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ORIGINAL ARTICLE

# Phytochemical Analysis and Cytotoxic Activity of *Cnidoscolus quercifolius* Pohl (Euphorbiaceae) against Prostate (PC3 and PC3-M) and Breast (MCF-7) Cancer Cells

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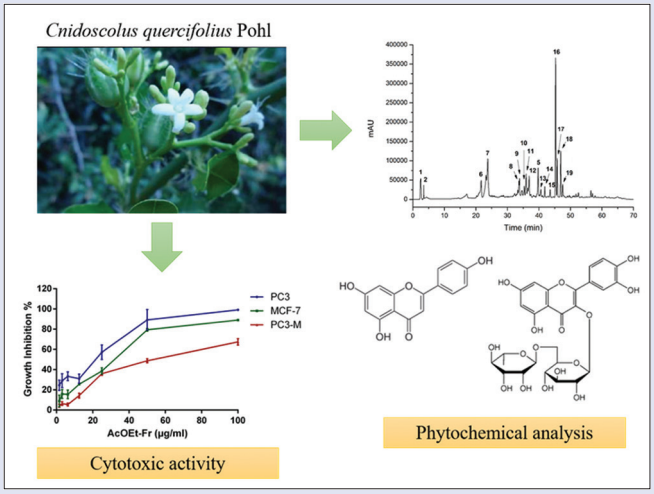
## ABSTRACT

**Background:** *Cnidoscolus quercifolius* is a Brazilian medicinal plant often found in the Caatinga biome. Previous studies have described several pharmacological properties for this plant, including antiproliferative effect. However, there are still few pharmacological and phytochemical reports involving this plant. **Objective:** In this report, it was described the cytotoxic effect of extract and fractions obtained from the leaves of *C. quercifolius*. It was also reported for the first time the identification of two flavonoids in this species. **Materials and Methods:** Ethanol extract (EE) and fractions hexane, chloroform-Fr, ethyl acetate (AcOEt-Fr) and methanol (MeOH-Fr) were evaluated against prostate (PC3 and PC3-M) and breast (MCF-7) cancer cell lines. A preliminary phytochemical analysis was performed by thin layer chromatographic, while the content of total phenolic compounds and flavonoids was determined by colorimetric assays. EE and bioactive fraction (AcOEt-Fr) were selected for analysis by high-performance liquid chromatography-diode-array detector (HPLC-DAD). **Results:** Phytochemical analysis revealed that the samples were positive for the presence of several classes of secondary metabolites, mainly phenolic derivatives and flavonoids. EE and AcOEt-Fr presented the highest phenolic and flavonoid content. HPLC-DAD analysis of EE and AcOEt-Fr allowed the identification of two flavonoids (rutin and apigenin) not yet described for this species. Concerning the cytotoxicity evaluation, only AcOEt-Fr demonstrated a strong cytotoxic effect against all cell lines, presenting the half maximal inhibitory concentration values between 15.75 and 46.97 µg/ml. **Conclusion:** The results suggest that flavonoids may play an important role in the cytotoxic effect observed for this species. In addition, this report contributed to the phytochemical knowledge of the species through the identification and quantification of flavonoids.

**Key words:** Breast cancer, cytotoxicity, flavonoids, medicinal plants, prostate cancer

## SUMMARY

- *Cnidoscolus quercifolius* Pohl, a Brazilian medicinal plant, has a cytotoxic effect against prostate (PC3 and PC3-M) and breast (MCF-7) cancer cell lines
- AcOEt-Fr, the most bioactive fraction, presented a high flavonoid content suggesting that these compounds may be responsible for the cytotoxic activity of the sample
- Flavonoids rutin and apigenin are being reported for the first time in *Cnidoscolus quercifolius*.




**Abbreviations used:** AcOEt-Fr: Ethyl acetate fraction; $CHCl_3$-Fr: Chloroform fraction; EE: Ethanol extract; Hex-Fr: Hexane fraction; $IC_{50}$: Half maximal inhibitory concentration; MeOH-Fr: Methanol fraction; TLC: Thin layer chromatography; UV: Ultraviolet.

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## INTRODUCTION

Recent reports have showed that cancer is one of the most impactful diseases due to its multifactorial nature and the difficulty of treatment.[1-3] It is a major public health problem, affecting both more and less economically developed countries.[4,5] According to the International Agency for Research on Cancer, there were 14.1 million new cancer cases and around 8.2 million cancer deaths in 2012 worldwide. Breast and prostate cancer are some of the most frequently diagnosed in women and men, with approximately 1.7 and 1.1 million new cases worldwide, respectively.[6]

The use of new chemotherapeutic agents has contributed to the treatment of breast and prostate cancer in several stages of the disease. However, many patients still show resistance to conventional drugs. The appearance of adverse effects is also frequent in this type of therapy,



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which makes it necessary to search for new molecules with anticancer potential.[7-10]

Natural products have been considered a promising source of new anticancer compounds. In fact, the use of secondary metabolites derived from plants has helped significantly in the discovery of molecules with innovative mechanisms of action.[11,12] Newman and Cragg examined the new drugs approved by the Food and Drug Administration in the United States between 1981 and 2010 and found that 34% of these drugs were obtained from natural products or semi-synthetic derivatives, including anticancer and immunosuppressive molecules.[13] Recent studies have confirmed the contribution of natural products as a source of new antitumor agents.[14,15]

*Cnidoscolus quercifolius* Pohl is a Euphorbiaceae species, popularly known as "faveleira" or "urtiga-branca," endemic to the Caatinga biome, in Brazil. Its leaves and stem-barks are used in folk medicine for the treatment of stomach problems, infections, inflammation, pain, and wound healing.[16] Previous pharmacological investigations have demonstrated the antinociceptive,[17] anti-inflammatory,[18] and antiproliferative[19,20] potential of extracts obtained from *C. quercifolius*. Most of these pharmacological activities are related to the presence of phenolic[17,18] and terpenoid[19,20] compounds. In this study, we describe the cytotoxic potential of *C. quercifolius* against prostate and breast cancer cell lines as well as phytochemical analysis, with the identification of flavonoids never reported in this species.

## MATERIALS AND METHODS

### Plant material

The leaves of *C. quercifolius* Pohl were collected in the city of Petrolina (Coordinates: 09° 03' 55.30" S and 40° 20' 06.90" W), State of Pernambuco, Brazil, in January of 2013. A voucher specimen (n° 19202) was deposited at the Herbário Vale do São Francisco of the Universidade Federal do Vale do São Francisco.

### Extraction and fractionation

The dried and pulverized leaves of *C. quercifolius* (482 g) were macerated with 95% ethanol for 72 h. The solvent was successively changed, and the solution was removed, filtered and concentrated under reduced pressure on a rotatory evaporator at 50°C, yielding 63 g of ethanol extract (EE, 13.07%). Subsequently, an aliquot of EE (15 g) was fractionated by vacuum liquid chromatography, using silica gel 60 as the stationary phase. Hexane (Hex), chloroform ($CHCl_3$), ethyl acetate (AcOEt) and methanol (MeOH) were used as mobile phase, in increasing order of polarity, resulting in the respective fractions: Hex-Fr (0.17 g, 1.13%), $CHCl_3$-Fr (2.06 g, 13.73%), AcOEt-Fr (1.98 g, 13.20%), and MeOH-Fr (10.34 g, 68.93%).

### Phytochemical screening

A solution of extract and fractions (1 mg/ml) were prepared in $CHCl_3$ and applied on thin layer chromatographic (TLC) plates of silica gel 60 $F_{254}$ in aluminum support (Merck®). TLC plates were eluted in different solvent systems as described by Wagner and Bladt,[21] seeking to highlight the main secondary metabolites groups [Table 1]. After elution, the plates were visualized under ultraviolet (UV) camera at wavelengths of 254 and 365 nm.

### Determination of total phenolic content

Total phenolic content was assayed using the Folin–Ciocalteu reagent, as reported by Slinkard and Singleton,[22] and only the volumes have been adjusted. An aliquot (40 µl) of diluted extract and fractions was added to 3.16 ml of distilled water and 200 µl of the Folin–Ciocalteu reagent. The mixture was shaken and allowed to stand for 6 min, before adding 600 µl of 20% sodium carbonate solution. The solutions were left at 20°C for 2 h, and the absorbance of each sample was determined at 765 nm (spectrophotometer Quimis®) against the blank. Total phenolic contents of all samples were expressed as mg gallic acid equivalents per g (mg GAE/g) through the calibration curve with gallic acid. The calibration curve range was 50–1000 mg/l ($R^2$ = 0.999). All spectrophotometric analyses were performed in triplicate.

### Determination of total flavonoid content

Total flavonoid content was determined using a colorimetric method described previously.[23] Briefly, 0.30 ml of extract and fractions solutions were mixed with 1.50 ml of distilled water in a test tube followed by addition of 90 µl of a 5% $NaNO_2$ solution. After 6 min, 180 µl of a 10% $AlCl_3.6H_2O$ solution was added and allowed to stand for another 5 min before 0.6 ml of 1 M NaOH was added. Then, 330 µl of distilled water was added to complete the total volume of the sample (3.0 ml) before measuring the absorbance. The absorbance was measured immediately against the blank at 510 nm in comparison with the standard prepared similarly with known (+)-catechin concentrations. The results were expressed as mg of catechin equivalents per g of extracts (mg CE/g) through the calibration curve with catechin ($R^2$ = 0.998). The calibration curve range was 50–1000 mg/l. All spectrophotometric analyses were performed in triplicates.

### High-performance liquid chromatography-diode-array detector analysis

High-performance liquid chromatography-diode-array detector (HPLC-DAD) analyses were performed to better characterize EE and polar fractions (AcOEt-Fr and MeOH-Fr). All samples were solubilized in MeOH, resulting in solutions at 10 mg/ml. After preparation, the solutions were filtered and analyzed on an HPLC apparatus (Shimadzu®), coupled to DAD detector, using an Eclipse plus C-18 column (4.6 mm × 250 mm, 5 µm, Agilent®), and a guard column (4.6 mm × 12.5 mm, 5 µm, Zorbax®), maintained at 37°C. The mobile phase was composed of 0.1% formic acid (solvent A) and acetonitrile (solvent B), in gradient mode as follows: 0–50 min

**Table 1:** Elution systems and revelators used to characterize the main secondary metabolites from extracts and fractions of *Cnidoscolus quercifolius* by thin layer chromatography

| Phytochemicals | Elution systems | Revelators |
|---|---|---|
| Alkaloids | Toluene:AcOEt:diethylamine (70:20:10, v/v) | Dragendorff reagent |
| Anthracene derivatives | AcOEt:MeOH:water (100:13.5:10, v/v) | 10% ethanolic KOH |
| Anthraquinones | Ethyl ether:AcOEt:formic acid (75:25:1, v/v) | Phosphomolybdic acid |
| Coumarins | Toluene:ethyl ether (1:1 saturated with 10% acetic acid, v/v) | 10% ethanolic KOH |
| Flavonoids and tanins | AcOEt:formic acid:acetic acid:water (100:11:11:26, v/v) | NEU reagent |
| Lignans | Chloroform:MeOH:water (70:30:4, v/v) | Vanillin phosphoric reagent |
| Mono and diterpenes | Toluene:AcOEt (93:7, v/v) | Vanillin sulfuric reagent |
| Triterpenes and steroids | Toluene:chloroform:ethanol (40:40:10, v/v) | Lieberman-Burchard reagent |

AcOEt: Ethyl acetate; MeOH: Methanol

(100% solvent A), 50–60 min (40% solvent A and 60% solvent B), and 60–70 min (100% solvent A). All analyses were performed with a flow rate of 1 ml/min and the injection volume for the samples was 50 µl. Analytical standards of phenolic acids and flavonoids (Sigma-Aldrich®) commonly found in plant species were analyzed under the same conditions. The identification of the compounds in EE and AcOEt-Fr was performed by comparing the retention time, and the maximum absorption wavelengths ($\lambda_{max}$) verified for each peak. The quantification of the identified compounds was also determined by HPLC-DAD through a calibration curve obtained for analytical standards.

## Cell culture and cytotoxicity assay

The cytotoxic activity of EE and fractions was evaluated in prostate (PC3 and PC3-M) and breast (MCF-7) cancer cell lines from the National Cancer Institute (NCI, United States of America), through the MTT test.[24] All cell culture experiments were performed at 37°C. Cells were grown in RPMI 1640 medium supplemented with 10% fetal calf serum and 1% of antibiotics, in a 5% $CO_2$ humidified atmosphere. The cells were plated at the concentration of 0.1 cells/ml × $10^6$ cells/ml (10000 cells per well) for all cell lines and afterward samples were solubilized in dimethyl sulfoxide (DMSO) and diluted in the cell culture medium (1.56, 3.13, 6.25, 12.5, 25, 50, and 100 µg/ml). After 72 h of incubation, the plates were centrifuged, and the supernatant was removed. Subsequently, 150 µl of an MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) solution (tetrazolium salt) was added, and the plates were incubated for 3 h. Absorbance was read after the dissolution of the precipitate with 150 µl of pure DMSO in a plate spectrophotometer at 595 nm. Absorbance values were converted to The half maximal inhibitory concentration ($IC_{50}$). Samples with $IC_{50}$ >100 µg/ml were considered weakly active. All experiments were performed in triplicate.

## Statistical analysis

All obtained data were analyzed using the GraphPad Prism® 6.0 software and expressed as mean ± standard deviation. $IC_{50}$ values were obtained by interpolation from nonlinear regression analysis with 95% of confidence level. $IC_{50}$ was defined as the concentration sufficient to obtain 50% of the maximum inhibitory effect on cell viability.

**Table 2:** Phytochemical characterization of extract (ethanol extract) and fractions (hexane fraction, chloroform fraction, ethyl acetate fraction, methanol fraction) from the leaves of *Cnidoscolus quercifolius*

| Phytochemicals | EE | Hex-Fr | $CHCl_3$-Fr | AcOEt-Fr | MeOH-Fr |
|---|---|---|---|---|---|
| Alkaloids | - | - | - | - | - |
| Anthracene derivatives | + | - | - | ++ | - |
| Anthraquinones | - | + | ++ | - | - |
| Coumarins | - | - | - | - | - |
| Flavonoids and tanins | + | - | - | + | + |
| Lignans | + | - | + | + | - |
| Mono and diterpenes | + | ++ | +++ | - | - |
| Triterpenes and steroids | + | - | + | - | - |

-: Not detected; +: Low presence; ++: Moderate presence; +++: Strong presence. EE: Ethanol extract; Hex-Fr: Hexane fraction; $CHCl_3$-Fr: Chloroform fraction; AcOEt-Fr: Ethyl acetate fraction; MeOH-Fr: Methanol fraction

**Table 3:** Identification of compounds in ethanol extract and ethyl acetate fraction after high-performance liquid chromatography-diode array detector analysis

| Peak | RT (min) | $\lambda_{max}$ (nm) | Compound or derivative |
|---|---|---|---|
| 1 | 2.46 | 264/345 | Quercetin derivative |
| 2 | 3.47 | 259 | Phenolic acid derivative |
| 3 | 20.29 | 255/353 | Rutin* |
| 4 | 21.58 | 265/345 | Quercetin derivative |
| 5 | 39.88 | 267/332 | Apigenin* |
| 6 | 21.63 | 266/297 | Phenolic acid derivative |
| 7 | 23.80 | 322 | Phenolic acid derivative |
| 8 | 33.67 | 287 | Phenolic acid derivative |
| 9 | 33.80 | 299 | Phenolic acid derivative |
| 10 | 35.36 | 288/312 | Chrysin derivative |
| 11 | 35.94 | 267/332 | Apigenin derivative |
| 12 | 36.89 | 289/317 | Chrysin derivative |
| 13 | 40.60 | 240/350 | Fisetin derivative |
| 14 | 41.86 | 268/341 | Apigenin derivative |
| 15 | 43.54 | 312 | Phenolic acid derivative |
| 16 | 45.26 | 268/331 | Apigenin derivative |
| 17 | 45.78 | 267/335 | Apigenin derivative |
| 18 | 46.88 | 267/344 | Apigenin derivative |
| 19 | 47.53 | 334 | Phenolic acid derivative |

*Rutin and apigenin were identified based on the comparison of RT and $\lambda_{max}$ of the peaks with the data corresponding to the analytical standards used. RT: Retention time; $\lambda_{max}$: Maximum absorption wavelengths; HPLC-DAD: High-performance liquid chromatography-diode array detector

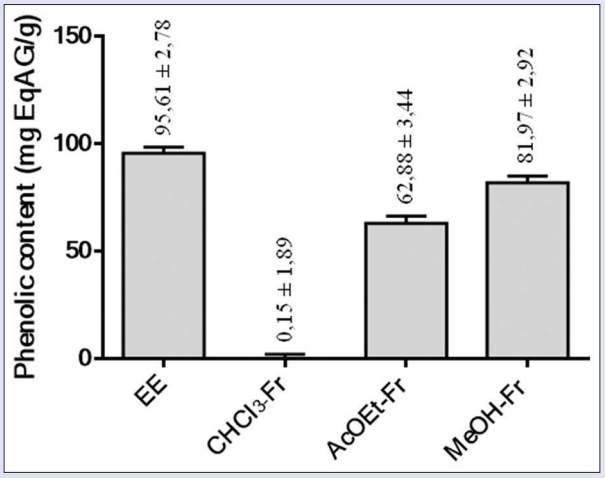


**Figure 1:** Determination of phenolic content of extract (EE) and fractions ($CHCl_3$-Fr, AcOEt-Fr, MeOH-Fr) from the leaves of *Cnidoscolus quercifolius*. Results are expressed as mean ± Standard deviation

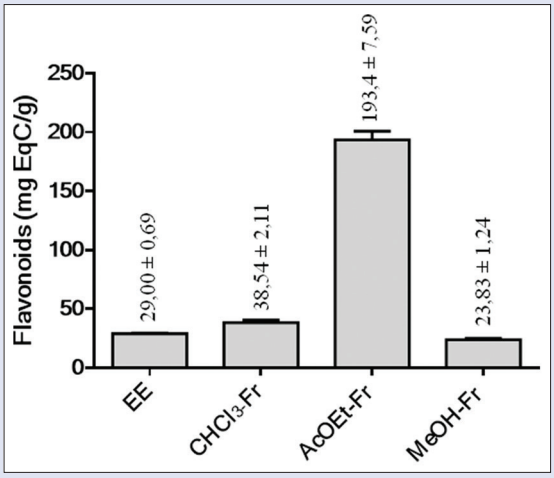


**Figure 2:** Determination of flavonoid content of extract (EE) and fractions ($CHCl_3$-Fr, AcOEt-Fr, MeOH-Fr) from the leaves of *Cnidoscolus quercifolius*. Results are expressed as mean ± Standard deviation

## RESULTS

Phytochemical screening revealed the presence of several classes of secondary metabolites in extracts and fractions, including flavonoids detected mainly in fractions of higher polarity (AcOEt-Fr and MeOH-Fr), as shown in Table 2. Anthracene derivatives, anthraquinones, lignans, terpenoids, and steroids were also detected in *C. quercifolius*.

Phenolic compounds content was determined by the Folin-Ciocalteau reagent test, whereas total flavonoid content was determined by the aluminum complexation assay. Figures 1 and 2 show that EE and AcOEt-Fr presented the highest phenolic and flavonoid content, respectively. However, such colorimetric assays do not provide sufficient information on the chemical structure of the compounds. Accordingly, EE and AcOEt-Fr were analyzed using HPLC using standards of various phenolic compounds and flavonoid commonly found in medicinal plants.

HPLC analysis revealed the presence of 19 substances [Figure 3], among which it was possible to identify rutin and apigenin [Table 3]. The content of these flavonoids in EE and AcOEt was also quantified and is shown in Table 4. Although several compounds were not identified, the other peaks had RTs close to the standards used, as well as UV absorption spectra characteristic of flavonoids and phenolic acids, and it was possible to infer that the other chemical constituents were quercetin, apigenin, chrysin, fisetin, and phenolic acids derivatives [Table 3].

To investigate the cytotoxic effect of *C. quercifolius*, PC3, PC3-M, and MCF-7 human cancer cell lines were used. Cells were treated for 72 h at different concentrations, and then cell viability was analyzed by the MTT assay. Table 5 shows that only AcOEt-Fr demonstrated strong cytotoxic activity against all cell lines, presenting $IC_{50}$ values between 15.75 and 46.97 µg/ml. In Figure 4, we show the percentage of inhibition of cell growth as a function of the tested concentration of the AcOEt-Fr in all tumor cell lines tested.

**Table 4:** Quantification of rutin and apigenin in ethanol extract and ethyl acetate fraction after high-performance liquid chromatography-diode array detector analysis

| Sample | Rutin (µg/mg) | Apigenin (µg/mg) |
|---|---|---|
| EE | 16.51±2.81 | 0.34±0.02 |
| AcOEt-Fr | - | 0.50±0.04 |

Quantification was performed by obtaining a calibration curve for rutin ($R^2$=0.981) and apigenin ($R^2$=0.998). Results are expressed as mean±SD. All analyses were performed in triplicate. AcOEt-Fr: Ethyl acetate fraction; EE: Ethanol extract; SD: Standard déviation

**Table 5:** Cytotoxic activity of extract (ethanol extract) and fractions (hexane fraction, chloroform fraction, ethyl acetate fraction and methanol fraction) from the leaves of *Cnidoscolus quercifolius* against PC3, PC3-M and MCF-7 cell lines

| Sample | $IC_{50}$ (µg/ml) | | |
|---|---|---|---|
| | PC3 (prostate) | PC3-M (prostate) | MCF-7 (breast) |
| EE | >100 | >100 | >100 |
| Hex-Fr | >100 | >100 | >100 |
| CHCl3-Fr | >100 | >100 | >100 |
| AcOEt-Fr | 15.75 (11.56-21.44) | 46.19 (40.88-52.20) | 46.97 (36.51-60.42) |
| MeOH-Fr | >100 | >100 | >100 |

$IC_{50}$ was defined as the concentration sufficient to obtain 50% of the maximum inhibitory effect on cell viability. All assays were performed in triplicate. EE: Ethanol extract; Hex-Fr: Hexane fraction; $CHCl_3$-Fr: Chloroform fraction; AcOEt-Fr: Ethyl acetate fraction; MeOH-Fr: Methanol fraction; $IC_{50}$: Half maximal inhibitory concentration

## DISCUSSION

Prostate and breast cancer have high prevalence rates in the world population for a long time. Conventional therapy is not always effective in combating these diseases, requiring the use of new molecules or combinations of various drugs. In this context, many research groups have investigated the potential of natural products as an alternative to the treatment of prostate and breast cancer.[25,26] In this study, we describe the cytotoxic potential of extracts and fractions obtained from *C. quercifolius*, a Brazilian medicinal plant, endemic to the Caatinga biome.

Preliminary phytochemical analysis demonstrated a large variety of secondary metabolites in EE and fractions [Table 2], mainly phenolic compounds and flavonoids [Figures 1 and 2]. AcOEt-Fr showed the highest content of flavonoids and interestingly, was the most active sample, exhibiting a significant cytotoxic activity against all cell lines [Table 5 and Figure 4]. These findings suggest that the flavonoids produced by *C. quercifolius* are probably responsible for its cytotoxic potential.

HPLC analysis was performed to identify flavonoids in EE and AcOEt-Fr. The chromatographic data obtained allowed the identification and quantification of rutin and apigenin for the first time in this species [Tables 3 and 4]. However, many flavonoids and phenolic acids derivatives could not be identified through this technique, suggesting that the species produces unusual compounds, which makes it necessary to conduct new phytochemical studies to isolate and characterize these molecules.

Flavonoids have been extensively described as cytotoxic agents against prostate and breast cancer cells.[27-29] In addition, the role of flavonoids in the chemoprevention of prostate and breast cancer is also well known.[30,31] A recent pharmacological investigation has shown that rutin efficiently reverses multidrug resistance and restores chemosensitivity to conventional chemotherapeutic drugs in human breast cancer cells.[32] Similarly, apigenin has shown synergistic effect in combinatorial therapy inducing apoptosis even in prostate cancer models resistant to conventional

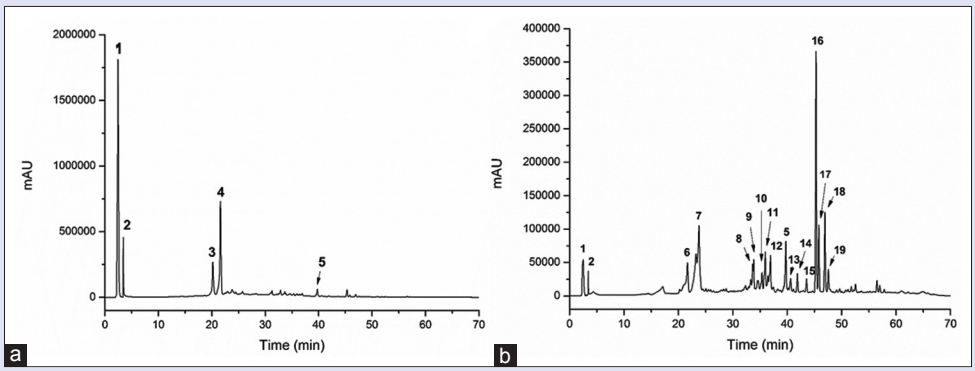


**Figure 3:** Chromatograms (320 nm) obtained after analysis of EE (a) and AcOEt-Fr (b) by high-performance liquid chromatography-diode-array detector

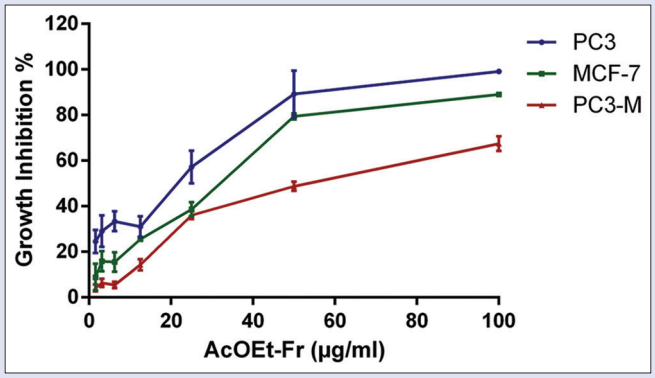


**Figure 4:** Growth inhibition of PC3, MCF-7 and PC3-M cells in the presence of AcOEt-Fr. Tumor cells were grown for 72h in a cell culture medium containing increasing concentrations of AcOEt-Fr (1.56-100 µg/ml). Results are shown as mean ± SD

anticancer agents.[33] In this sense, these flavonoids possibly contributed to the cytotoxic effect of *C. quercifolius* presented in this report.

## CONCLUSION

In summary, AcOEt-Fr showed relevant cytotoxic activity against human prostate and breast cancer cells. Phytochemical analyzes showed high flavonoid content in this fraction, including the identification of rutin and apigenin, flavonoids known for their potential in the treatment and prevention of prostate and breast cancer. The results obtained in this investigation suggest that these flavonoids play an important role in the cytotoxic effect observed for this species. However, additional phytochemical and pharmacological studies should be performed to better characterize its chemical composition and the mechanisms of action involved.

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Nil.

## Conflicts of interest

There are no conflicts of interest.

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