Evaluation of Terpenes Psiadin, Plectranthone, and Saudinolide as Selective Anti-Cancer Agents against Hepatic Carcinoma Cells

Khaled Y. Orabi 1,* , Mohamed S. Abaza 2 , Rajaa Al-Attiyah 3 and Yunus A. Luqmani 4

1 Department of Pharmaceutical Chemistry, Faculty of Pharmacy, Kuwait University, Safat 13110, Kuwait; ky.orabi@ku.edu.kw
2 Department of Biological Sciences, Faculty of Science, Kuwait University, Safat 13060, Kuwait; abazams@yahoo.com
3 Department of Microbiology and Immunology, Faculty of Medicine, Kuwait University, Safat 13110, Kuwait; rj.alattiyah@hsc.edu.kw
4 Department of Pharmaceutical Chemistry, Faculty of Pharmacy, Kuwait University, Safat 13110, Kuwait; yunus.luqmani@ku.edu.kw

* Correspondence: ky.orabi@ku.edu.kw; Tel.: +965-2 463-6158

Abstract: Plant-derived terpenes have aroused considerable interest as chemotherapeutic agents for a variety of diseases. This study aimed at the isolation and purification of the scarce terpenes psiadin, plectranthone and saudinolide from their respective plants, followed by the determination of antiproliferative activity, against hepatic cancer cell lines (HepG2, Hep3B), and the potential molecular mechanisms. Time- and dose-dependent cytotoxicity, evaluated using MTT and colony-forming assays, were exhibited by psiadin and plectranthone against the cancer cells. Flow cytometry showed that these two terpenes blocked cell cycle progression and induced mitochondrial-mediated apoptosis, particularly through increased cytochrome c and disruption of mitochondrial membrane potential. Additionally, they initiated the generation of reactive oxygen species as well as inhibiting NF-κB. Psiadin lowered several essential cyclins and cyclin-dependent kinases and reduced RB activation. It was concluded that psiadin, in particular, has a significant therapeutic potential with the biggest advantage of differentiating between cancer and normal cells which is acutely lacking in current cytotoxic drugs. Its precise mode of action needs further investigation but appears predominantly to cause cell cycle arrest by interfering with cyclin production. It will be important to determine, in future studies, whether these terpenes will similarly inhibit other cancer cell lines and retain its activity against tumors in vivo.

Keywords: Terpenes; Hepatic carcinoma; Apoptosis.

1. Introduction

The global cancer burden is escalating significantly, with projections of 28 million new cases and 16 million cancer deaths by 2040 [1]. Post-surgical cancer chemotherapy relies heavily on a variety of general cytotoxic agents. However, most are associated with collateral damage to normal tissues that are frequently severe and intolerable, limiting their usefulness. Thus, there is a great need for new drugs that are more cancer-selective with lesser undesirable side effects [2]. Natural products hold historical significance in various folkloric medicines and form the basis of many modern-day drugs. Phytochemicals are the only sources for several medications that are almost impossible to get from other sources. Also, they serve as prototypes by providing pharmacophores for many synthetic drugs [3]. Indeed, a significant proportion of current chemotherapeutics is either natural or derived from them [4]. Moreover, conjugation of toxic natural products to monoclonal antibodies or polymeric
carriers can lead to more efficacious targeted therapies [5-9]. Between 1981 and 2019, 247 such chemical entities were approved as anticancer drugs [2].

Of these phytochemicals, terpenes, including cembranoids, are the largest group with a wide range of biological activities, two prominent examples with the anticancer application being paclitaxel and limonene. A large number of terpenes have been identified in extracts of plants from the genera of families such as Euphorbiaceae, Lamiaceae, and Asteraceae, that are endogenous to the Arabian Peninsula and extensively used in local folkloric medicine [10-13]. This study we focused our attention on three terpenes; psiadin, plectranthone, and saudinolide (Figure 1a).

Psiadin is a diterpene isolated from the aerial parts of Psiadia arabica Jaub. et Spach. (Asteraceae) [14]. Over seventy compounds, including flavonoids, phenylpropanoids, coumarins, and terpenoids, have been isolated from the genus Psiadia, of which only the flavonoids are reported to have bioactivity [15]. Crude extracts and essential oils of Psiadia species have demonstrated antimicrobial [16, 17], anti-viral [18, 19], antiplasmodial [20, 21], and anti-inflammatory activities. [21, 22].

Phytochemical studies on Plectranthus species indicate the presence of eudesmane sesquiterpenes, labdane, abietane, ent-kaurane diterpenoids and triterpenoids [23]. The plant itself has a folkloric record of use in southern and western regions of Arabia as a disinfectant and deodorant, and for digestive and respiratory diseases. Thymol, the main constituent responsible for the culinary uses of the fresh plant, is responsible for its antibacterial property [13]. The sesquiterpene plectranthone has been identified in Plectranthus cylindraceus Hochst. ex Benth (Lamiaceae) [23].

The 6,7-secolabdane diterpene saudinolide was identified as a constituent of Clutyia richardiana L. (Euphorbiaceae). This plant is characterized by its unpleasant odor and intense bitter taste [10]. Some of the isolated diterpenes, saudin for example, have a significant hypoglycemic effect in non-alloxanized fasted mice [11, 12].

Using standard chromatographic and spectral analyses protocols, we isolated, purified and identified these three terpenes from their respective plant sources and determined their effects on human hepatic cancer cell lines. The data suggest that plectranthone and, more especially psiadin have selective cytotoxic activity towards the cancer cells. They also induced greater cell death rates at equivalent concentrations than several drugs currently utilized in cancer chemotherapy through various discussed mechanisms.

2. Results

2.1. Extraction, isolation and, identification of terpenes

Psiadia arabica chloroform extract was solvent-portioned and chromatographed to yield 1380 mg of impure psiadin (Figure 1a). Crystallization from methylene chloride/ethyl acetate gave 813 mg (0.041% yield) of pure psiadin as colorless needles.

Psaidin: colorless needles; mp 164-165 °C; [α]D -120° (c 0.1, CHCl3); UV (MeOH) λmax (log ε) 282 (2.14) nm; IR (KBr) νmax 3422, 1690 cm⁻¹; 1H NMR (CDCl3, 600 MHz) see Table 1; 13C NMR (CDCl3, 150 MHz) see Table 1; HRESMS m/z; 301.3257 [M-H2O+H]+, 283.3109 [M-2H2O+H]+.

Similarly, the 95% ethanol extract of Plectranthus cylindraceus was purified to afford, after crystallization from ethyl acetate/petroleum ether, 1400 mg of plectranthone (0.13% yield) as pure colorless prisms.

Plectranthone: colorless prisms; mp 134-135 °C; [α]D -36.9° (c 0.03, CHCl3); UV (MeOH) λmax (log ε) 237 (3.13) nm; IR (KBr) νmax 2900, 1725, 1670 cm⁻¹; 1H NMR (CDCl3, 600 MHz) see Table 1; 13C NMR
(CDCl$_3$, 150 MHz) see Table 1; HRESMS $m/z$ 277.2827 [M-CH$_3$COOH+H]$^+$, 217.2472 [M-2CH$_3$COOH+H]$^+$. The ethyl acetate extract of Cluytia richardiana aerial parts gave, after chromatography and crystallization, 450 mg (0.028% yield) colorless granules identified as the diterpene saudinolide.

Saudinolide: colorless granules; mp 231-235 °C; [α]$^D + 98°$ (c 0.142, CHCl$_3$); UV (MeOH) $\lambda_{max}$ (log $\varepsilon$) 220 (4.30), 265 (3.90) nm; IR (KBr) $\nu_{max}$ 3520 (OH), 1780 ($\gamma$-lactone), 1720 ($\delta$-lactone), 1510, 1450, 1380 (br), 1200, 1190, 1050, 970, 860, 830, 735 cm$^{-1}$; $^1$H NMR (CDCl$_3$, 600 MHz) see Table 1; $^{13}$C NMR (CDCl$_3$, 150 MHz) see Table 1; HRESMS $m/z$ [MH]$^+$ 389.2544 [C$_{20}$H$_{20}$O$_8$ + H]$^+$.

**Table 1.** $^1$H and $^{13}$C NMR Data (CDCl$_3$) for Psiadin, Plectranthone and Saudinolide

|   | Psiadin | Plectranthone | Saudinolide |
|---|---------|---------------|-------------|
| # | $\delta_c$, multiplicity | $\delta_H$, multiplicity ($J$ in Hz) | $\delta_c$, multiplicity | $\delta_H$, multiplicity ($J$ in Hz) | $\delta_c$, multiplicity | $\delta_H$, multiplicity ($J$ in Hz) |
| 1 | 48.6, CH$_2$ | 1$_{eq}$: 2.61, d (14.0) 1$_{ax}$: 2.72, d (14.0) | 47.8, CH$_2$ | 2.06, d (15.8) 2.55, d (15.8) | 73.0, CH | 5.93, m |
| 2 | 212.7, C | --- | 198.4, C | --- | --- | --- |
| 3 | 55.9, CH$_2$ | 3$_{eq}$: 2.38, dd (14.0, 1.5) 3$_{ax}$: 2.06, dd (14.0, 1.5) | 128.7, CH | 5.90, br s | 172.7, C | --- |
| 4 | 43.7, C | --- | 158.0, C | --- | 47.7, CH | 2.9, dq (1.3, 8.0) |
| 5 | 50.5, CH | 1.22, m | 44.0, CH | 3.01, br s | 72.5, C | --- |
| 6 | 20.9, CH$_2$ | 6$_{eq}$: 1.65, m 6$_{ax}$: 1.42, m | 71.6, CH | 5.56, br s | 42.3, CH$_2$ | 2.61, d (15.0) 2.54, d (15.0) |
| 7 | 40.4, CH$_2$ | 1.56, m | 43.2, CH | 0.90, m | 108.4, C | --- |
| 8 | 44.0, C | --- | 20.9, CH$_2$ | 1.38, m 1.60, m | --- | --- |
| 9 | 55.0, CH | 1.10, m | 28.8, CH$_2$ | 1.48, m 1.48, m | 89.7, C | --- |
| 10 | 46.6, C | --- | 38.6, C | --- | 100.6, CH | 5.93, s |
| 11 | 18.5, CH$_2$ | 1.54, m | 29.1, CH | 1.48, m | --- | --- |
| 12 | 32.7, CH$_2$ | 1.46, m | 21.1, CH$_3$$^a$ | 0.88, d (6.7)$^a$ | 174.7, C | --- |
| 13 | 43.6, CH | 2.60, br s | 21.2, CH$_3$$^a$ | 0.86, d (6.7)$^a$ | 44.3, C | --- |
| 14 | 39.1, CH₂ | 14αβ: 1.84, dd (11, 1.5) 14α: 1.10, m | 69.7, CH₂ | 3.86, d (11.0) 4.40, d (11.0) | 126.2, CH | 5.91, d (3.9) |
| 15 | 44.7, CH₂ | 2.01, m | 23.2, CH₃ | 1.99, s | 132.3, CH | 6.22 dd (3.9, 8.0) |
| 16 | 154.7, C | --- | 21.5, CH₃ | 2.03, sᵇ | 48.8, C | --- |
| 17 | 103.6, CH₂ | 4.82, s 4.76, s | 21.5, CH₃ | 2.04, sᵇ | --- | --- |
| 18 | 70.9, CH₂ | 3.85, s | 170.8, Cᵇ | --- | --- | --- |
| 19 | 65.4, CH₂ | 3.74, d (10.5) 3.54, d (11.0) | 171.2, Cᵇ | --- | --- | --- |
| 20 | 19.3, CH₃ | 1.06, s | --- | --- | 141.9, CH | 7.68 d (1.0) |
| 2' | --- | --- | --- | --- | 124.3, C | --- |
| 3' | --- | --- | --- | --- | --- | --- |
| 4' | --- | --- | --- | --- | 108.7, CH | 6.52, dd (1.0, 1.8) |
| 5' | --- | --- | --- | --- | 144.4, CH | 7.48, t (1.8) |
| OH | --- | 5.5, br s | --- | --- | --- | 4.10, d (1.3) |
| OH | --- | 5.6, br s | --- | --- | --- | --- |
| C4-CH₃ | --- | --- | --- | --- | 15.5, CH₃ | 1.41, d (8.0) |
| C13-CH₃ | --- | --- | --- | --- | 19.6, CH₃ | 1.19, s |
| C16-CH₃ | --- | --- | --- | --- | 16.5, CH₃ | 1.46, s |

*a,b* Assignments may be interchanged within the same column

2.2. Effect of the isolated terpenes on the growth of hepatocellular cancer cells

Cytotoxicity of the tested terpenes towards normal human cells were tested using varying concentrations for 24-144 h. Psiadin exhibited minimal effect until the concentration exceeded 100 µg/mL when there was about 10% cytotoxicity after 24 h, increasing to 30% only with more prolonged exposure. Plectranthone and saudinolide toxicity did not exceed 30% at the highest concentration and longest culture time (data not shown). Treatment of the cancer cell lines with the three terpenes showed differential effects as assessed by MTT and colony-forming assays (Figure 1b - d). Psiadin exhibited significant time- and dose-dependent cytotoxicity on both cell lines with very similar efficacy. After 24 h exposure, about 50% of cells died at 100 µg/mL and, after 72 h exposure, there was over 50% cell death with 10 µg/mL, with total cell kill observed with 60-80 µg/mL. The cells' ability, pre-exposed to psiadin for 24 h, to form colonies was reduced in a dose-dependent manner starting at 20 µg/mL for both cell lines. Plectranthone was also cytotoxic but required higher concentrations; for Hep3B, cells it achieved 50% cell kill at 125 µg/mL compared with 325 µg/mL for HepG2 cells after 72 h exposure. Similarly, it inhibited colony-forming ability to a much greater extent.
in Hep3B cells. Saudinolide, in contrast, had a minimal impact on HepG2 cells and 20-30% cytotoxicity on Hep3B at the highest concentration tested after 72 h exposure. For this reason, it was not tested in the colony-forming assay and further experiments excluded saudinolide in view of its lack of appreciable effect.

In a separate series of experiments, we compared the efficacy of psiadin and plectranthone with four drugs that are currently used in the clinical management of various cancers. These results are illustrated in Figure 1c. As already observed in earlier experiments, on HepG2 cells, psiadin, after 24 h, achieved 75% cell kill at 120 μg/mL, extending to 100% after 72 h with 75 μg/mL. Similar concentrations of ellipticine (ELP) and camptothecin (CPT) achieved 15-25% cell kill after 24 h and 40-70% after 72 h, while 5-fluorouracil (5FU) and doxorubicin (DOX) showed no effect at 24 h and only about 10% toxicity after 72 h. Hep3B cells gave similar results. Plectranthone after 72 h achieved a cell kill of approximately 50% in HepG2 cells. This was more than with ELP and 5FU but less than with DOX and CPT. In Hep3B cells, plectranthone was the most effective of the five drugs.
Figure 1. (a) Chemical structures of the isolated terpenes. (b) HepG2 and Hep3B cells were treated with different terpenes in a time- and dose-dependent manner, and cell viability was determined by MTT assay and expressed as % cytotoxicity. (c) HepG2 and Hep3B cells were treated with different concentrations of psiadin, plectranthone (PC2) or standard chemotherapeutic drugs (5-fluorouracil, doxorubicin, camptothecin and ellipticine) for 24 h and 72 h. Then, cell viability was measured by MTT assay and expressed as % cytotoxicity. (d) Colony formation assay was performed to determine HepG2 and Hep3B cells viability, following treatment with psiadin and plectranthone (PC2) and incubation for 10-14 days. Each data point represents mean ± SEM for triplicate samples expressed as percentage of treated to untreated condition. Asterisks denote significant difference from the control/vehicle, with \( p < 0.01 \) (*) and \( p = 0.05 \) (**).
2.3. Effect of psiadin and plectranthone on cell cycle distribution of HepG2 and Hep3B cells

The percentage of cells in each stage of the cell cycle was determined by flow cytometry following 24 h exposure to increasing concentrations of the two terpenes. These data (from one representative experiment, repeated three times) are shown in Figure 2a. Treatment of HepG2 cells with psiadin resulted in increased accumulation of cells in G1/G0 and in G2/M phases with consequently fewer cells were entering the S phase as compared with vehicle (DMSO) treated cells. With plectranthone, we did not observe any significant change in cell cycle distribution. In the case of HepB3 cells treated with psiadin, the percentage of cells in G1/G0 did not change, whereas they increased by about 40%, with less cells in S-phase with increasing drug concentration. With plectranthone, on the other hand, there was a small increase in the percentage of cells in G1/G0 with less in S phase and G2/M.

2.4. Effect of psiadin and plectranthone on apoptosis in HepG2 and Hep3B cells

Figure 2b illustrates the data from one representative experiment (repeated three times with overall similar outcome) on the effect of treating cells with 100 and 400 μg/mL of psiadin. For psiadin treated HepG2, there was a large decrease in the proportion of viable cells from about 91 to 37% and very substantial increases in early apoptotic, late apoptotic, and necrotic cells. After plectranthone treatment, there was a smaller fall in viable cells of about from 90 to 75%, with corresponding rise in late apoptotic and necrotic cells. For Hep3B cells, psiadin reduced living cells from 81 to just 2%, increased early apoptotic cells from 2 to 5%, late apoptotic cells from 12 to 82%, and necrosis from 5.4% to 11%. Plectranthone induced apoptosis in both HepG2 and Hep3B only at high concentrations (≥ 600 μg/mL; data not shown).
Figure 2. Psiadin and plectranthone modulate cell cycle and induce apoptosis in hepatic cancer cells. (a) Alterations in the cell cycle distribution were seen in HepG2 and Hep3B cells treated with the indicated concentrations of psiadin and plectranthone for 24 h. At least three samples were analyzed, and 20,000 events were scored for each sample. The vertical axis represents the relative number of events and the horizontal axis represents the fluorescence intensity. (b) HepG2 and Hep3B cells were treated with the indicated concentrations of psiadin for 24 h, followed by staining with Annexin V-FITC/PI, and then analyzed by flow cytometry. B1: % necrosis, B2: % late apoptosis, B3: % living cells, B4: % early apoptosis.

2.5. Changes in mitochondrial membrane potential induced by psiadin and plectranthone

A fluorescent dye from Abcam was used for measuring the change (Δψm), in the mitochondrial membrane potential (MMP), a key indicator of severe cellular damage. The MitoNIR dye accumulates in the mitochondria. In apoptotic cells, the NIR stain intensity is decreased due to the collapse of MMP. Cells stained with NIR dye can be visualized with a flow cytometer at red excitation and far-red emission (FL4 channel).
The results of one experiment (representative of three independent determinations) are shown in Figure 3a. The shift in the spectral emission of the dye indicates the dissipation of the MMP. (calibration was performed using CCCP). Both psiadin and plectranthone induced a change in the Δψm. The effect was more pronounced in Hep3B cells.

2.6 Effect of psiadin and plectranthone on NFκB DNA-binding activity

Figure 3b shows the measurements for the binding of nuclear extracts of HepG2 and Hep3B cells to immobilized DNA sequences representing the NFκB binding sites. Specific binding was confirmed with the purified NFκB protein included with the kit. Cell extracts treated with DMSO vehicle showed a significant level of binding for both cell extracts. Both drugs significantly inhibited this binding to an approximately similar degree.

2.7 Measurement of reactive oxygen species generation

As shown in Figure 3c, neither cell line under control conditions exhibited any observable fluorescence using a ROS detection kit. However, treatment with either psiadin or plectranthone resulted in the detection of intense fluorescence in both cell populations indicating ROS presence.
Figure 3. Psiadin and plectranthone induce mitochondrial-mediated apoptosis, inhibit NF-kB, and initiate ROS generation in HepG2 and Hep3B cells. (a) Mitochondrial membrane potential detection by MitoNIR dye staining after treating the cells with the indicated concentrations of each terpene for 24 h. The produced fluorescence intensity was measured by flow cytometry in the FL4-H channel. (b) NFkB DNA-binding activity where cells were treated with the indicated concentrations of psiadin (PS) or plectranthone (PC2) for 24 h. Histobars represent mean ± SEM of three independent determinations. NSB: non-specific binding, PC: positive control, Veh: vehicle, dsDNA: specific competitor. Drug treatments significantly differ from control/vehicle with p > 0.01 (*) and p = 0.02 (**). (c) ROS generation where HepG2 and Hep3B were treated with the indicated concentrations of each terpene for 48 h, and then examined with inverted immunofluorescent microscope.
2.8. Protein expression

Using western blotting, the levels of various proteins known to be involved in cell cycle regulation and apoptotic processes, and some associated with cell signaling pathways, were measured. We limited this study to psiadin as the most effective of the three compounds under investigation; to one of the two cell lines since they had so far shown broadly similar behavior (Figure 4). Panel A shows the expression of proteins known to be associated with the cell cycle. Psiadin substantially reduced CDK4 in psiadin-treated cells. CDK6 was also lower, though to a lesser extent. Moreover, psiadin increased the level of p-KIP, a CDK interacting protein/kinase inhibitory protein. Panel B shows several pro-apoptotic factors; AIF and BID were unchanged, whereas BAK was increased. Among the caspases tested, no change was seen in Procaspase, Caspase 3 or 8. Cleaved Caspase 9 was either unchanged or lower. Among the anti-apoptotic factors (Panel C), there was a small reduction in CIAP 2, no change in CIAP 1 or MCL a pro-survival member of the BCL2 family, increase in FLIP. We examined a few mitochondrial proteins (Panel D) and did not find much change in SMAC, a very small increase in BCLX, and a large increase in Cyt C. We also looked at the RB protein because of its well-established role as a tumor suppressor and cell cycle regulation (Panel E). There was a significant reduction in the levels of both total and phosphorylated forms of the RB protein. Examples of some essential proteins involved in cell signaling pathways are shown in Panel F. Total and, phosphorylated forms of AKT did not change. Nor did we observe any changes in MEK or p-MEK. Finally, in Panel G, it can be seen that PARP was unchanged.
Figure 4. Expression of proteins implicated in various cellular functions measured in HepG2 cells treated with psiadin (PS) or vehicle control (Veh). Protein extracts were electrophoresed on polyacrylamide gels, electroblotted onto PVDF membranes, cut into thin strips, and incubated with appropriate primary antibodies, followed by secondary antibodies and visualized by autoradiography with ECL reagents as described in Methods. For each group of proteins, KIP was used as loading control. Blots are representative of at least three independent experiments yielding very similar results.

3. Discussion
Standard cancer chemotherapy relies on a limited number of favored drugs to inhibit various aspects of cancer cell growth. However, both the substantial ‘collateral damage’ to normal tissues due to lack of specificity as well as the emergence of intrinsic or acquired drug resistance [24] presents a serious therapeutic problem. The need for new drugs has prompted studies evaluating possible anticancer agents in natural resources. Natural products with diverse bioactivities are becoming an important source of novel agents with pharmaceutical potential. The potential for plant extracts to act as anticancer agents is due to their abilities to inhibit tumor growth, angiogenesis, and metastasis with few side effects [2]. In this study we isolated, confirmed the identities of three terpenes; psiadin, plectranthone and saudinolide, and tested them against two commonly used hepatic carcinoma cell lines.

The designated plants were collected, authenticated and voucher specimens were processed appropriately. Their coarsely powdered aerial parts were extracted and purified as detailed above.

Psiadin was isolated as colorless needles. The IR spectrum of psiadin showed absorption bands for carbonyl and hydroxyl groups. The presence of a ketone carbonyl group was further supported from the $^{13}$C NMR spectra, which exhibited 20 carbon resonances; one of them is a ketone carbonyl, which resonated as a singlet at $\delta$ 212.7. Its molecular formula was determined as C$_{20}$H$_{30}$O$_{3}$ based on the ion peak at m/z 319.3400 [M+1]$^+$. The NMR spectral data (Table 1) showed signals characteristic of exocyclic methylene group ($\delta$ 4.82, 4.76, each resonated as a singlet and integrated for 1H, H-17; $\delta$ 103.6, t) and a single methyl group ($\delta$ 1.06, s, 3H, H-20; $\delta$ 19.3, q). Additionally, the presence of two hydroxymethyl groups was confirmed. One group (H-18) resonated at $\delta$ 3.85 as a singlet and integrated for 2H ($\delta$ 70.9, t), and the other one (H-19) resonated at $\delta$ 3.74, 1H, d, J=10.5 Hz, and at $\delta$ 3.54, 1H, d, J=10.5 Hz ($\delta$ 65.4, t). These data are consistent with a kaur-16-ene diterpene structure and identical to those previously reported for psiadin [25].

Plectranthone was isolated as colorless prisms. The molecular formula of this compound was determined as C$_{10}$H$_{16}$O$_{2}$ on the basis of the ion peak at m/z 277.2827 [M - CH$_2$COOH+1]$^+$ and the NMR data. Its IR spectrum showed absorption bands for two different carbonyl functions, an $\alpha,\beta$-unsaturated ketone (1670 cm$^{-1}$) and an ester band (1725 cm$^{-1}$). The $^{13}$C NMR spectrum (Table 1) showed 19 carbon resonances, including five quartets, four triplets, five doublets, and five singlets.

The presence of an isopropyl group was indicated by the fact that two of the methyl carbons resonated at $\delta$ 21.1 and 21.2 and correlated with two doublets, each of which integrated for three protons and resonated at $\delta$ 0.88 (f = 6.7 Hz) and 0.86 (f = 6.7 Hz). The two olefinic carbons of the $\alpha,\beta$-unsaturated ketone resonated as a singlet ($\delta$ 158.0, C-4) and a doublet ($\delta$ 128.7, C-3), with the carbonyl carbon absorbing at $\delta$: 198.4. The $^1$H NMR spectrum of this compound exhibited two AB coupling systems. One of these was due to the protons of the isolated methylene group at C-1 and the other one was ascribed to the pair of protons at C-14. Other 2D NMR data confirmed the identity of this compound to be the eudesmane sesquiterpene plectranthone [13].

On the other hand, saudinolide was isolated as colorless granules. This compound, possessing the molecular formula C$_{20}$H$_{31}$O$_{6}$, was found to have a $\gamma$-lactone ($\lambda_{\text{max}}$ 1780 cm$^{-1}; \delta$: 174.7), a $\delta$-lactone ($\lambda_{\text{max}}$ 1720 cm$^{-1}; \delta$: 172.9), and a mono-substituted furan ring. Additionally, $^1$H and $^{13}$C NMR data (Table 1) indicated the presence of a hydroxyl group at C-5 ($\lambda_{\text{max}}$ 3520 cm$^{-1}; \delta$: 72.5, C), a disubstituted double bond at C-14(15) ($\delta$: 14 126.2, $\delta$: 13 132.3), and an oxide bridge at C-7(10) ($\delta$: 7 108.4; $\delta$: 10 100.6). Furthermore, a COSY experiment suggested the presence of the system -CH=CHCH(O)-, and this was confirmed by the HSQC experiment, which showed signals at ($\delta$: 73.0 (CH2), 44.3 (C), 126.2 (CH),...
132.3 (CH), and 48.8 (C) assigned to C-1, C-13, C-14, C-15, and C-16, respectively. C-10 showed a high degree of oxygenation as indicated by the characteristic resonances at $\delta$ 5.93 (s) and $\delta$ 100.6 (CH). This last piece of information, together with the fact that the C-7 signal occurred at $\delta$ 108.4, suggested the gross structure of saudinolide. Other NMR data were in accordance with those previously assigned to the diterpene saudinolide [12].

The results presented in this study showed that none of the tested terpenes had appreciable toxicity against normal human cells. Saudinolide, at the concentrations tested, was also relatively ineffective against the cancer cells and was not further considered. Psiadin and plectranthone showed appreciable activity against both cancer lines, with psiadin being the more effective killing agent. To put this into more therapeutic context, we compared the effect of the two terpenes with several agents that are currently used in clinical practice. In both cases they compared very favorably in their efficacy, particularly psiadin, showing itself to be significantly more effective than standard chemotherapy drugs such as DOX and 5FU.

To determine how these terpenes may exert their inhibitory actions, we looked at several selected cellular processes involved in proliferation/cell death and established cancer progression hallmarks. Examination of the cell cycle stage showed that psiadin and plectranthone had different effects on the two cell lines. In HepG2 cells, psiadin caused cell cycle arrest in both the G1/G0 and G2/M phases, thereby preventing cells from initiating DNA replication needed for cell division. In Hep3B cells, the blockade was seen only in the G2/M phase suggesting differences in cellular response to this compound. Plectranthone did not seem to have any significant degree of influence on the cell cycle in either cell line, suggesting that its effect on proliferation was mediated differently from psiadin. Eukaryotic cells are dependent upon the production of various cyclin proteins, which through interaction with corresponding cyclin-dependent kinases facilitate cells’ progression through cell cycle checkpoints, which ultimately leads to cell division. Mitogenic signals, such as those mediated through the ERK/MEK pathway, result in the association of cyclins D with CDK4/6 leading to increased phosphorylation and, thereby, deactivation of the tumor suppressor RB protein [26]. This then relieves its repression of transcription factor E2F, allowing it to transcribe cyclins A and E, combining with CDK2 to take the cell through the G1 and G2/M checkpoints. We observed a general decrease in all the cyclins that were measured as well as in CDK4 in HepG2 cells treated with psiadin, indicating complete disruption of the cycling process. There was a small reduction in CDK2 which is thought to drive cells’ progression into S and M phase [27]. An interacting factor p-KIP [28] was increased by psiadin. This is known to inhibit the interaction of CDK4/6 with cyclin D which complex is involved in RB phosphorylation. It may also block CDK2 interaction with cyclin E which allows cells to exit from G1 into G0.

Failure to progress through cell cycle checkpoints is thought to lead to apoptosis, and as with other natural products [29, 30] this seems to be the consequence of psiadin exposure. Consistent with the MTT assay data, the viability of both cell lines was drastically reduced with a concurrent increase in cells undergoing both early and late phases of apoptosis as well as direct necrosis. Apoptosis is often divided into the extrinsic and intrinsic or mitochondrial pathways [31] involving activation of several caspases in the formation of an apoptosome, [32] also involving BCL2 family members such as BAX and BAK and release of pro-apoptotic proteins including cyt C [33]. Thus, psiadin in HepG2 cells elevated BCLX and BAK and substantially increased cyt C but surprisingly did not appear to increase caspases. Psiadin also did not affect the level of poly (ADP-ribose) polymerase (PARP),
which is often involved in DNA repair. The mitochondrial membrane potential ($\Delta \Psi_m$), generated by proton pumps (Complexes I, III and IV), is critical for maintaining the physiological function of the respiratory chain to generate ATP [34]. Opening the mitochondrial permeability transition pore (MPTP) leads to the fairly catastrophic collapse of the $\Delta \Psi_m$ and subsequent release of cyt C into the cytosol, which in turn triggers other downstream events in the apoptotic cascade as mentioned above. Our data show that both psiadin and plectranthone mimicked the effects of a mitochondrial uncoupler (4-(trifluoromethoxy)phenyl carbonohydrazonoyl dicyanide) in producing some reduction in the $\Delta \Psi_m$ in both cell lines, indicating that these compounds induce end-stage mitochondrial dysfunction. This has been observed with a number of common anti-cancer drugs [35]. The effect was most pronounced in HepB3 cells with psiadin. In HepG2 cells, psiadin caused increased production of mitochondrial proteins Cyt C and BCLX but not of AIF or BID, which are other BCL2 pro-apoptotic proteins.

It has been reported that resistance to apoptosis may be dependent on activation of NF-κB, [36] via its many target genes [37], and its action in preventing mitochondrial-mediated apoptosis through neutralization of ROS. Consistent with this are our findings that both terpenes, psiadin and plectranthone, which cause apoptosis, also significantly reduced NF-κB binding of nuclear extracts to a consensus DNA response element for both cell lines. In addition to this, we also observed a very significant production of ROS induced by both drugs and in both cell lines. In this regard, we observed a small psiadin-induced decrease in CIAP 2 known to be NF-κB regulated. Among other anti-apoptotic molecules, there was no change in FLIP or MCL. We observed no change in the survival factor AKT. Similarly, there was no effect on the levels of the ERK pathway intermediate MEK or of p38.

4. Materials and Methods

4.1. Plant material

The aerial parts of three plants; *Psiadia arabica* Jaub. et Spach. (known locally as “Tabbak”), *Plectranthus cylindraceus* Hochst. ex Benth (known locally as “Al-Shar”) and *Cluytia richardiana* L. (known locally as “Sa’eer”) were collected from Wadi Al Uss, near Abha, Saudi Arabia between March and April, 2012. The plant identities were authenticated by Dr. Sultan-ul-Abdeen and voucher specimens (# 10331, 10352 and 10362, respectively) were deposited at the herbarium of the College of Pharmacy, King Saud University, Riyadh, Saudi Arabia. All plants names have been checked with [http://www.theplantlist.org](http://www.theplantlist.org).

4.2. Extraction and isolation

*Psiadia arabica*. The powdered aerial parts (2 kg) were exhaustively extracted with chloroform in a Soxhlet for 72 h. Evaporation *in vacuo* gave a 260 g greenish residue that was partitioned between 3 L chloroform and water (3 × 1 L). The chloroform fraction, after evaporation, was partitioned between 10% aqueous methanol (2 L) and 3 portions each of 1 L of *n*-hexane. The combined hexane layers were washed with 100 ml of the methanol layer. Evaporation of the combined methanol layers gave 185 g as a gummy residue. This residue was chromatographed, in portions, on silica gel columns (each was 550 g, 25 × 7.5 cm) using CHCl₃-MeOH, 4:1, yielding 1380 mg of the impure diterpene psiadin, which upon crystallization from methylene chloride-EtOAc gave 813 mg of pure psiadin as colorless needles.
**Plectranthus cylindraceus.** The dried powdered aerial parts (1.12 kg) were percolated at room temperature with 95% EtOH (3 × 4 L), and the extract was evaporated *in vacuo* to leave 22 g of residue, which was chromatographed over silica gel (500 gm, 4 × 130 cm), using increasing concentrations of EtOAc in *n*-hexane as an eluent, to yield, after crystallization from EtOAc-petroleum ether, 1400 mg of plectranthone as colorless prisms.

**Cluytia richardiana.** The dried powdered aerial parts (1.6 kg) were percolated successively at room temperature in petroleum ether (60-80 °C) and then in EtOAc. Upon drying *in vacuo*, the EtOAc fraction afforded 65 g of greenish-white precipitate, which was filtered off to yield 26 g of the precipitate. This precipitate was subjected to chromatographic purification over flash silica gel (2.6 kg). Elution with petroleum ether (60-80 °C)-EtOAc (8.5:1.5) yielded 560 mg saudinolide. Crystallization from EtOAc-petroleum ether afforded 450 mg as colorless granules.

### 4.3. Terpenes identification

Melting points were determined in open capillary tubes using a Mettler 9100 electrothermal melting point apparatus and were uncorrected. IR spectra were recorded using a JASCO FTIR-4100 spectrophotometer. UV spectra were measured in MeOH using a UV-160 IPC UV-visible dual-beam spectrophotometer. The ¹H and ¹³C NMR spectra were obtained on a Bruker Advance II 600-MHz spectrometer operating at 600 and 150 MHz, respectively. Both ¹H and ¹³C NMR spectra were recorded in CDCl₃, and the chemical shift values were expressed in δ (ppm) relative to the internal standard TMS. For the ¹³C NMR spectra, spectral editing was determined by DEPT. 2D NMR data were obtained using the standard pulse sequence of the Bruker 600 for COSY, HSQC and HMBC. High resolution ESMS were obtained using a double-focusing magnetic sector mass spectrometer (GC-MS DFS, Thermo).

### 4.4. Cell lines

Human hepatic carcinoma cells Hep3B and HepG2, were obtained from the American Type Culture Collection (ATCC; VA, USA). They were cultured in 90% Leibovitz’s L15 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS) and grown at 37°C in an incubator with 5% CO₂ atmosphere.

### 4.5. Chemicals and reagents

Trypsin, Leibovitz’s L-15 and EMEM medium, FBS, and penicillin/ streptomycin solution (100×) were obtained from Mediatech (Herndon, VA, USA). Annexin V-FITC apoptosis detection kit was obtained from BD Hoffmann-La Roche Inc. (NJ, USA). DNA extraction kit was obtained from Beckman & Coulter (FL, USA). Nuclear/cytosol fractionation kit was obtained from BioVision, Inc. (Mountain View, CA, USA). Antibodies used in this study and their sources are listed in the Supplementary Table. All other reagents were purchased from Sigma Chemicals (MO, USA).

### 4.6. MTT assay

Cell viability was determined at various times (24–144 h) using MTT (3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide) assay, which is based on the conversion of MTT to formazan crystals by mitochondrial dehydrogenases [38]. Briefly, cells were seeded at 27 × 10³ cells/well in 96-well plates and incubated at 37 °C for 24 h. The cells were then treated with various
concentrations of each terpene under investigation (or 0.2% DMSO vehicle for control condition) for various times (24-144h). MTT solution (5 mg/ml) was added to the incubated cells (20 μl/well); the cells were then incubated for another 4 h and the medium was discarded. DMSO (200 μl) was added to each well, and the absorbance measured in a micro-plate reader at 492 nm. All samples were assayed in triplicate in three independent experiments. The results (mean ± SEM) are expressed as % cytotoxicity; by dividing the densitometric readings for the drug treated conditions by that of the DMSO treated control.

In a separate series of experiments, the effect of each of the terpenes on the cancer cell lines was compared with camptothecin (CPT), 5-fluorouracil (5FU), doxorubicin (DOX) and ellipticine (ELP) over 72h of drug exposure.

As we later determined that the terpenes had effects on mitochondrial function and therefore might affect the MTT assay which depends on a reaction occurring within the mitochondria we also used the MTX assay as well as doing direct cell counting for a number of drug treated samples and obtained similar results. Therefore, we concluded that the reaction involving MTT was not affected.

4.7. Colony forming assay

Briefly, cancer cells (10^5/well) were plated in a 24-well plate and incubated at 37°C for 18 h. Thereafter, the cells were treated with serial doses of the test compounds (or DMSO vehicle for controls) and incubated at 37°C for 24 h. After that, cells were collected by trypsinization, washed with Hank’s Balanced Salt Solution (HBSS), counted and plated in 24-well plates at 500 cells/well, and incubated at 37°C for 10-14 days. The resultant colonies were washed with cold phosphate-buffered saline (PBS), fixed with 100% methanol, and stained with 0.1% crystal violet. The colonies were counted using an inverted microscope [39].

4.8. Cell cycle analysis

The distribution of cell-cycle phases (G_0/G_1, S and G_2/M) was determined using flow cytometry by measuring the DNA content of nuclei labeled with propidium iodide as described previously [40]. Briefly the cancer cell lines were plated at 2.5 x 10^5 cells/ml in 24-well plates and incubated at 37°C for 18 h. The cells were then treated with psiadin (100-400 μg/ml) and plectranthone (200-800 μg/ml) or DMSO vehicle for 24 h. Cells were collected by trypsinization, washed with cold PBS and counted. Cells were processed using a DNA-prep kit (Beckman & Coulter, USA). For this end, the cells were treated with a cell-membrane permeabilizing agent followed by propidium iodide and RNase. The samples were incubated at room temperature for 15 min before analysis by flow cytometry (FC500, Beckman & Coulter, USA). The percentages of cells in different cell cycle phases were calculated using the statistical software package, advanced DNA cell-cycle software from Phoenix Flow System (San Diego, USA).

4.9. Apoptosis assay

Induction of apoptosis was determined by annexin V-FITC apoptosis detection kit (BD Hoffmann La Roche Inc, USA) according to the manufacturer’s instructions. Briefly, cancer cells were plated (2.5 x 10^5 cells/ml) in 24-well plate and incubated at 37°C for 18 h. Following treatment with psiadin (100-400 μg/ml), plectranthone (200-800 μg/ml) or DMSO vehicle for 24 h, cells were re-
suspended in 100 µl of annexin V fluorescein and propidium iodide in HEPES buffer. Following incubation in darkness at room temperature for 15 min, cells were analyzed by flow cytometry.

4.10. Measurement of mitochondrial membrane potential

To determine whether mitochondrial damage occurs as an early event in psiadin or plectranthone-induced apoptosis, changes in mitochondrial membrane potential (ΔΨm) were measured using flow cytometry with NIR Mitochondrial Membrane Potential Assay Kit (Ab112149) from Abcam (UK). Cancer cells were plated at 2.5 x 10^5 cells/ml in 24-well plates at 37°C for 18 h followed by treatment with psiadin (50, 100 µg/ml) and plectranthone (200, 400 µg/ml) for 24 h. The adherent cells were gently detached with 0.5 mM EDTA to keep them intact and washed once with serum-containing media prior to incubation with NIR dye loading solution. MitoNIR Dye (5 µl) was added and the cells incubated at 37°C for 15-30 min then centrifuged at 1000 rpm for 4 min, re-suspended in 1ml of assay buffer and fluorescence intensity monitored in the FL4 channel (Ex / Em = 635/660) using a flow cytometer gated on the cells of interest. Experimental conditions were standardized using the mitochondrial uncoupler, ((3-chlorophenyl)hydrazono)malononitrile, (CCCP) from Abcam (Ab141229) as a positive control.

4.11. NFκB DNA-binding activity

Cancer cells were plated at 2.5 x 10^5 cells/ml into 24-well plates at 37°C for 18 h, then treated with psiadin (50, 100 µg/ml), plectranthone (200, 400 µg/ml) or DMSO vehicle for 24 h. Cells were harvested and nuclear extracts prepared using a cytosol/nuclear fractionation kit from BioVision (CA, USA) as per the manufacturer’s instructions. NF-κB DNA-binding activity was determined using an NF-κBp65 Transcription Factor Assay Kit (ab133112; Abcam, UK) which utilizes a 96-well plate with a specific DNA sequence containing the NF-κB response element immobilized onto the bottom of the wells. Following the manufacturer’s protocol, nuclear cell extracts were added to wells and incubated with shaking for 1h at room temperature. Following washing 5 times with wash buffer, NF-κB specific primary antibody was added to wells for 1h, subsequent washing as above, then addition of goat anti-rabbit HRP conjugated secondary antibody for another 1h. After further extensive washing and incubation for 15-45 min with developer solution, absorbance at 450 nm was determined in a plate reader.

4.12. Measurement of reactive oxygen species generation

Cancer cells were plated at 2.5 x 10^5 cells/ml into 24-well plates at 37°C for 18 h. Media was removed and cells gently washed with HBSS (2-3 times) followed by addition of 100 µl of 1x 2’7’-dichlorofluorescein diacetate (DCFH-DA) (Sigma, USA) in PBS for 30-60 min in the dark at 37°C. Solution was then removed and cells were washed with HBSS (2-3 times). The DCFH-DA loaded cells were then treated with psiaidin (50, 100 µg/ml), plectranthone (200, 400 µg/ml) or DMSO vehicle for 24 h. Cells were then examined by inverted fluorescence microscopy.

4.13. Western blot analysis

Cancer cells were cultured in 6 well plates (5 x 10⁶ cells/ml) to approximately 75-80% confluency and then treated with psiadin (75 µg/ml) or DMSO vehicle. After 24h exposure cells were harvested and recovered by centrifugation for 5 min at 1000 x g. Fresh cell pellets were washed with PBS and
then re-suspended into 300 μl of homogenization buffer containing 50 mM HEPES, 50 mM NaCl, 5 mM EDTA, 1% Triton X-100, 10 μg/ml leupeptin, 10 μg/ml aprotinin and 100 μg/ml PMSF. The Pierce BCA protein assay dye reagent (Pierce, Rockford, USA) was used to determine protein concentration in the cell lysate. About 30 μg of protein was mixed with an equal volume of 2 x sample loading buffer containing 100 mM Tris-Cl (pH 6.8), 4% (w/v) SDS, 0.2% (w/v) bromophenol blue, 20% (v/v) glycerol and 200 mM dithiothreitol, and heated at 90°C for 10 min. Lysates were loaded onto 10% SDS-polyacrylamide gels and electrophoresed at 150 V for 1 h. Proteins were then transferred onto a PVDF membrane and, using the molecular weight markers lane as a guide, cut into narrow strips (in order to economize on antisera) in the size range of the expected protein to be detected. Each strip was then treated with 2% BSA for 1 h before being incubated overnight at 4°C with various primary antibodies (listed in Supplementary Table). After removal of the antisera the membrane was washed and subsequently incubated with anti-HRP-conjugated secondary antibody (1:500) for 1 h, and signals developed with Super Signal ECL and visualized with Kodak X-ray film. Bands were quantified by densitometry and intensity calculated proportional to that obtained for β actin on the same blot.

4.14. Statistical analysis

Differences between mean values of tested terpenes was analyzed by the student’s t-test or one-way ANOVA followed by Bonferroni post-hoe test using GraphPad Prism 8. P<0.05 was considered statistically significant.

5. Conclusions

In summary, we have examined the anti-cancer potential of three terpenes purified from natural products. Saudinolide was not useful, and plectanthrone was effective in some processes but only at very high concentrations. Psiadin, on the other hand, could be a very promising lead compound. It showed little activity against normal cells but was significantly more effective at killing two hepatic cancer cell lines than several currently used chemotherapeutic drugs. We examined several processes commonly associated with cancer cell metabolism but were unable to pinpoint any specific cellular target so that it may have a wide range of activity. Although, cytotoxic agents often result in many changes that are secondary to their primary effect, however, we did observe disruption of a number of specific cellular pathways, most prominently cell cycle proteins, stimulation of mitochondrially mediated apoptotic pathways, very pronounced induction of ROS, and inhibition in expression and function of the RB protein. In future experiments it would be worthwhile to examine genome–wide expression and perform whole proteome analysis of psiadin-treated cells. It will also be necessary to test the effects of psiadin on other cell lines to determine its general applicability. Also, in vivo animal studies, which were beyond the present investigations’ scope, would be the next step to see whether psiadin is active against tumors, having established its effects in vitro.

Supplementary Materials: Table S1: Antibodies used in this project along with their target proteins and sources.

Author Contributions: Conceptualization, K.Y.O. and M.S.A.; methodology, K.Y.O, M.S.A., R.A. and Y.A.L.; validation, K.Y.O, M.S.A. and Y.A.L.; formal analysis, K.Y.O and Y.A.L.; investigation, K.Y.O., M.S.A. and Y.A.L.; resources, K.Y.O. and M.S.A.; data curation, K.Y.O., M.S.A. and Y.A.L.; writing—original draft preparation, K.Y.O. and Y.A.L.; writing—review and editing, K.Y.O., M.S.A. and Y.A.L.; visualization, K.Y.O.; supervision, K.Y.O.; project administration, K.Y.O.; funding acquisition, K.Y.O. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by of Kuwait University Research sector, grant number PC01/12. and grant SRUL.02/13 to the Research Unit for Genomics, Proteomics and Cellomics Studies. The spectral analyses were
obtained at the Research Sector Project Unit (RSPU), Faculty of Science, Kuwait University, supported by Grant numbers GS01/01 and GS01/03.

Acknowledgments: Ms. Samar Faggal is acknowledged for her excellent technical assistance with the protein expression analysis.

Conflicts of Interest: The authors declare no conflict of interest.

References:
1. American Cancer Society Global Cancer Facts and Figure. 2020; pp https://www.cancer.org/research/cancer-facts-statistics/global.html
2. Newman, D. J.; Cragg, G. M., Natural Products as Sources of New Drugs over the Nearly Four Decades from 01/1981 to 09/2019. J Nat Prod 2020, 83 (3), 770-803.
3. Ulbricht, C. E.; Chao, W., Phytochemicals in the oncology setting. Curr Treat Options Oncol 2010, 11 (3-4), 95-106.
4. Cragg, G. M.; Pezzuto, J. M., Natural Products as a Vital Source for the Discovery of Cancer Chemotherapeutic and Chemopreventive Agents. Med Princ Pract 2016, 25 Suppl 2, 41-59.
5. Lee, K. W.; Bode, A. M.; Dong, Z., Molecular targets of phytochemicals for cancer prevention. Nat Rev Cancer 2011, 11 (3), 211-8.
6. Tan, A. C.; Konczak, I.; Sze, D. M.; Ramzan, I., Molecular pathways for cancer chemoprevention by dietary phytochemicals. Nutr Cancer 2011, 63 (4), 495-505.
7. Mates, J. M.; Segura, J. A.; Alonso, F. J.; Marquez, J., Anticancer antioxidant regulatory functions of phytochemicals. Curr Med Chem 2011, 18 (15), 2315-38.
8. Neergheen, V. S.; Bahorun, T.; Taylor, E. W.; Jen, L. S.; Aruoma, O. I., Targeting specific cell signaling transduction pathways by dietary and medicinal phytochemicals in cancer chemoprevention. Toxicology 2010, 278 (2), 229-41.
9. Karikas, G. A., Anticancer and chemopreventing natural products: some biochemical and therapeutic aspects. J BUON 2010, 15 (4), 627-38.
10. Mossa, J. S., Medicinal Plants of Saudi Arabia. King Saud University Press: Saudi Arabia, 2000.
11. Mossa, J. S.; El-Denshary, E. S. M.; Hindawi, R.; Ageel, A. M., The Hypoglycaemic Effect of Saudin. International Journal of Crude Drug Research 1988, 26 (2), 81-87.
12. Mossa, J. S.; Muhammad, I.; Al-Yahya, M. A.; Mirza, H. H.; El-Feraly, F. S.; McPhail, A. T., Five New Modified 6,7- Secolabdane Diterpenoids from Cluytia richardiana. Journal of Natural Products 1996, 59 (3), 224-231.
13. Orabi, K. Y.; Mossa, J. S.; Muhammed, I.; Alloush, M. H.; Galal, A. M.; El-Feraly, F. S.; McPhail, A. T., New eudesmane sesquiterpenes from Plectranthus cylindraceus. J Nat Prod 2000, 63 (12), 1665-8.
14. El-Domiaty, M. M.; El-Feraly, F. S.; Mossa, J. S.; McPhail, A. T., Diterpenes from Psiadia arabica. Phytochemistry 1993, 34 (2), 467-471.
15. Mahadeo, K.; Grondin, I.; Kodja, H.; Soulange Govinden, J.; Jhaumeer Laulloo, S.; Frederick, M.; Gauvin-Bialecki, A., The genus Psiadia: Review of traditional uses, phytochemistry and pharmacology. J Ethnopharmacol 2018, 210, 48-68.
16. Aumeeruddy-Elalfi, Z., Antimicrobial, antibiotic potentiating activity and phytochemical profile of essential oils from exotic and endemic medicinal plants of Mauritius. Industrial crops and products 2015, v. 71, pp. 197-204-2015 v.71.
17. Govinden-Soulange, J.; Magan, N.; Gurib-Fakim, A.; Gauvin, A.; Smadja, J.; Kodja, H., Chemical composition and in vitro antimicrobial activities of the essential oils from endemic Psiadia species growing in mauritius. *Biol Pharm Bull* 2004, 27 (11), 1814-8.

18. Fortin, H.; Vigor, C.; Lohezic-Le Devehat, F.; Robin, V.; Le Bosse, B.; Boustie, J.; Amoros, M., In vitro antiviral activity of thirty-six plants from La Reunion Island. *Fitoterapia* 2002, 73 (4), 346-50.

19. Robin, V.; Boustie, J.; Amoros, M.; Girre, L., In-vitro Antiviral Activity of Seven Psiadia Species, Asteraceae: Isolation of Two Antipoliovirus Flavonoids from Psiadia dentata. *Pharmacy and Pharmacology Communication* 1998, 4 (1), 61-64.

20. Jonville, M. C.; Kodja, H.; Humeau, L.; Fournel, J.; De Mol, P.; Cao, M.; Angenot, L.; Frederick, M., Screening of medicinal plants from Reunion Island for antimalarial and cytotoxic activity. *J Ethnopharmacol* 2008, 120 (3), 382-6.

21. Jonville, M. C.; Kodja, H.; Strasberg, D.; Pichette, A.; Ollivier, E.; Frederick, M.; Angenot, L.; Legault, J., Antiplasmodial, anti-inflammatory and cytotoxic activities of various plant extracts from the Mascarene Archipelago. *J Ethnopharmacol* 2011, 136 (3), 525-31.

22. Recio, M. C.; Giner, R. M.; Márquez, S.; Rios, J. L.; Marston, A.; Hostettmann, K., Screening of tropical medicinal plants for antiinflammatory activity. *Phytotherapy Research* 1995, 9 (8), 571-574.

23. Ali, N. A. A.; Wurster, M.; Denkert, A.; Arnold, N.; Faday, I.; Al-Didamony, G.; Lindequist, U.; Wessjohann, L.; Setzer, W. N., Chemical Composition, Antimicrobial, Antioxidant and Cytotoxic Activity of Essential Oils of Plectranthus cymindraceus and Meriandra benghalensis from Yemen. *Natural Product Communications* 2012, 7 (8), 1934578X1200700834.

24. Luqmani, Y. A., Mechanisms of drug resistance in cancer chemotherapy. *Med Princ Pract* 2005, 14 Suppl 1, 35-48.

25. Mossa, J. S.; El-Domiaty, M. M.; Al-Meshal, I. A.; El-Feraly, F. S.; Hufford, C. D.; McPhail, D. R.; McPhail, A. T., A flavone and diterpene from Psiadia arabica. *Phytochemistry* 1992, 31 (8), 2863-2868.

26. Giacinti, C.; Giordano, A., RB and cell cycle progression. *Oncogene* 2006, 25 (38), 5220-7.

27. Tadesse, S.; Caldon, E. C.; Tilley, W.; Wang, S., Cyclin-Dependent Kinase 2 Inhibitors in Cancer Therapy: An Update. *J Med Chem* 2019, 62 (9), 4233-4251.

28. Quereda, V.; Portlan, E.; Cañamero, M.; Dubus, P.; Malumbres, M., An essential role for Ink4 and Cip/Kip cell-cycle inhibitors in preventing replicative stress. *Cell Death Differ* 2016, 23 (3), 430-441.

29. Zhong, Z.; Chen, X.; Tan, W.; Xu, Z.; Zhou, K.; Wu, T.; Cui, L.; Wang, Y., Germacrone inhibits the proliferation of breast cancer cell lines by inducing cell cycle arrest and promoting apoptosis. *European Journal of Pharmacology* 2011, 667 (1), 50-55.

30. Park, H. S.; Kim, G. Y.; Nam, T. J.; Deuk Kim, N.; Hyun Choi, Y., Antiproliferative activity of fucoidan was associated with the induction of apoptosis and autophagy in AGS human gastric cancer cells. *J Food Sci* 2011, 76 (3), T77-83.

31. Yoon, J. H.; Gores, G. J., Death receptor-mediated apoptosis and the liver. *J Hepatol* 2002, 37 (3), 400-10.

32. Sun, S.-Y.; Hail, N., Jr; Lotan, R., Apoptosis as a Novel Target for Cancer Chemoprevention. *INCI: Journal of the National Cancer Institute* 2004, 96 (9), 662-672.

33. Mignotte, B.; Vayssiere, J.-L., Mitochondria and apoptosis. *European Journal of Biochemistry* 1998, 252 (1), 1-15.

34. Zorova, L. D.; Popkov, V. A.; Plotnikov, E. Y.; Silachev, D. N.; Pevzner, I. B.; Jankauskas, S. S.; Babenko, V. A.; Zorov, S. D.; Balakireva, A. V.; Juhaszova, M.; Sollott, S. J.; Zorov, D. B., Mitochondrial membrane potential. *Anal Biochem* 2018, 552, 50-59.
35. Varga, Z. V.; Ferdinandy, P.; Liaudet, L.; Pacher, P., Drug-induced mitochondrial dysfunction and cardiotoxicity. *Am J Physiol Heart Circ Physiol* 2015, 309 (9), H1453-67.

36. Rahman, K. M.; Ali, S.; Aboukameel, A.; Sarkar, S. H.; Wang, Z.; Philip, P. A.; Sakr, W. A.; Raz, A., Inactivation of NF-kappaB by 3,3'-diindolylmethane contributes to increased apoptosis induced by chemotherapeutic agent in breast cancer cells. *Mol Cancer Ther* 2007, 6 (10), 2757-65.

37. Karin, M., Nuclear factor-kappaB in cancer development and progression. *Nature* 2006, 441 (7092), 431-6.

38. Mosmann, T., Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J Immunol Methods* 1983, 65 (1-2), 55-63.

39. Abaza, M. S. I.; Al-Saffar, A.; Al-Sawan, S.; Al-Attiyah, R., c-myc Antisense Oligonucleotides Sensitize Human Colorectal Cancer Cells to Chemotherapeutic Drugs. *Tumor Biology* 2008, 29 (5), 287-303.

40. Abaza, M. S.; Bahman, A. M.; Al-Attiyah, R. J., Valproic acid, an anti-epileptic drug and a histone deacetylase inhibitor, in combination with proteasome inhibitors exerts antiproliferative, pro-apoptotic and chemosensitizing effects in human colorectal cancer cells: underlying molecular mechanisms. *Int J Mol Med* 2014, 34 (2), 513-32.