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Global yield of the Supercritical CO_2 extraction from *Cordia verbenacea* DC - Anticancer and antimycobacterial activities

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ABSTRACT - In the present work supercritical fluid extraction (SFE) from *Cordia verbenacea* DC was studied. The effects of pressure and temperature on the global yield were investigated using the Surface Response Methodology. Extracts of *C. verbenacea* were also prepared by hydrodistillation and Soxhlet using ethanol. The chemical compositions of the extracts were determined by GC-FID. The anticancer and antimycobacterial activities of the extracts were evaluated. The results show that pressure and temperature significantly affected the global yield (p_{value} < 0.05). The maximum global yield was obtained at 300 bar and 50°C (4.25 % dry basis – d.b.). The extract obtained by Soxhlet using ethanol showed the highest yield (8.13 ± 0.01 % d.b). β-caryophyllene was identified as the major compound in the volatile fraction. The anticancer activity of the SFE extracts varied with the operational condition. The SFE extract obtained at 200 bar and 40 °C showed the best results and it is similar to that presented by the extracts obtained by hydrodistillation and Soxhlet extraction. The SFE extracts showed a lower antimycobacterial activity.

KEYWORDS - Anticancer and antimycobacterial activities, *Cordia verbenacea*, supercritical fluid extraction.

INTRODUCTION

During the last decades there is an increasing interest in the use of substances obtained from plants. They produce a broad spectrum of metabolites, some of them having tremendous potential in the pharmaceutical, cosmetic and food industries (1,2,3.) Brazil has an important role in this field, since it has the largest biodiversity in the world, with more than 55000 catalogued species from a total of the 350000 to 550000 species present in the world (0).

Cordia verbenacea DC is among the species that has pharmacological applications. It is indigenous species in Brazil that grows in regions from 500 to 1000 meters above the sea level 5. The popular name of this plant in Brazil is "Erva-baleeira" (6). This plant was erroneously classified as *Cordia curassavica* (Jacq.) Roem. & Schult (6).

In folk medicine, *C. verbenacea* is used as anti-inflammatory, anti-arthritic, analgesic, and anti-ulcer 6. The *C. verbenacea* leaves infusion are used in the treatment of rheumatism, rheumatoid arthritis, muscle and back pain, prostatitis, neuralgia and concussions (6). Pharmacological studies have validated its anti-inflammatory activity (6,8). The leaves extracts have anti-inflammatory and analgesic activities (9). The *C. verbenacea* volatile oil, obtained by hydrodistillation, can inhibit the growth of some gram-positive bacteria (*Bacillus cereus*, *B. subtillis*, *Staphylococcus aureus*, *S. epidermitis*) and some yeasts (*Candida albicans*, *C. dubliniensis*, *C. glabrata*, *C. guillermondii*, *C. lusitaneae*, *C. parapsilosis*, *C. stellatoidea*, *C. tropicallis* and *Cryptococcus* sp.) (10). The Aché Pharmaceutical Laboratories, one of the largest pharmaceutical groups of Brazil, launched in June of 2005 the Acheflan® after seven years of studies in partnerships with important Brazilian Universities. The Acheflan® formulated using *C. verbenacea* essential oil containing 2.3 – 2.9 % of alpha-humuleno is indicated for the treatment of chronic tendonitis and myofascial pains (11).

Lins et al. 12 identified the presence of some flavonols such as sitosterol, 5-hidroxy-3,6,7,3',4'-pentametoxyflavone (artemetin) and 5,6'-dihidroxy-3,3',4',6,7-pentametoxiflavone. The artemetin has

been identified as the compound responsible for the anti-inflammatory activity of *C. verbenacea* 8. However, Bayeux et al. 9, showed that the anti-inflammatory activity of C. *verbenacea* extracts is attributed to its chemical compounds besides artemetin. Van de Velde 13 identified two triterpenes: Cordialin A & B. In the volatile oil, the following compounds were identified α-pinene, β-phellandrene, citronellol acetate, β-elemene, trans-caryophyllene, β-gurjunene, α-humulene, allo-aromadendrene, bicyclogermacrene, δ-cadinene, spathulenol and epoxy-caryophyllene 10.

The conventional methods used to produce *C. verbenacea* extracts are Soxhlet using ethanol 6, 12 ketone 8, dichloromethane 9, and hydrodistillation 10.

In general, the active compounds present in plants are at low concentrations and thus it is desirable to use a methodology that results in maximum yields with minimum changes in the functional properties of the extract. Supercritical fluid extraction (SFE) represents an alternative for the conventional processes. This technology exploits the high solvation power, low viscosity and high mass diffusivity presented by supercritical fluids. Thus, SFE can result in high yields of the desired compound with high mass transfer rates. The most used supercritical fluid is carbon dioxide. Carbon dioxide can be used in food, beverage or pharmaceutical applications 14, 15, 16, since it is a non expensive solvent, non toxic, non flammable, straightforward to find and can be readily separated from the extract. Definitions and discussions of supercritical fluid extraction can be found in the literature 17, 18.

In this technology, the selection of the extraction operational conditions (temperature and pressure) have a strong influence in both the global yield and the extract composition, since the solubility of each component is affected by these variables. The global yield is defined as the maximum amount of solute that can be extract at a given extracting pressure and temperature. This value is important in the estimation of the cost to produce extracts by SFE 18, 19.

There is no report in the literature related to the supercritical fluid extraction (SFE) of solutes from *C. verbenacea*. Thus, the main objective of the present work was to determine the influence of both extraction temperature and pressure on the extract global yield, using supercritical CO_2 as solvent. The key components of the extracts were identified and investigated for their potential anticancer and antibacterial activity. The results were compared to the ones obtained with extracts produced by the conventional methods (hydrodistillation and ethanol Soxhlet).

MATERIALS AND METHODS

Raw material

The *Cordia verbenacea* leaves used in this work were cultivated in the Experimental Farm of the CPQBA (Chemical, Biological and Agricultural Pluridisciplinary Research Center) - UNICAMP (Campinas, Brazil). The leaves were dried in a tray drier with air circulation (Fabber-Primar, model 170, Piracicaba, Brazil) at 40 °C and comminuted in a knife mill (Stephan, model UM 140, Brazil). The particles were packed in plastic bag and stored in a domestic freezer (Brastemp, model 7501, São Paulo, Brazil) at –10°C.

Raw Material Characterization

The humidity of the particles was measured using the xylol distillation method 20. The particle size distribution (PSD) was determined using a vibratory sieve shaker (Bertel, model 1868, São Paulo, Brazil); sieves of meshes 24, 48, 60, 80 and 100 (series Tyler, USA) were used. Particles from 24 – 60 meshes were selected for the SFE assays.

Soxhlet Extraction and Hydrodistillation

The organic solvent extraction was carried out in a conventional Soxhlet apparatus using ethanol as solvent (PA, Lot K30916283231, Merck, Darsmtadt, Germany). Eight grams (8×10^{-3} kg) of grounded raw material were packed into a Filter Paper # 42 (JProlab, Curitiba, Brazil). This cartridge was introduced in the Soxhlet apparatus containing 200 mL of solvent. The system was maintained under reflux using a 500 mL heating mantle (Fisaton, model 102, São Paulo, Brazil). The total reflux time was 4 hours. After cooling, the solvent was separated from the solute using a rotovap (Heidolph Instruments, model Laborota 4001, Germany) with a vacuum controller (Heidolph Instruments, model Rotavac Control, Germany). The resulting extract was weighed using an analytical balance (Sartorius, model A200S, Goettingen, Germany).

The hydrodistillation was accomplished in a Schilcher like apparatus. Eighty grams (80×10^{-3} kg) of grounded raw material and 1.5 L of distilled water were mixed in a 2 L glass flask. The system was boiled using a 2 L heating mantle (Fisaton, model 102, São Paulo, Brazil) for 3 hours. The essential oil was collected from the top of the condensed vapor using a Pasteur pipette. The extract was centrifuged (Heraeus Instruments, model Biofuge-haemo, Osterode, Germany) at 10,000 rpm for 10 min in order to separate small water

droplets present in the essential oil. The essential oil was recovered from the centrifuge vial using a Pasteur pipette, weighed and stored in the refrigirator for further analysis.

Supercritical Carbon Dioxide Extraction – Global yield (X_o)

Supercritical fluid extraction was accomplished in the Speed SFE system (Applied Separations, model 7071, Allentown, USA) using CO_2 (Gama Gases Especiais, 99.0 % purity, Campinas, Brazil) as solvent.

The effects of extraction pressure and temperature on the global yield were studied using the Surface Response Methodology. The ANOVA test was performed using the STATISTICA 5.0. (StatSoft, Inc., Tulsa, USA). For pressure, the low and high level were 100 and 300 bar, respectively; for temperature, 30°C and 50°C, respectively. The axial point, corresponding to 59 bar and 26°C, was changed for 78 bar and 26°C, due to equipment limitations.

The bed of raw material was prepared by manual packing of the grounded particles ($3.37 \pm 0.01 \times 10^{-3}$ kg) inside of the 5 mL extraction column (Thar Designs, CL 1165, Pittsburgh, USA). A glass wool plug was placed at both sides of the extraction column in order to avoid the drag of small particles by the solvent. A static period of 5 minutes was used to allow the contact between the particles and the supercritical solvent. The CO_2 mass flow rate was 6.8×10^{-5} kg/s. The extract was collected in a cooled glass flask (ethylene glycol at temperatures lower than 5 °C) to reduce the amount of volatile compounds in the outlet gas stream. The CO_2 was allowed to flow into the extraction column until there was no noticeable extract leaving the system (approximately 1 hour). The tubing line after the extraction column was washed with ethyl acetate to recover the extract deposited on it. The solvent was separated from the extract using a rotovap (Heidolph Instruments, model Laborota 4001, Germany) with a vacuum controler (Heidolph Instruments, model Rotavac Control, Germany).

Characterization of the extracts

The quantification of the components present in the extracts was done using the external standard method 21. The quantitative analysis was done in a GC-FID (Shimadzu, model 17A, Kyoto, Japan) equipped with a fused silica capillary column DB-5 (30 m × 0.25 mm × 0.25 mm, J&W Scientific, Folsom, USA). The carrier gas was helium (1.7 mL/ min, 99.9 % purity, White Martins Gases Industriais, Campinas, Brazil); a split ratio of 1/30 was used. The temperatures of the injector and of the detector were 240°C and 280°C, respectively. The column was heated to 50°C for 5 min and programmed at 5°C/min to 280°C, and heated for 5 min. One microliter of the samples was injected (5×10^{-6} kg of extract diluted in 1×10^{-6} m^3 ethyl acetate P.A., Lot K30929523229, Merck, Darmstadt, Germany). The identification of the major compound was done by comparison of the retention time with that of the following standard: β-caryophyllene (P.A., Lot 38H2503, Sigma, USA).

The samples analyzed in the GC-FID were also tested by thin layer chromatography (TLC). TLC plates (20 × 10 cm, 0.25 mm thickness) coated with silica gel 60 F_{254} (Lot 940378601, Merck, Darmstadt, Germany) were used as stationary phase. The mobile phase was composed by 90% of hexane (P.A., Lot K27512774012, Darmstadt, Germany) and 10% of ethyl acetate (P.A., Lot K30929523229, Merck, Darmstadt, Germany). The plates were sprayed with a solution of anisaldehyde: glacial acetic acid: concentrated sulphuric acid (0.5:50:1) and heated to 100ºC for the visualization of the substances.

Functional Properties

Anticancer Activity

Briefly, the anticancer activity was determined as follows 23: Experiments were performed using the following human cancer cell lines: K562 (leukemia), MCF7 (breast), NCIADR (breast expressing the multidrug resistance phenotype), NCI460 (lung), UACC62 (melanoma), OVCAR (ovary), HT29 (colon), PCO3 (prostate), and 786 (kidney). The National Cancer Institute, Frederick, MD (NCI), kindly donated these cell lines, and stock cultures were kept in liquid nitrogen. Cells were cultured in 25 mL flasks (Nunc Brand Products, Roskilde, Denmark) containing 5 mL of RPMI 1640 (Gibco BRL, Life Technologies, São Paulo, Brazil) with 5% fetal bovine serum (Gibco BRL, Life Technologies). The sulforodamine B (SRB) assay was performed according to the method of Skehan 24. The cells were fixed by means of protein precipitation with 50% trichloroacetic acid (TCA) (Sigma Chemical Co.) at 4 °C (50 μL/well, final concentration = 10%) for 1 hour. The supernatant was then discarded, and the plates were washed five times with tap water. The cells were stained for 30 minutes with 0.4% the SRB (Sigma Chemical Co.) dissolved in 1% acetic acid (50 μL/well) (Sigma Chemical Co.) and subsequently washed four times with 1% acetic acid to remove unbound stain. The plates were air-dried, and bound protein stain was solubilized with 150 μL of 10 mM Trizma buffer (Sigma Chemical Co.). The optical density was read on an automated spectrophotometer

plate reader at 540 nm. The assays were performed in triplicates. For cells growing in suspension (e.g., leukemia), the same method was employed, but the TCA concentration was 80%, in order to fix the cells to the bottom.

Antimycobacterial Activity

The antimycobacterial activity or the minimum inhibitory concentration (MIC) of the extracts was measured in a Middlebrook 7H9 medium inoculated with *M. tuberculosis* H_{37}Rv-ATCC 27294 using the microplate Alamar Blue assay (MABA) 25.

RESULTS AND DISCUSSION

Global yield - The extract global yield for the Cordia verbenacea + CO_2 system is presented in Table 1. The maximum and minimum yields were obtained at 300 bar / 50 °C (4.25 % d.b.) and 78 bar / 40 °C (0.11 % d.b.), respectively. At 100 bar the global yield decreased from 1.60 % d.b. to 0.47 % d.b., for temperatures of 30 and 50 °C, respectively. At 300 bar the global yield increased from 2.87 % d.b. to 4.25 % d.b., at the same temperatures. This behavior is closely related to the CO_2 physical properties changes, mainly the density. At 100 bar, the CO_2 density considerably decreases with temperature (from 771.5 kg/m^3 at 30 °C to 384.33 kg/m^3 at 50 °C), therefore, the decrease in CO_2 solvation power promoted the decrease of the global yield. Conversely, the opposite effect is observed at 300 bar. The density decrease with temperature is lower at high pressures (from 947.98 to 870.43 kg/m^3, at 30 °C and 50 °C, respectively), therefore the effect of the increase in solute vapor pressure is more significant. Similar behaviors were observed for the pressure and temperature effects in the extract global yield of grape seeds 26 and dandelion leaves 27.

The analysis of variance (ANOVA) indicates that the statistical model can describe well the experimental data. The correlation coefficient was R^2 = 0.9712. The most important effects were the pressure (p_{value} = 0.0001), the quadratic term of pressure (p_{value} = 0.0014), the pressure / temperature interaction (p_{value} = 0.0119), and temperature (p_{value} = 0.0461), for a significance level of 95 %. The pressure and temperature effects on dandelion leaves SFE extract global yield (pressures from 150 to 450 bar and temperatures from 35 to 65 °C) were studied using a complete factorial design 3^2 27. The ANOVA indicated that the pressure and pressure/temperature interaction terms were highly significant, for a confidence level of 95%. On the other hand, the statistical model suggests that there is a pressure where the global yield reaches a maximum value. Similar behavior was observed for turmeric 28 and onion 29 global yields. In order to compare the SFE yields, the global yields for Soxhlet extraction with ethanol and hydrodistillation were determined as 8.13 ± 0.01 % (d.b.) and 0.55 ± 0.06 % (d.b.), respectively.

The chemical composition of the obtained extracts changed considerably. The main compound found in all extracts was β-caryophyllene, as reported in the literature 10. In spite of the higher yield of the ethanol Soxhlet method, the volatile oil obtained in the hydrodistillation was high amount of β-caryophyllene content (Table 2). The chemical composition of the SFE extracts was a function of the both extraction pressure and temperature: at 100 bar, the content of volatile oil in the extract increased with temperature; at 300 bar, the opposite was observed. This fact was observed by the β-caryophyllene relative purity. The relative purity was defined as the ratio between the β-caryophyllene and extract masses (kg β-caryophyllene / kg extract × 100). At 100 bar / 50°C, the β-caryophyllene purity was 23.6 %; in the volatile oil obtained by hydrodistillation a similar purity was determined (21.8 %). The β-caryophyllene purity drastically decreased with the increase in CO_2 density. At 100 bar / 30 °C, the β-caryophyllene purity was 3.16 %. At 300 bar, the β-caryophyllene purity was 2.64 and 2.52 %, for 30 °C and 50 °C, respectively.

Functional Properties

Figure 1 presents the anticancer activity results of the *C. verbenacea* extract obtained by SFE. The anticancer activity of the extracts obtained by hydrodistillation and Soxhlet extraction has been presented in Figure 2. In order to compare the results, the extracts were considered actives if the growth inhibition is larger than 50% (dashed line). The cytostatic effect was observed for extract concentrations larger than 0.25 μg/mL, except for the leukemia (K562) and kidney cancer (786-0) cells, which needed a minimum extract concentration of 2.5 μg/mL. The cytolytic effect was observed for extract concentration of 250 μg/mL, excepted for prostate cancer cells (PC0.3). The results obtained for the hydrodistillation extract was similar to the SFE ones. The cytolytic effect of the ethanol Soxhlet extract was observed for extract concentration of 25 μg/mL for kidney, ovarian, leukemia, and breast cancer cells.

The cytocide effect was observed only for some few cancer cells (leukemia and ovarian) at extract concentration of 25 μg/mL.

Table 1. Influence of temperature and pressure on the global yield from C. verbenacea obtained by supercritical CO_2 Extraction.

Pressure (bar)	Temperature (°C)	Global Yield (X_o) (% dry basis)
300	30	2.87
300	50	4.25
100	30	1.60
100	50	0.47
341	40	3.36
200	54	3.90
78	40	0.11
200	26	2.35
200	40	3.36
200	40	3.35
200	40	3.32

X_o = *kg extract / kg dry raw material*

Table 2. Comparison of yields of β-caryophyllene from C. verbenacea obtained by different methods of Extraction.

Extraction methods	X_o β-caryophyllene (% d.b.)	Purity (%)*
Hydrodistillation	0.12 ± 0.01	21.8
Soxhlet (Ethanol)	0.0894 ± 0.0001	1.09
SFE		
100 bar / 30°C	0.05	3.16
100 bar / 50°C	0.11	23.62
300 bar / 30°C	0.07	2.52
300 bar / 50°C	0.11	2.65
200 bar / 40°C	0.13	3.99

For extracts obtained at 100 bar / 50°C and 300 bar / 50°C, the cytotoxic effect for the majority of the cells were observed for extracts whole concentrations were larger than 2.5 μg/mL, and the cytocide effect for extract concentrations were larger than 250 μg/mL. Thus, the anticancer activity was not affected by the increase in the extraction temperature.

In the antibacterial activity test, the extracts obtained from SFE method did not exert any significant antibacterial effects against *M. tuberculosis* (MIC = 128). Similar results were reported for rosemary extracts 23. The MIC of *C. verbenacea* extract was larger than the ones observed for ginger and turmeric extracts (31.25 μg/mL)23. Thus, the *C. verbenacea* extract exhibited low antibacterial activity against *M. tuberculosis*.

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Figure 1. Anticancer activity as a function of cancerous cellular ancestries for Cordia verbenacea extract obtained using CO_2 at the indicated conditions.

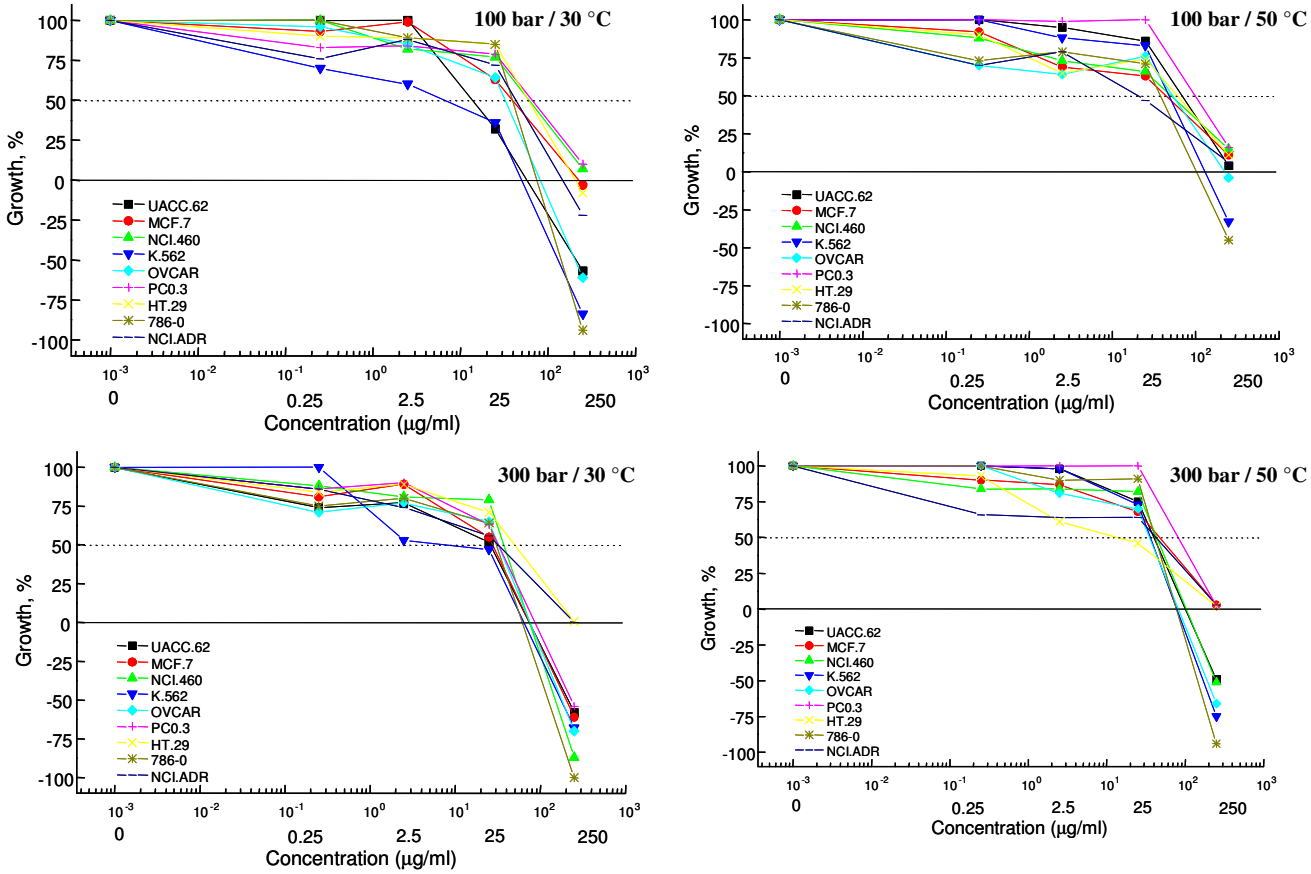
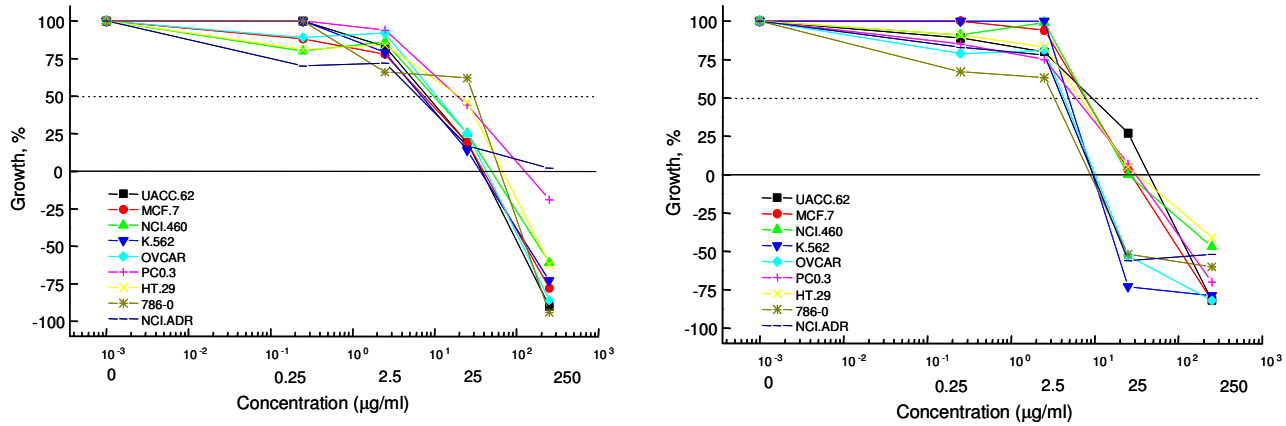


Figure 2. Anticancer activity as a function of cancerous cellular ancestries for Cordia verbenacea extract obtained by hydrodistillation (A) and Soxhlet extraction (B).



A B

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