384.

Ralph, S.J., S. Rodríguez-Enríquez, J. Neuzil, E. Saavedra and R. Moreno-Sánchez, 2010. The causes of cancer revisited: Mitochondrial malignancy and ROS-induced oncogenic transformation - Why mitochondria are targets for cancer therapy. Mol. Aspects Med., (in press).

Ramirez-Mares, M.V., S. Fatell, S. Villa-Trevino and E.G. De Mejia, 1999. Protection of extract from leaves of *Ardisia compressa* against benomyl-induced cytotoxicity and genotoxicity in cultured rat hepatocytes. Toxicol. *In Vitro*, 13: 889-896.

Ramirez-Mares, M.V. and E.G. De Mejia, 2003. Comparative study of the antioxidant effect of ardisin and epigallocatechin gallate in rat hepatocytes exposed to benomyl and 1-nitropyrene. Food Chem. Toxicol., 41: 1527-1535.

Ramirez-Mares, M.V., S. Chandra and E.G. De Mejia, 2004. *In vitro* chemopreventive activity of Camellia sinensis, Ilex paraguariensis and *Ardisia compressa* tea extracts and selected polyphenols. Mutat. Res., 554: 53-65.

Schweizer, F., 2009. Cationic amphiphilic peptides with cancer-selective toxicity. Eur. J. Pharmacol., 625: 1-3.

Shureiqi, I., P. Reddy and D.E. Brenner, 2000. Chemoprevention: General perspective. Crit. Rev. Oncol. Hematol., 33: 157-167.

Siddhuraju, P. and K. Becker, 2003. Antioxidant properties of various solvent extracts of total phenolic constituents from three different agroclimatic origins of drumstick tree (*Moringa oleifera* Lam.) leaves. J. Agric. Food Chem., 51: 2144-2155.

Siddiqui, I.A., M. Saleem, V.M. Adhami, M. Asim and H. Mukhtar, 2007. Tea beverage in chemoprevention and chemotherapy of prostate cancer. Acta Pharmacol. Sin., 28: 1392-1408.

Sumino, M., T. Sekine, N. Ruangrungsi, K. Igarashi and F. Ikegami, 2002. Ardisiphenols and other antioxidant principles from the fruits of *Ardisia colorata*. Chem. Pharm. Bull., 50: 1484-1487.

Wu, N., Y. Zu, Y. Fu, Y. Kong, J. Zhao, X. Li, J. Li, M. Wink and T. Efferth, 2010. Antioxidant activities and xanthine oxidase inhibitory effects of extracts and main polyphenolic compounds obtained from *Geranium sibiricum* L. J. Agric. Food Chem. (in press).