Research Article

Mitra Abolmaesoomi, Sarni Mat Junit, Johari Mohd Ali, Zamri Bin Chik and Azlina Abdul Aziz*

Effects of polyphenolic-rich extracts from Citrus hystrix on proliferation and oxidative stress in breast and colorectal cancer

Abstract

Objectives: The anti-proliferative effects of Citrus hystrix have been reported. However, information on breast and colorectal cancer is limited especially the mechanistic aspects. In this study, the antioxidant activities of hexane, ethyl acetate, methanol and water extracts of C. hystrix leaves and their growth inhibitory effects on colorectal (HCT 116) and breast cancer (MCF 7, MDA-MB 231 and HCC 1937) cells were analysed.

Methods: Antioxidant and oxidative stress status were measured using non-cellular and cellular assays. Caspase and gene expression were utilized to determine anti-proliferative effects. Polyphenolic content was analysed using LC-IT-TOF/MS.

Results: The water extract showed the highest polyphenolic content and antioxidant activities (FRAP, DPPH, ABTS, superoxide anion radical scavenging, ferrous ion chelation, cellular antioxidant assay). The ethyl acetate extracts of C. hystrix (CH-EA) demonstrated the highest anti-proliferative activity against all cancer cell lines (IC50<100 μg/mL). Increase in ROS was observed in CH-EA-treated HCT 116, MDA-MB 231 and HCC 1937 cells (p<0.05). Increase in caspase activities and upregulation of Bax, Bcl-2, Cdk-1, TP53 and TNF-α expression in HCT 116 cells indicated activation of apoptosis by CH-EA. LC-IT-TOF/MS analysis indicated presence of quercetin and rutin in CH-EA.

Conclusions: CH-EA showed anti-proliferative effects, possibly through modulation of oxidative stress and apoptosis.

Keywords: anti-proliferative; apoptosis; breast cancer; caspase; Citrus hystrix; colorectal cancer; oxidative stress.

Introduction

Cancer is the third most common cause of death in Malaysia. Colorectal and breast cancer are the most frequent cancers in Malaysian males (16.3%) and females (32.1%), respectively [1].

Excess reactive oxygen species (ROS) are linked to development of diseases such as cancer, diabetes mellitus and cardiovascular diseases. Antioxidants are protective against oxidative damage caused by ROS. Plants are rich sources of antioxidants such as polyphenols, capable of protecting against diseases including cancer. Approximately 75% of chemotherapy agents are derived from natural products [2].

Citrus hystrix (Family-Rutaceae) or makrut lime, is an aromatic herb that is widely used in Southeast Asian cuisine. Traditionally, it is used as a remedy for heart disease, dizziness and indigestion [3]. Its biological properties included anti-microbial, anti-clastogenic and anti-cancer [4–8]. The leaves contain flavonoids, coumarin, saponins and terpenoids [7, 9] and volatile compounds such as α-pinene, camphene, limonene, copaene, linalool, and citronellol [10].

Studies on the anti-proliferative properties of C. hystrix are of great interest. Essential oils from the fruits and
leaves of *Citrus hystrix* showed anti-proliferative activities against human mouth epithelial carcinoma (KB) and murine leukaemia (P388) cells [11]. The methanolic leaf extracts showed cytotoxicity against human leukaemia (HL-60) cells [12] while the ethyl acetate and hexane extracts inhibited growth of leukaemia, cervical cancer and neuroblastoma cells while showing no cytotoxicity on normal human peripheral blood mononuclear cells [8, 13, 14]. The anti-proliferative effects of *C. hystrix* on breast cancer (MDA MB 231) cells was recently reported [15]. Nevertheless, its effects on other breast cancer cells as well as the optimal solvent extract that confer the anti-proliferative effects remain unknown. Furthermore, the anti-proliferative effects of *C. hystrix* on colon cancers have not been reported and the molecular mechanisms are unexplored.

In this study, the growth inhibitory effects of the leaves of *C. hystrix* on colorectal and breast cancer cells were investigated and the potential mechanism of action explored. Information obtained can provide a better understanding on the antioxidant and anti-proliferative activities of the extracts as well as the molecular mechanisms involved in the apoptotic effects of the selected extracts.

**Materials and methods**

**Preparation of leaf extracts of *C. hystrix***

The leaves of *C. hystrix* (voucher specimen KLU49455) were sourced from a local market and deposited at Universiti Malaya’s Herbarium, by Dr. Yong Kien Thai.

The dried leaf powder was extracted sequentially using hexane, ethyl acetate, methanol and water at a ratio of 1:10 (g:mL) with each extraction performed three times, for 8 h in a shaker-incubator (145 rpm, 40 °C). The supernatant was pooled and dried using a rotary evaporator (Buchi, Switzerland). The extract was dissolved in 10% DMSO and kept at −20 °C. The water extracts were lyophilized (Labconco, UK).

**Polyphenolic and flavonoid content**

For analysis of polyphenolic content, plant extracts (2000 μg/mL), 10% Folin-Ciocalteu reagent (100 μL) and 1 M Na2CO3 (70 μL) were mixed and their absorbance was read at 765 nm after 2 h [16]. Gallic acid was the standard.

For analysis of flavonoid content, equal volumes of plant extracts (2000 μg/mL), aluminum trichloride (10% w/v) and potassium acetate (1 M) were mixed and their absorbance was read at 325 nm after 2 h [14]. Quercetin was the standard.

**Ferric reducing antioxidant power (FRAP)**

Reagents for the assay consisted of 10 mM 2,3,6-tripryridyl-s-trazine (TPTZ) in 40 mM HCl, 20 mM FeCl3.H2O and 300 mM acetate buffer (pH 3.6) [18]. Warm FRAP reagent (300 μL) was mixed with 10 μL of sample (2000 μg/mL) and absorbance was read at 593 nm after 4 min incubation. Iron sulphate (FeSO4) was the standard.

**1,1-Diphenyl-2-picryl hydrazyl (DPPH) radical scavenging**

DPPH solution (100 μM) and plant extracts (0–2000 μg/mL) were incubated for 30 min [19]. Absorbance was measured at 515 nm. Results were expressed as EC50 value (μg/mL).

**2,2′-Azinobis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS·+) radical scavenging**

ABTS+ reagent (200 μL) was reacted with 10 μL of plant extracts (0–2000 μg/mL) [20]. Absorbance was read at 734 nm after 15 min. Results were expressed as EC50.

**Superoxide anion radical (O2−) scavenging**

Equal volumes of plant extracts (0–2000 μg/mL), nitro blue tetrazolium (0.15 mM), NADH (0.468 mM) and phenazine methosulphate (60 M) were mixed, incubated in the dark for 10 min and absorbance read at 570 nm [21]. Results were presented as EC50 (μg/mL).

**Ferrous (Fe2+) ion chelating activity**

FeCl3 (0.5 mM), ferrozine (2.5 mM) and distilled water were added at a ratio of 2.5:1:1:8, respectively [22]. Absorbance was read at of 562 nm following a 10-min incubation. EDTA was used as positive control. Results were expressed as EC50 (μg/mL).

**Cellular antioxidant assay (CAA)**

HCT 116 cells (5 × 104), in a 96-well plate, were incubated for 1 h with the plant extracts (0–1,500 μg/mL) and 25 μM dichloro-dihydro-fluorescein diacetate (DCFH-DA) [23]. The cells were rinsed with PBS and 2,2′-azobis(2-amidinopropane) dihydrochloride (ABAP, 0.6 mM) was added. Fluorescence intensity (excitation 485, emission 538 nm) was measured for 60 min at 5-min interval using a multimode reader (Tecan, Switzerland). The relative fluorescence unit vs time was plotted and CAA was determined [24]. Quercetin was the positive control.

**Cell culture**

Colon cancer cell line (HCT 116) and breast cancer cell lines (HCC 1937 BRCA1-deficient, MCF 7 ER-positive and MDA-MB 231 triple negative breast cancer) were used for the anti-proliferative study (ATCC, USA). Cytotoxicity of the extracts were tested on normal colon (CCD 841) and liver (WRL 68) epithelial cells.
DMEM supplemented with 10% FBS and 1% penicillin-streptomycin were used for culturing the cells. HCC 1937 cells were cultured in RPMI media supplemented with 10% FBS and 1% penicillin-streptomycin. All cells were maintained in a humidified atmosphere (37 °C and 5% CO₂).

**Cell viability**

Cells seeded in 96-well plates (10⁴ cells per well) were treated with the plant extracts (0–500 µg/mL). After 48 h, cell viability was measured using MTT, at 595 nm. The cells were treated with MTT and the formazan crystals were solubilized in DMSO. The concentration that corresponded to 50% inhibition of cell growth (IC₅₀) was calculated from the dose-response curve. To determine if the plant extracts could inhibit growth of the cancer cells, an initial cell viability analyses were performed at a concentration of 500 µg/mL of the plant extracts. Camptothecin and 5-fluorouracil (5-FU) were the positive controls.

**Caspase 3/7**

HCT 116, HCC 1937 and MDA-MB 231 cells (7 × 10⁴ cells/well), attached overnight, were treated for 24 and 48 h with the IC₅₀ concentration of ethyl acetate extract of C. hystrix (EA-CH). Caspase activity was measured using the ApoTox-Glo™ Triplex Assay (Promega, USA). The pan caspase inhibitor, carbobenzoxy-valyl-alanyl-aspartyl-[O-methyl]-fluoromethylketone (zVAD.fmk) was used to validate the effects of the plant extracts on caspase 3/7. HCT 116 cells (10⁴ cells/well) were treated with CH-EA at the IC₅₀ concentration together with 10 nM of zVAD.fmk and incubated for 24 h. MTT assay estimated the cell viability.

**Reactive oxygen species (ROS)**

CH-EA (IC₅₀ concentration) was added to HCT 116 cells (5 × 10⁴ cells/well) in the presence of 25 µM of DCFH-DA. Fluorescence readings (excitation 485, emission 538) were measured (Tecan Infinite M1000 Pro, Switzerland) following a 90-min incubation at 37 °C [23].

**Gene expression (RT-qPCR)**

Regulation of apoptotic genes (Bax, Bcl-2, TP-53, TNF-a, Cdk-1, Cdk-2 and Fas) was analysed using RT-qPCR using StepOne™ Real-Time PCR System (Applied BioSystem, USA). HCT 116 cells (2 × 10⁶) were treated with IC₅₀ concentration of CH-EA for 48 h. Cell detachment was performed using trypsin prior to tcRNA extraction. tcRNA extraction was performed using the RNeasy® Plus Mini Kit (Qiagen, Germany). Good RNA quality is depicted by A260/A280 ratio of above 1.8 (NanoDrop™ 2000, Thermo Scientific, USA).

tcRNA was reverse transcribed using Tetro cDNA synthesis kit (Bioline, USA). Total RNA concentration was 1,000 ng. The PCR mixture contained: 50 ng cDNA, 200 nM primers, ROX passive reference dye and THUNDERBIRD® SYBR® qPCR Mix (Toyobo, Japan). PCR was performed under the following conditions: activation of Taq DNA Polymerase (20 s, 95 °C), 40 cycles of denaturation (3 s, 95 °C), primer annealing (30 s, 60 °C). The reference gene was GAPDH. The primers used are indicated in Table S1.

**LC-IT-TOF/MS analysis**

A Shimadzu Ultra-Fast Liquid Chromatography (UFLC) system coupled with a photodiode array (PDA) detector and Ion Trap TOF/Mass Spectrometer (Shimadzu, Japan) was used. A Water Bridge BEH C₁₈ column (PN 186003085, 50 × 2.1 mm 2.5 µm) was utilised. Water and acetonitrile (containing 0.1% formic acid) were the mobile phases. Samples were analysed using a 0–100% gradient of acetonitrile over 14 min, at a flow rate of 0.25 mL/min. The column temperature was 40 °C and concentration of samples was 1 ppm.

**Statistical analysis**

Statistical analyses were performed using the SPSS statistical software, version 23 (SPSS Inc., Chicago, Illinois, USA). Means among groups were compared using Tukey’s Honestly Significant Difference test and one-way analysis of variance (ANOVA). Level of significance was set at p<0.05. Gene expression data was analysed using independent sample t-test with the confidence interval percentage set at 95%.

**Results**

**Polyphenolic content and yield**

The methanolic extract had the highest yield which was 2.5, 3.5 and 11 folds higher than the water, hexane and ethyl acetate extracts, respectively (Table 1). The water extract of C. hystrix contained the highest polyphenolic content whereas the hexane extract contained the highest flavonoid content.

**Antioxidant activity of C. hystrix**

The water extract of C. hystrix had the highest antioxidant potential (Table 1) and demonstrated the highest FRAP and ABTS radical scavenging activities. Only the water and methanol extracts achieved 50% inhibition of the DPPH radicals, with the former showing more than 4 folds scavenging ability than the latter. Only the water extracts showed superoxide anion radical scavenging and cellular antioxidant activities. The water extract was unable to chelate ferrous ions but the ethyl acetate and hexane extracts showed ferrous ion chelating activities. However, their EC₅₀ values were much higher than the positive control, quercetin.
Table 1: Yield, polyphenolic, flavonoid and antioxidant activities of the leaves of *C. hystrix* extracted with hexane, ethyl acetate, methanol and water.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Yield (%)</th>
<th>Polyphenol, mg GAE/g</th>
<th>Flavonoid, mg QE/g</th>
<th>FRAP, mmol Fe⁺²/g</th>
<th>ABTS⁺ radical scavenging activity EC₅₀, μg/mL</th>
<th>DPPH radical scavenging activity EC₅₀, μg/mL</th>
<th>Superoxide anion scavenging activity EC₅₀, μg/mL</th>
<th>Ferrous Ion Chelating Activity EC₅₀, μg/mL</th>
<th>CAA EC₅₀ (μg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hexane</td>
<td>7.05 ± 0.00³</td>
<td>7.60 ± 0.92²</td>
<td>0.35 ± 0.01³</td>
<td>1.92 ± 0.04²</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>846.17 ± 11.39</td>
<td>ND</td>
</tr>
<tr>
<td>Ethyl acetate</td>
<td>2.28 ± 0.00³</td>
<td>23.65 ± 0.23³</td>
<td>0.15 ± 0.00³</td>
<td>3.77 ± 0.02³</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>225.21 ± 9.05</td>
<td>ND</td>
</tr>
<tr>
<td>Methanol</td>
<td>25.23 ± 0.00³</td>
<td>36.82 ± 0.60³</td>
<td>0.04 ± 0.00³</td>
<td>5.01 ± 0.10</td>
<td>ND</td>
<td>ND</td>
<td>1763.15 ± 22.72 ²</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Water</td>
<td>9.85 ± 0.00³</td>
<td>64.18 ± 1.30³</td>
<td>0.07 ± 0.00³</td>
<td>9.45 ± 0.05³</td>
<td>782.60 ± 48.27 ²</td>
<td>380.13 ± 2.56³</td>
<td>766.50 ± 23.88</td>
<td>ND</td>
<td>281.57 ± 14.57</td>
</tr>
<tr>
<td>Quercetin</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>9.54 ± 0.11³</td>
<td>73.34 ± 0.64</td>
<td>64.84 ± 0.69</td>
<td>163.80 ± 0.17</td>
<td>NA</td>
<td>15.60 ± 0.82</td>
</tr>
<tr>
<td>EDTA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>ND</td>
<td>63.39 ± 5.7³</td>
<td>NA</td>
</tr>
</tbody>
</table>

Results are expressed as means ± standard deviation (n=3). Values with different lower case letters (a,b,c,d,e) are significantly different at p<0.05 between different solvents. NA, not analysed; ND, not determined; ABTS, 2,2'-azinobis (3-ethylbenzothiazoline-6-sulphonic acid) radical scavenging capacity, expressed as EC₅₀, concentration of the extracts (μg/mL) required to inhibit 50% of the radicals; FRAP, ferric reducing antioxidant power, expressed as mmol Fe⁺²/g; DPPH, 1,1-diphenyl-2-picrylhydrazyl radical scavenging activity and superoxide anion (O₂⁻) scavenging activity are expressed as EC₅₀, concentration of the extracts (μg/mL); CAA, cellular antioxidant assay, expressed as EC (μg/mL).

Cell viability analysis was performed using an initial cell viability study using a high concentration of the plant extracts indicating that only the ethyl acetate (CH-EA) and hexane (CH-HX) extracts showed antiproliferative activities against the cancer cells tested as described in Table 2. To determine if oxidative stress could influence the cell viability based on the antioxidant activities. This extract was chosen based on the anti-proliferative effects of the plant extract, the cell viability studies were performed using the Caspase 3/7 assay.这体験を選択した主な理由は、植物抽出物の抗プロリフェラティブ性が体験により、細胞内でのコスラーゼ活性を示す点に対する影響である可能性があるためです。
Table 2: High dose MTT analyses of *C. hystrix* crude extracts.

<table>
<thead>
<tr>
<th>Extract</th>
<th>HCT 116</th>
<th>MCF-7</th>
<th>MDA-MB-231</th>
<th>HCC 1937</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hexane</td>
<td>8.49 ± 0.88</td>
<td>23.06 ± 2.45</td>
<td>21.43 ± 2.50</td>
<td>77.40 ± 1.55</td>
</tr>
<tr>
<td>Ethyl acetate</td>
<td>6.78 ± 0.50</td>
<td>20.81 ± 1.16</td>
<td>17.62 ± 0.97</td>
<td>10.82 ± 0.15</td>
</tr>
<tr>
<td>Methanol</td>
<td>84.65 ± 3.38</td>
<td>134.45 ± 3.77</td>
<td>103.33 ± 1.55</td>
<td>62.80 ± 0.75</td>
</tr>
<tr>
<td>Water</td>
<td>94.96 ± 1.08</td>
<td>88.83 ± 7.97</td>
<td>77.99 ± 3.52</td>
<td>88.19 ± 0.20</td>
</tr>
</tbody>
</table>

Results are expressed as mean ± SD (n=3).

Table 3: Cytotoxicity analyses of the ethyl acetate and hexane extracts of *C. hystrix* against colon cancer cell line; HCT 116 and breast cancer cells lines; MCF 7, MDA-MB 231 and HCC 1937.

<table>
<thead>
<tr>
<th>Sample</th>
<th>10_{50}, µg/mL</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HCT 116</td>
</tr>
<tr>
<td><em>C. hystrix</em>_{hexane}</td>
<td>84.28 ± 0.11a</td>
</tr>
<tr>
<td><em>C. hystrix</em>_{ethyl acetate}</td>
<td>62.16 ± 1.41b</td>
</tr>
<tr>
<td>Camptothecin</td>
<td>NA</td>
</tr>
<tr>
<td>5-FU</td>
<td>14.55 ± 0.84d</td>
</tr>
</tbody>
</table>

Results were expressed as mean ± SD (n=3). Values with different lowercase letters (a–d) within the same column are significantly different at p<0.05 among extracts. ND, not determined, NA, not analysed, 5-FU, fluorouracil.

significantly increased cellular ROS in HCT 116, MDA-MB 231 and HCC 1937 cells compared to untreated control cells, with fold changes of more than 2.80 (Figure 1E). Cellular ROS was significantly higher in MDA-MB 231 and HCC 1937 cells but lower in HCT 116 cells compared to 5-FU.

**Gene expression with qRT-PCR**

The expression of *Bax*, *Bcl-2*, *Cdk-1*, *TP-53* and *TNF-α* genes was significantly upregulated while the expression of *Cdk-2* and *Fast1* was unchanged (Figure 2). The highest fold change was observed in *Bax* (6.43 ± 0.02) followed by *TP-53* (2.86 ± 0.11) and *Bcl-2* (2.85 ± 0.58).

**LC-IT-TOF/MS analysis**

LCMS analysis identified quercetin (positive mode: m/z 303.07) and rutin (negative mode: m/z 609.16) in CH-EA (Figure 3). Identification was performed by comparing retention times, precursor and product ions (m/z values) of the sample with authentic flavonoid standards.

**Discussion**

Although the antioxidant potential of *C. hystrix* using different extraction methods have been reported [3, 25, 26], the combination of antioxidant activities together with analysis of their anti-proliferative effects have not been done. The leaves of *C. hystrix* have higher antioxidant potential compared to the fruit peels [27]. The highest extraction yield was in the methanol extracts which implies that most of the phytochemicals in the leaves are relatively polar. Sequential extraction was chosen as studies have reported increased efficiency of this method compared to single solvent extraction [13]. This approach is especially useful when the sample amount is limited.

Polyphenolic-rich plants have potent antioxidant properties and protective against diseases such as cancer and heart diseases, thus it is common practice to measure both polyphenolic content and antioxidant activities, when investigating the antioxidant potential of plants [28]. The polyphenolic content of the water extract of *C. hystrix* in this study was higher than previously reported for the methanol [26] and ethanol crude extracts [25]. The water extract also had the highest antioxidant activity, potentially contributed by the high amount of polyphenols. There is positive correlation between polyphenolic content and antioxidant activities of plants [29].

A cell-based antioxidant assay (CAA assay) was also incorporated to include cellular effects such as absorption and metabolism of the phytochemicals. This also allows comparisons of antioxidant activities with the *in vitro* chemical assays. HCT 116 cells, which are intestinal epithelial cells are suitable for this assay as they provide...
intestinal absorption capacity. The water extract of *Citrus hystrix* was the only extract with cellular antioxidant activity, similar to the non-cellular antioxidant assays. This implied the ability of the HCT 116 cells to take up the bioactive compounds and react intracellularly with ROS.

Although the water extract of *Citrus hystrix* had the highest antioxidant potential, it did not show high anti-proliferative effects. Instead, CH-EA was the most potent against the four cancer cell lines, with IC_{50} values less than 100 μg/mL. When solvents of varying polarities were used for the extraction of phytochemicals, the highest antioxidant and anti-proliferative activities may not be observed in the same solvent extracts [24]. This is the first report on the anti-proliferative effects of *Citrus hystrix* on HCT 116 cells. This extract was reported to show cytotoxicity against leukemia, cervical cancer and neuroblastoma cells [13, 14].

**Figure 1:** Caspase 3/7 activation in (A) HCT 116 cells (B) MDA-MB 231 and (C) HCC 1937 cells treated with the ethyl acetate extract of *C. hystrix* (CH-EA) for 24 and 48 h. (D) Percentage viability of HCT 116 cells treated with the ethyl acetate extract of *C. hystrix* and 10 nM of pan-caspase inhibitor zVAD.fmk for 24 h and (E) ROS determination in HCT 116, MDA-MB 231 and HCC 1937 cells treated with the ethyl acetate extract of *C. hystrix* (CH-EA) for 90 min. *Indicates values significantly different compared to untreated control (p<0.05). #Indicates values significantly different compared to 5-FU (p<0.05).
A recent study reported that the hexane extract of *C. hystrix* had the highest anti-proliferative effects against MDA MB 231 cells (IC$_{50}$ 317.63 ± 2.00 μg/mL) compared to the ethyl acetate extract [15]. The ethyl acetate and hexane extracts in our study were more potent at inhibiting growth of the MDA MB 231 cells (IC$_{50}$<50 μg/mL). The differences could be due to several factors including sources and growth conditions of the plant as well as extraction methods.

Apoptosis analyses and ROS levels were measured to determine if the anti-proliferative effects occurred through these mechanisms. Increased activities of caspases 3/7 in HCT 116, MDA-MB 231 and HCC 1937 cells by CH-EA indicated induction of programmed cell death which was validated using zVAD.fmk in HCT 116 cells. CH-EA could induce the activation of pro-apoptotic proteins in T47D breast cancer cells [14].

Redox imbalance, in favour of increased ROS, is seen in cancer cells and is believed to contribute to cancer induction. Polyphenols such as catechin, quercetin, kaempferol, rutin and myricetin, with some of these reported to be present in *C. hystrix*, could combat cancers including colorectal cancer [30]. Polyphenols can have both antioxidant and pro-oxidant activities. The pro-oxidant effects of polyphenols have been reported to contribute towards cancer cell death, potentially via inducing toxic levels of ROS in cancer cells. An example is the ROS-mediated *p53*-dependant apoptosis [31]. The more than 2.80-fold increase in ROS observed in the CH-EA-treated cells in this study suggest pro-oxidant effects.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Fold Change</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bax</td>
<td>6.43</td>
</tr>
<tr>
<td>Bcl-2</td>
<td>2.85</td>
</tr>
<tr>
<td>Cdk-1</td>
<td>1.96</td>
</tr>
<tr>
<td>TP-53</td>
<td>2.86</td>
</tr>
<tr>
<td>TNF-α</td>
<td>1.31</td>
</tr>
<tr>
<td>Cdk-2</td>
<td>1.05</td>
</tr>
<tr>
<td>Fas-1</td>
<td>0.83</td>
</tr>
</tbody>
</table>

**Figure 2:** Gene expression analyses of HCT 116 cells treated with the ethyl acetate extract of *C. hystrix*. The bar chart shows the gene expression patterns (expressed as the fold change relative to the untreated control) of selected genes involved in apoptosis (with respect to GAPDH, a housekeeping gene). *p<0.05, **p<0.01, ***p<0.001.

**Figure 3:** Mass spectrometry spectrum of the ethyl acetate extract of *C. hystrix*. (A) Quercetin, (B) Rutin.
HCT 116 cells were used for the gene expression study as the anti-proliferative effects of *C. hystrix* have not been tested on these cells. Bax and Bcl-2 are pro- and anti-apoptotic proteins, respectively and increase and decrease of these proteins, respectively, contribute to apoptosis. Although the gene expression of both Bax and Bcl-2 increased in this study, the ratio of Bax to Bcl-2 is more than 2-fold and may indicate pro-apoptosis. The increase in Bax caused release of cytochrome c from the mitochondria into the cytosol, leading to activation of caspase 3 and subsequently apoptosis [32].

Expression of other cancer-related genes were also measured in HCT 116 cells. There was a significant upregulation of TP53. The nuclear factor p53 is a tumour suppressor that stops cell cycle or activates apoptosis when cells are damaged [31]. High cellular stress results in high p53 concentration, which promotes the formation of mitochondrial ROS and induces apoptosis [33]. The upregulation of TP53 seen in HCT 116 cells could cause the increase in ROS observed in this study. The expression of TNF-α was also significantly increased. Binding of TNF-α to its receptor, TNF-R1 activates the caspase-dependent apoptotic cascade and its regulation is also responsible in the activation of signal transduction pathways; MAP kinases, NF-κB and caspases [34].

The LC-MS analysis of the ethyl acetate extract of *C. hystrix* indicated the presence of quercetin and rutin, as previously reported [3, 35]. Quercetin could induce cell cycle arrest in G2/M phase, reduce cyclin A, induce expression of Cdc-2 and p21 and inhibit the β-catenin/Tcf signaling pathway, while rutin could damage DNA, induce apoptosis, change expression level of Bax, Bcl-2 and caspase-9 [30, 36]. These two polyphenols might contribute towards the anti-proliferative effects seen in HCT 116 cells.

**Conclusions**

Data from this study demonstrated the antioxidant and anti-proliferative nature of the extracts of *C. hystrix*. Although the water extract has the highest antioxidant activity, the anti-proliferative activities were limited to the ethyl acetate extract. The ethyl acetate extract inhibited the growth of breast cancer and colorectal cancer cells by the activation of caspase 3/7 and induction of ROS. This study was the first to report on the anti-proliferative effects of *C. hystrix* on HCT 116 cells. Quercetin and rutin could be responsible for the molecular events observed in HCT 116 cells. The potential of CH-EA to be developed as cancer therapeutics or adjuvant therapy especially on colorectal cancer merits further investigation.

**Research funding:** None declared.

**Author contributions:** All the authors have accepted responsibility for the entire content of this submitted manuscript and approved submission.

**Competing interests:** Authors state no conflict of interest.

**References**


34. Dash S, Sahu AK, Srivastava A, Chowdhury R, Mukherjee S. Exploring the extensive crosstalk between the antagonistic cytokines- TGF-β and TNF-α in regulating cancer pathogenesis. Cytokine 2021;138:155348.


Supplementary Material: The online version of this article offers supplementary material (https://doi.org/10.1515/tjb-2022-0062).