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Plant-derived antimicrobials have similar cytotoxic effects on cancerous and non-cancerous cells

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Abstract

Essential oils and their active components, referred here as plant derived antimicrobials (PDAs), have been used for their antimicrobial, anti-inflammatory and antioxidant properties. Many reports also document PDAs' cytotoxic effects on cancerous cells, raising the hope that they could be used for cancer treatments. Due to the lack of specificity, we hypothesize that PDAs are cytotoxic to both cancerous and non-cancerous cells. *Trans*-cinnamaldehyde (TCA), carvacrol, and eugenol were assessed for their cytotoxicity on cancerous HeLa cells and normal skin fibroblasts (CCD-1123Sk, CCD) by MTT and LDH assays, flow cytometry, and reverse transcription quantitative PCR (RT-qPCR). After 24 hours of treatment, carvacrol and TCA significantly decreased cell viability (by more than 50%) at 100 µg/ml, whereas eugenol was ineffective up to 400 µg/ml. Cell detachment and significantly increased apoptosis were observed with 100 µg/ml of TCA on both cell types. RT-qPCR for apoptotic genes (*Bcl2*, *Casp3* and *Casp8*) and necrosis genes (*Mkl1*, *Ripk1* and *Ripk3*) did not show significant differences between control and treated cells of both types, with the exception of eugenol-treated HeLa cells in which expression of *Bcl2*, *Mkl1* and *Ripk1* was significantly higher than controls. Taken together, we conclude that the three PDAs studied here exhibited similar cytotoxic effects on both cancerous and non-cancerous cells.

Introduction

Essential oils have been used for centuries around the world in foods, cosmetics and as antimicrobials and pharmaceuticals to prevent and treat diseases¹. The bioactive constituents of essential oils are secondary plant metabolites and their derivatives². An active ingredient of cinnamon oil, for example, is cinnamaldehyde, which predominately occurs in the trans form, *trans*-cinnamaldehyde (TCA)³. Similarly, eugenol (EU) and carvacrol (CAR) are the main active ingredients of the essential oils from oregano (*Origanum vulgare*) and clove (*Syzgium aromaticum*)⁴, respectively. These plant compounds, referred together here as plant-derived antimicrobials (PDAs), have been found to possess a plethora of biological activities including anti-microbial, anti-inflammatory, anti-oxidant, and anti-cancerous effects^{5,6}.

The anti-cancerous activity of PDAs has been extensively tested in many cancer cell lines such as HeLa and HCT15 cells⁷⁻¹⁰. Using the colorimetric assay of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide), TCA has been reported to exhibit time- and dose-dependent inhibition on the metabolic activities of HeLa cells^{11,12}. TCA appeared to lead to apoptosis as shown by activated caspase cascade, DNA fragmentation, and loss of mitochondrial potentials in various tumor cell lines such as HCT15, SK-MEL-2, and K562 cells^{13,14}. Carvacrol exhibited effects comparable to TCA on cancer cell lines^{15,16} and additionally arrested human prostate cancer cells at G0/G1¹⁷. This anti-proliferative effect of carvacrol was also seen in primary rat neurons and N2a neuroblastoma cells¹⁸. Likewise, eugenol acted in HeLa cells as an anti-proliferative and cyto-lytic agent in a dose-dependent manner as shown by MTT and LDH (Lactate Dehydrogenase assay), respectively^{19,20}. Moreover, eugenol also appears to be anti-metastatic by downregulating genes such as *MMP2* and *MMP9*, which are correlated to metastasis²¹.

Despite extensive reports that PDAs are anti-cancerous, virtually all studies lacked non-cancerous cell lines as the control, leading to the speculation that PDAs are specifically anti-cancerous. We hypothesize conversely that, due to common mechanisms of cytotoxicity and the lack of a specific targeting pathway, PDAs should have the same effects on non-cancerous cells as on cancerous cells. Here we directly compared cytotoxicity of three PDAs (TCA, carvacrol and eugenol) on cancerous cells (HeLa) and non-cancerous fibroblast cells (CCD-1123Sk; CCD) to support our hypothesis.

Materials and Methods

Chemicals and reagents

All chemicals and reagents were purchased from Sigma (St Louis, MO) unless noted otherwise. 10% stock solutions of TCA, carvacrol (CAR), and eugenol (EU) were made using dimethyl sulfoxide (DMSO) as the vehicle and stored at 4°C. Working concentrations of each PDA, ranging from 0-800 µg/ml, were made by diluting the stock solution with Iscove's Modified Dulbecco's Medium (IMDM) on the day of usage.

Cell culture

CCD-1123Sk (CCD) and HeLa S3 cells (ATCC CCL-2.2) were obtained from ATCC (Manassas, VA). Both types of cells were maintained in IMDM with 10% fetal bovine serum (FBS, Thermo Fisher Scientific, Hampton, NH) and L-glutamine (2 mM, GlutaMAX, Gibco) in 5% CO₂ in humidified air at 37°C. The same medium was used for all experiments, and

antibiotics were not used. For every experiment, the same passage number for the two cell types were used and cells were not used after the 7th passage from receipt of the cell lines.

MTT assay for Metabolic Activity

The MTT assay measures cellular metabolic activity, an indicator for cell viability. The Vybrant MTT Cell Proliferation Assay Kit (Cat No: V13154, Thermo Fisher, Waltham, MA) was purchased and used by following the procedure of the manufacturer. HeLa and CCD cells (10⁴ cells/200µl/well) were seeded in 96-well plates and cultured for 24 hours for proper attachment. Subsequently, the medium was replaced with that containing working concentrations of each PDA. The final concentrations of TCA, EU and CAR were 0, 12.5, 25, 50, 100, 200, 400 and 800 µg/ml. Two technical replicates were included for each treatment, and the experiment was conducted 3 times. PDA treatment was removed after 24 hours by replacing PDA medium with 100 µl of fresh medium. Then 10 µl of 12 mM of MTT solution was added to each well, mixed, and incubated at 37°C in 5% CO₂. After 4 hours, 75 µl of medium was removed and 50 µl of 100% DMSO added per well and thoroughly mixed to dissolve the purple formazan crystals. Plates were incubated for 10 minutes at 37°C, wells subjected to additional mixing and removal of air bubbles, and absorbance was read at 540 nm using a plate reader (BioTek Synergy 2).

LDH Assay for Cytotoxicity

The CyQUANT LDH Cytotoxicity Assay (Thermo Fisher, Cat No: C20301) was purchased to determine the release of lactate dehydrogenase (LDH) into the medium, a measure of cell death/plasma membrane damage. Seeding and treatment of cells were similar to the MTT assay,

with the exception of cell number (5×10^3 cells/well) for the optimal functioning of the assay. First, the spontaneous and maximum LDH activity were set up as follows: 24 hours after cell seeding, 10 μ l of sterile water (spontaneous LDH control) or lysis buffer (maximum LDH control) were added to cells not treated with PDAs. The reactions were mixed and incubated for 45 minutes at 37°C in 5% CO₂. Subsequently and from all sample wells (spontaneous and maximum LDH controls and PDA-treated tests), 50 μ l of culture medium were transferred to a new 96-well plate. To this 50 μ l of LDH reaction mixture was added, mixed, and incubated for 30 minutes at room temperature. To terminate the reactions, 50 μ l of stop solution were added, mixed, and bubbles removed by centrifugation. Absorbance of the reaction mixtures was taken at 490 nm and 680 nm. To determine LDH activity, the 680 nm absorbance value was subtracted from that of the 490 nm. Then cytotoxicity was calculated using the formula:

$$\text{Cytotoxicity (\%)} = \left(\frac{\text{compound treated LDH activity} - \text{Spontaneous LDH activity}}{\text{Maximum LDH activity} - \text{Spontaneous LDH activity}} \right) * 100\%$$

Apoptotic Assay and Cell Morphology

Mitochondrial Membrane Potential Apoptosis Kit was purchased from Thermo Fisher (Cat No. V35116). For flow cytometry, 5×10^5 cells per well were plated in 6-well plates and incubated for 24 hours at 37°C in 5% CO₂. The medium was then replaced with 2 ml IMDM containing 100 μ g/ml of TCA – a level chosen because it induced cytotoxicity in prior experiments. Cells were incubated with the TCA treatment for 4 hours, then harvested by trypsinization, centrifugation, and re-suspension in 1 ml of IMDM. To stain live cells, 4 μ l of 10 μ M MitoTracker Red working solution were added and incubated at 37°C in 5% CO₂ for 30 minutes. Cells were then washed with phosphate-buffered saline to remove the red stain. To stain

apoptotic cells, 5 μ l of Annexin V dye was added to cells suspended in 1X Annexin binding buffer and incubated for 5 minutes at room temperature. Next, 400 μ l of 1X Annexin binding buffer was added, mixed gently, and placed on ice. Cells were analyzed by flow cytometry (BD LSRFortessa X-20) at 530 and 585 nm.

RNA Isolation and Real-time Quantitative PCR (RT-qPCR)

RNA was isolated from the frozen samples using RNeasy Mini Kit. For TCA and CAR treatment, samples were collected from control and 100 μ g/ml-treated wells, whereas for eugenol treatment, samples were collected from control and 400 μ g/ml-treated wells. For cDNA synthesis, a BioRad iScript cDNA Synthesis Kit was used. Briefly, 5X iScript reaction mix (4 μ l), iScript reverse transcriptase (1 μ l of 200 U/ μ l), RNA template (50 ng), and nuclease-water was mixed to a total reaction volume of 20 μ l. The cDNA synthesis was conducted in a PCR thermocycler (MJ Research 200) at 25°C for 5 min, 46°C for 20 min, and inactivation at 95 °C for 1 min. After cDNA synthesis, two rounds of PCR were performed. The first round of PCR was 12 cycles of amplification (MJ Research 200) in 96 well plate with respective primers using Dream Taq PCR reagents (Thermo Fisher, Waltham, MA). The reaction setup was 10X Dream Taq buffer (2 μ l), forward and reverse primers (0.5 μ l each of 10 μ M), dNTP (2 μ l of 2 mM), Polymerase (0.5 μ l of 5 U/ μ l), cDNA in 2 μ l, and 13 μ l of water. The temperature profile for the reaction was: 95°C for 1 min, 60°C for 30s, and 65°C for 5s (for 12 cycles), and finally 4°C. The second round of PCR was qPCR and carried out in an Applied Biosystems 7500 Fast Real-Time PCR System. Reaction mixtures contained forward and reverse primer (0.5 μ l of 10 μ M each), 1 μ l of 100% DMSO, 6 μ l of nuclease-free water, 10 μ l of 2X SYBR Green, and 2 μ l of PCR products from the first round. The temperature profile for the reaction was: 95°C hold for 10 min,

40 rounds of cycling (95°C for 3s, 60°C for 30s), and final melt curve program (95°C for 15s, 60°C for 1 min and 95°C for 15s, 60°C for 15s). The primers (Table S1) for genes *Bcl2*, *Casp3*, *Casp8*, *Mkl*, *Ripk1* and *Ripk3* were either designed or sourced from previously published studies^{22–24}.

Statistical Analysis

All experiments were conducted with two technical replicates and repeated three times. Two-way ANOVA was performed in R with cell type and levels of PDAs as the main factors. PDAs were not compared among each other. Pair-wise comparisons of means between different levels of PDAs were made using Tukey's post-hoc analysis. A P value of less than 0.05 was considered statistically significant.

Results

Effects of PDAs on Cellular Metabolic Activity Is Dose-dependent

All PDAs (TCA, EU and CAR) reduced cellular metabolism, a measure of cell viability, in a dose-dependent manner as determined by the MTT assay. Cancerous cells (HeLa) and non-cancerous fibroblasts (CCD) were equally affected by the treatments (Figure 1). Specifically, TCA induced a gradual decrease in metabolic activity with increasing concentrations. This effect did not become significant on untreated controls until 100 µg/ml, when the metabolic activities of HeLa and CCD cells were reduced by more than half, to 48.7% and 48.13% of controls, respectively ($P \leq 0.05$; Figure 1a). The effect of TCA appears to level off from 200 µg/ml up to

166 the maximum dose tested at 800 μ g/ml, where metabolic activity was reduced to approximately
167 30% of that of untreated controls.

168 Similar to TCA, carvacrol also reduced metabolic activity on both cell types in a dose-dependent
169 manner. Significant differences were observed at 100 μ g/ml with viability declining to 40.8%
170 and 59.9% of controls for HeLa and CCD cells, respectively ($P \leq 0.05$; Figure 1b). Finally,
171 eugenol appeared to be less effective than TCA and carvacrol, and did not exert a significant
172 effect on the metabolic activity up to 400 μ g/ml ($P \leq 0.05$; Figure 1c), when metabolic activity
173 was reduced to 30.8% and 41.34% of controls for HeLa and CCD cells, respectively.

174 Interestingly, while not significant, both CAR and EU increased cell viability at low levels (12.5
175 and 25 μ g/ml). This is potentially due to the anti-inflammatory effect of these PDAs, while
176 higher levels of PDAs became cytotoxic.

Figure 1: Effects of TCA (a), carvacrol (b) and eugenol (c) on the metabolic activity (MTT assay) of HeLa and CCD cells after 24 hours of treatment. The metabolic activity of treated cells was expressed as percentages over controls without PDA treatments. Bars with different alphabet labels within cell types are significantly different ($P \leq 0.05$).

CAR and EU Induced Cytotoxicity

Carvacrol and eugenol, but not TCA, disrupted membrane integrity, a measure of cytotoxicity, in a dose-dependent manner as measured by LDH assay. HeLa and CCD cells were equally

affected by the treatments. Specifically, carvacrol exhibited increased cytotoxicity on both cell types in a dose-dependent manner. Significant differences were observed at 100 $\mu\text{g/ml}$, with cytotoxicity increasing to 33.78% and 15.11% of untreated controls for HeLa and CCD cells, respectively ($P \leq 0.05$; Figure 2b). Eugenol, however, appeared to be less cytotoxic than carvacrol and did not exert a significant effect on viability until 400 $\mu\text{g/ml}$ ($P \leq 0.05$; Figure 2c), when cytotoxicity was 35.07% and 27.37% of controls for HeLa and CCD cells, respectively. The results on carvacrol and eugenol are consistent with those from the MTT assay. Additionally, as with the MTT assay, no significant difference was observed in the response of HeLa and CCD cells to carvacrol and eugenol treatments.

Interestingly, TCA only showed significant cytotoxicity at 200 $\mu\text{g/ml}$ on both cell types ($P \leq 0.05$; Figure 2a). Above this concentration significant differences could not be observed (400 and 800 $\mu\text{g/ml}$; Figure 2a). This may not be surprising because TCA has been reported to interfere with LDH activity, which in turn, may have caused the large variation in the results of this assay²⁵. Consequently, we believe that TCA was still exerting cytotoxicity by causing membrane damage to both cell types at all levels above 200 $\mu\text{g/ml}$, yet we did not obtain consistent readings due to the interference of TCA with the assay itself (Figure 2a).

Figure 2: Cytotoxicity (LDH assay) of TCA (a), carvacrol (b) and eugenol (c) on HeLa and CCD cells after 24 hours of treatment. Cytotoxicity on treated cells was expressed as percentages over un-treated controls. Bars with different alphabet labels within cell types are significantly different ($P \leq 0.05$).

Apoptosis Is Involved in PDA-mediated Cell Death

In response to PDA treatment, both cell types rounded up, detached from the dish, and shrank in size, thereby exhibiting typical morphology of cell death (data not shown). To determine if

212 apoptosis was involved, we quantified Annexin V staining by flow cytometry. We found that
213 when incubating cells with various concentrations of PDAs for 24 hours, most of the cells were
214 dead rather than apoptotic (data not shown). We then selected TCA as a representative at the
215 concentration of 100 $\mu\text{g/ml}$, the lowest level to induce significant viability change and incubated
216 both HeLa and CCD cells for 1, 2, and 4 hours (data not shown). A TCA treatment of 4 hours
217 allowed us to catch apoptosis in progress. The effects of TCA on apoptosis are shown in Figure
218 3. In each scatterplot, the top left quadrant (q1) showed live cells stained red and positive for
219 mitochondrial membrane and negative for Annexin V. The bottom right quadrant (q4) showed
220 apoptotic or dead cells which stained negative for MitoTracker and positively for Annexin V. A
221 basal level of apoptotic cells of approximately 5% was present in both cell types before
222 treatments (Figure 3c). The progression of apoptosis was captured after 4 hours of TCA
223 treatment in both cell types (Figures 3a and 3b). The percentages of apoptotic cells increased to
224 13.1% and 22.3% for HeLa and CCD, respectively ($P \leq 0.05$).

Figure 3: Flow cytometry analysis for apoptosis in HeLa (a) and CCD cells (b) after TCA (0 and 100 $\mu\text{g/ml}$) treatment for 4 hours. In each scatterplot, live cells (red dots in q1) were stained positive for Mito tracker (red) and negative for Annexin V which indicated positive

mitochondrial activity and apoptotic cells, respectively. Apoptotic cells (dark blue dots in q4) were Annexin V and Mito tracker positive. c) The mean percentages of apoptotic HeLa and CCD cells before and after TCA treatment. While both cell types responded to TCA and no significant difference was found between the cell type, with CCD cells appearing to have an accelerated response.

Real-Time PCR Analysis of Expression of Apoptotic and Necrotic Genes

To determine if PDAs induced changes in the mRNA levels of apoptotic and necrotic genes, three representative genes of each pathway (*Bcl2*, *Casp3*, *Casp8* for apoptosis and *Mkl1*, *Ripk1*, *Ripk3* for necrosis) were chosen for analysis in treated HeLa and CCD cells. TC and CAR treatments were not associated with significant changes in expression of genes studied between control and treated on either cell types (Figure 4a, 4b and Table S2). In cells treated with EU, HeLa cells showed a significant increase in mRNA levels of *Bcl2*, *Mkl1* and *Ripk1* in treated group ($P \leq 0.05$). CCD cells did not show any significant differences between the control and treated groups (Figure 4c and Table S2).

a *trans*-Cinnamaldehyde

b Carvacrol

c Eugenol

244

245

Figure 4: Relative levels of mRNAs for apoptotic and necrotic genes in HeLa and CCD cells treated with (a) TC, (b) CAR, and (c) EU. Expression levels are expressed in log (RQ, relative quantity) values. Asterisk (*) represents a significant difference between control and treated.

Discussion

In this study we chose HeLa and fibroblasts (CCD), both established cell lines, as representatives of cancerous and non-cancerous cells to determine their responses to PDA treatments. We demonstrated that with increasing concentrations, TCA, carvacrol, and eugenol caused decreased cell viability and increased cytotoxicity ($P \leq 0.05$). We also showed that apoptosis was involved in these processes.

Out of the three compounds studied, TCA and carvacrol appeared to be more effective than eugenol. TCA and carvacrol significantly induced a reduction in cell viability at a much lower concentration (100 $\mu\text{g/ml}$) than eugenol (400 $\mu\text{g/ml}$) as shown by the MTT assays. Carvacrol and eugenol maintained these effective and significant doses in inducing cytotoxicity as determined by the LDH assays. TCA, however, did not cause cytotoxicity until 200 $\mu\text{g/ml}$. It is possible that TCA was more effective in inhibiting cell viability than inducing cytotoxicity. However, from the large variation of the data, we believed that TCA may somehow interfere with the LDH assay. This assumption was supported by previous findings that pre-treatment of cinnamaldehyde decreased LDH activities in rats²⁵.

The MTT assay measures the metabolic activity of mitochondria through the action of succinate dehydrogenase²⁶, whereas LDH assay measures the LDH release to the medium after cell

268 membrane damage²⁵. Carvacrol and eugenol each exerted significant effects on both MTT and
269 LDH assays at the same treatment levels (100 µg/ml), while it took more TC to elicit a
270 significant effect on LDH than on MTT. This led us to believe that TCA modulates the
271 mitochondria function but does not lead to dismantling of the cell membrane until there is a high
272 concentration.

273 Following PDA treatments, both cell types rounded up, detached from the dish, and shrank in
274 size, exhibiting typical morphology of cell death. To determine if apoptosis was involved, we
275 quantified Annexin V staining by flow cytometry which readily revealed significant apoptosis at
276 100 µg/ml of TCA, a concentration that significantly reduced cell viability. The percentages of
277 apoptotic cells, ~20%, were much lower than those of non-viable cells (~70%) at the same TCA
278 concentration in the MTT study. This was likely a result of the difference in treatment duration,
279 4h vs. 24h, between the two types of experiments. More cells would be progressively going
280 through apoptosis as the treatment extended to 24h.

281 Based on prior findings using cancer cells only including HeLa^{19,20,27}, we expected the PDA
282 treatments to affect the expression of genes in the apoptotic pathway in both cell types. No prior
283 studies had explored the role of necrosis by PDA treatments. The only indication of necrosis was
284 the report of a smear pattern of DNA fragmentation when human oral cancer cells were treated
285 with eugenol²⁸. We therefore aimed to determine expression of genes in apoptosis and necrosis
286 in both cell types. However, our real-time qPCR did not reveal any significant difference
287 between control and treated samples with the exception of eugenol-treated HeLa cells. While
288 these results support claims from Wafai et al. on HeLa cells²⁹, this could not be seen in CCD
289 cells. We believe that this may be caused by the specific doses of PDAs used in our study.
290 Although we chose levels that produced statistical changes in cell viability and cytotoxicity,

291 major alterations, albeit statistically insignificant due to the large variations, already occurred at
292 much lower doses of PDAs. It is likely that the PDA levels used in the gene expression
293 experiments injured most of the cells so rapidly through membrane damages and energy
294 deficiency that they could not respond by modulating the apoptotic or necrotic pathways. This
295 possibility was supported by the low concentrations of RNAs extracted from the samples.

296 The effects of TCA, eugenol or carvacrol on HeLa cells have been documented in six previous
297 studies. Lee et al.⁸ found that TCA was four times more effective than eugenol in inducing
298 cytotoxicity, a similar finding to ours. In fact, Lee et al. also found that TCA was equally
299 effective (similar IC₅₀) to chemotherapy drugs cisplatin and mitomycin C. Carvacrol and
300 eugenol were found to reduce viability and/or cause cytotoxicity as well as apoptosis^{19,20,27,30}.
301 Other studies also reported potentiating effects of eugenol on a chemotherapy drug or
302 radiation^{20,27}. However, none of these prior studies included control non-cancerous cells. Our
303 finding that PDAs affected fibroblasts and HeLa cells similarly in all aspects of viability,
304 cytotoxicity and apoptosis provided sufficient evidence to question whether the previously
305 reported data on HeLa cells were specific or not.

306 It is also worth-noting that the concentration of PDAs previously reported to reduce cell viability
307 and to cause cytotoxicity varied tremendously. For example, in one study, as low as 5.4 µg/ml of
308 TCA caused a 50% change in cytotoxicity of HeLa cells⁸. In studies of eugenol, it was reported
309 that 50 µM eugenol (approximately 8.21 µg/ml) inhibited the viability of HeLa cells by 75%
310 with 24h of treatment³⁰. Yet in another study, it took 750 µM (approximately 123.2 µg/ml) of
311 eugenol for 24h to produce the same results²⁰. A level between these extremes, at 350 µM
312 (approximately 57.5 µg/ml) was also reported to have a similar effect on the viability of HeLa
313 cells in 24h²⁷. Similar to our findings, it was reported that 100 µg/ml of carvacrol reduced cell

314 viability and increased cytotoxicity of HeLa cells to 25% and 40% of the untreated controls,
315 respectively¹⁶. It is unclear why such big variations in effective PDA concentration were found
316 against HeLa cells in previous studies. In our study, we observed large variations at the lower
317 concentration ranges of PDAs. These lower levels sometimes produced positive effects on cell
318 viability, most likely due to their antioxidant properties. These variations prevented us from
319 detecting statistical significance until higher PDA levels were applied when most cells were dead
320 upon treatments.

321 While searching through the literature on the anti-cancerous effects of PDAs among other cancer
322 cell lines, we came upon only two studies that included non-cancerous cell controls. However,
323 these studies documented conflicting results. Aydin et al compared healthy rat neurons and N2a
324 neuroblastoma cancer cells on their proliferative response to carvacrol treatment. Using the MTT
325 assay they observed that both cell types were affected similarly¹⁸. In contrast, using human breast
326 epithelial cells (non-cancerous) and their ras-transformed counterparts, Yan et al. reported that
327 eugenol inhibited proliferation and intracellular ATP level of only the ras-transformed cells³¹.
328 Cancer cells have been intensively studied for the Warburg effect of metabolism, i.e. the
329 preferential utilization of the glycolysis pathway even in the presence of oxygen and fully
330 functioning mitochondria³². More recently, however, in breast cancer cells of the mouse model,
331 it was discovered that mitochondrial electron transport was necessary to drive *de novo*
332 pyrimidine synthesis to overcome cell-cycle arrest³³. While many cancer cells do indeed employ
333 oxidative phosphorylation, ATP generation was found dispensable for tumorigenesis³³. In Yan et
334 al., eugenol was found to inhibit gene expression of the oxidative phosphorylation pathway in
335 ras-transformed breast epithelial cells, but such inhibition was not determined on non-cancerous

control cells, raising the question if eugenol can be used to target cancer cells by inhibiting the dispensable oxidative phosphorylation pathway for ATP generation³¹.

Conclusion

We conclude that although TCA, carvacrol and eugenol have been reported to be anti-cancerous, they exert similar effects on a non-cancerous fibroblast cell line in regards to viability, cytotoxicity and mechanism of death as they do in the HeLa cell model.

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CONFLICT OF INTEREST

The authors declare no conflict of interests.

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442 **AUTHOR'S CONTRIBUTION**

443 SR, DZ, FS, SS, WH conducted the experiments.

444 KV, ERT and XT designed the study.

445 XT obtained research funding.

446 All authors contributed to the drafting/editing of the manuscript.

447 **SUPPLEMENTS**

448 **Table S1:** Primers used for real-time qPCR

Genes	Primer Sequences
<i>Actin</i>	F 5'- CCTCGCCTTTGCCGATCC-3' R 5'- CGCGGCGATATCATCATCC-3'
<i>Bcl2</i>	F 5'-GATGTGATGCCTCTGCGAAG-3' R 5'-CATGCTGATGTCTCTGGAATCT-3'
<i>Casp3</i>	F 5'-GCTCTGGTTTTTCGGTGGGT-3' R 5'- GAGTCCATTGATTCGCTTCCA-3'
<i>Casp8</i>	F 5'-AGAGTCTGTGCCCAAATCAAC-3' R 5'-GCTGCTTCTCTCTTTGCTGAA-3'
<i>Ripk1</i>	F 5'-GGCATTGAAGAAAAATTTAGGC-3' R 5'-TCACAACTGCATTTTCGTTTG-3'
<i>Ripk3</i>	F 5'-GCAACATAGGAAGTGGGGCT-3' R 5' -GGTCCCAGTTCACCTTCTCG-3'
<i>Mkl1</i>	F 5' -AGAGCTCCAGTGGCCATAAA-3' R 5' -TACGCAGGATGTTGGGAGAT-3'

449

450

451 **Table S2:** P-values for the real-time qPCR results on HeLa and CCD cells. Comparisons were
 452 made using two-way ANOVA (PDAs and treatments).

	TC		CAR		EU	
	HeLa	CCD	HeLa	CCD	HeLa	CCD
<i>Bcl2</i>	0.475	0.745	0.475	0.745	0.007*	0.691
<i>Casp3</i>	0.477	0.428	0.477	0.428	0.089	0.935
<i>Casp8</i>	0.664	0.692	0.664	0.692	0.090	0.764
<i>Mkl1</i>	0.845	0.743	0.845	0.743	0.004*	0.520
<i>Ripk1</i>	0.674	0.246	0.674	0.246	0.002*	0.584
<i>Ripk3</i>	0.777	0.492	0.777	0.492	0.113	0.649

453 *=significant difference between treated and untreated within the same PDA.

454

Figures

Figure 1

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Figure 2

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Figure 3

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Figure 4

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