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Turmeric and Chinese goldthread synergistically inhibit prostate cancer cell proliferation and NF-kB signaling

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ABSTRACT:

Background: Pre-clinical studies using bioactive compounds from botanicals appear to offer some protection against cancer. Research using single bioactives contributes greatly to our understanding of their mechanism of action, but *in vitro* studies demand concentrations that are higher than achievable in humans (μM). However, maintaining these bioactives in the presence of other compounds originally derived from the food or extract of origin may synergistically lower the bioactive dose so translatability becomes feasible. The objective of this study was to determine if bio-efficacy of phytonutrients can be enhanced when used in combination even at doses that are ineffective for any compound when used in isolation.

Methods: The anti-proliferative and molecular effects of herbs (turmeric and Chinese goldthread) and their bioactives (curcumin and *ar*-turmerone, berberine and coptisine, respectively) were determined in isolation and in combination. Using CWR22Rv1 and HEK293 cells, cell proliferation (as assessed by the MTT assay) and NF-κB promoter activity (using a luciferase reporter construct) were evaluated and synergy of action was assessed by the Chou-Talalay method utilizing CompuSyn® software.

Results: Turmeric and Chinese goldthread act synergistically (combination index<1) when inhibiting cell proliferation with all cell lines tested. The synergy of action of combinations of companion bioactives from the same herb (i.e., curcumin/*a*r-turmerone and berberine/coptisine) and bioactives from different herbs (i.e., curcumin/berberine) help to explain why turmeric and Chinese goldthread are more effective than their major bioactives in isolation. At the molecule level, curcumin+*a*r-turmerone and curcumin+coptisine synergistically attenuated TNFα-stimulated NF-κB promoter activity. Even compounds with poor efficacy become more biologically active in the presence of companion compounds. Importantly, the effects of

combining any two bioactives or herbal extracts were highly synergistic at concentrations approaching physiological significance (nanomolar).

Conclusions: These results suggest that bioactives in combination (as plant extracts or isolated compounds) are highly synergistic at the cellular and molecular level at physiologically relevant concentrations. These data help to explain why complex mixtures of botanicals may be more efficacious than their bioactives in isolation.

Keywords: Synergy; Chou-Talalay; turmeric; Chinese goldthread; curcumin; berberine; *ar*-turmerone; coptisine; prostate cancer; NF-κB

BACKGROUND:

Research exploring the use of natural products, such as botanicals, herbs, herbal extracts, and their isolated bioactives are becoming increasingly appealing because of their potential efficacy in attenuating growth and viability of cancer cells with few observed side effects in humans when these agents are taken orally. The combination of multiple botanicals may prove to be more efficacious as compared with their use in isolation. These effects may be related to the fact that when bioactives are used in combination, their synergy could reduce the concentration needed for effectiveness to one that is physiologic. For example, polyherbal mixtures can be quite effective in inhibiting cancer cell proliferation when individual herbal extracts have no effect (see Supplemental Figure 1). Furthermore, *in vitro* studies demand concentrations (often in the 20-100 μM range) [1, 2] that are several orders of magnitude higher than nM concentrations achievable in humans. The difference between these effects seen *in vitro* using micromolar concentrations when compared with the nanomolar concentrations measured in humans raises the issue of the translational relevance of some of the *in vitro* studies, which likely accounts for at least part of the disappointing outcomes in clinical trials [3]. Tables 1 and 2 summarize this point where the mean IC_{50} concentrations observed in a variety of cancer cell lines are 41 μM, 21 μM, >243 μM and 29 μM for the bioactives berberine, curcumin, *ar*-turmerone and coptisine, respectively, while the serum/plasma concentrations in humans provided oral doses of curcumin are in the nanomolar range. With one exception, the serum/plasma levels of curcumin are <200 nM at doses as high as 12 g (Table 2). Maintaining an individual bioactive in the presence of other bioactive compounds (originally derived from the food or extract of origin) may turn isolated compounds that have weak bioactivity to those that have substantially more activity at lower doses. This would make translation to humans more feasible. For instance, the World Cancer Research Fund/American Institute for Cancer Research reports that consumption of individual nutrients could in some instances result in vastly different outcomes when compared with their foods of origin [4]. This concept of the importance of the complex source versus isolated nutrients is echoed by the American Cancer Society [5]. Importantly, this concept can be equally applied to combinations of foods or botanicals.

To investigate the synergistic action of botanicals, we used CWR22Rv1 and HEK293 cells. CWR22Rv1 cells are an *in vitro* model of castrate-resistant prostate cancer (PCa) [6] and complement our research investigating the effects of a mixture of bioactive botanicals on *in vitro*

Table 1. In vitro antiproliferative effects of berberine, curcumin, coptisine and ar-turmerone in a variety of cell lines based on IC_{50} concentrations (μM).

Berberine [41]

Cell Line	IC_{50}
HeLa	20
L1210	10
A431	75
DU145	100
U937	15
MCF7	20
CL1-5	7.5
Colo205	80
C6	10
U-87	20
VSMC	200
B16	3
RPMI 8226	5
MDA-MB231	25
NPC-HK1	200
EAC	2
YES-6	3
NIH-3T3	30
A7r5	23
HepG2	39
Hep3B	45
Sk-Hepl	10
PLC/PRF/5	40
K562	43
U937	28
P3H1	24
Raji	1.8
L929	120
Mean	41

Curcumin

Cell Line	IC_{50}	Ref	
KBM-5	3.8	[42]	
Jurkat	4.3	[42]	
U266	7.6	[42]	
A549	17	[42]	
U87	15	[46]	
T98G	31	[46]	
PC3	32	[48]	
LNCaP	53	[49]	
DU-145	30	[50]	
MCF-7	20	[51]	
MCF7/LCC2	20	[51]	
MCF7/LCC9	20	[51]	
Mean	21		

***ar*-Turmerone**

Cell Line	IC_{50}	Ref
MDA-MB-231	>100	[33]
	50	[34]
	>462	[35]
MCF-7	>100	[33]
	>50	[34]
	>462	[35]
HepG2	>100	[33]
	300	[36]
	>462	[35]
Hep3B	564	[36]
Huh-7	472	[36]
U937	185	[52]
K562	185	[32]
L1210	116	[32]
U937	111	[32]
RB1-2H3	162	[32]
Mean	>243	

Coptisine

Cell Line	IC_{50}	Ref
MDA-MB-231	78	[43]
Hela	35	[44]
MDA-MB-468	63	[43]
HepG2	203	[45]
	3.5	[47]
Hep3B	5.4	[47]
SK-Hep1	1.4	[47]
PLC/PRF/5	6.6	[47]
K562	7.2	[47]
U937	1.5	[47]
Raji	0.6	[47]
P3H1	11	[47]
LoVo	2.7	[53]
LoVo/Dx	12.5	[53]
HT29	1.5	[53]
Mean	29	

and *in vivo* models of castrate-resistant PCa [7-9]. The HEK293 cell line is a transformed human embryonic kidney cell that is widely used as a tool for expression vectors [10]. In this case, HEK293 cells were transfected with a recombinant plasmid expressing a luciferase gene under the control of a multimerized NF-κB responsive-promoter stimulated with TNFα [11].

In Traditional Chinese Medicine [12], Chinese goldthread and turmeric are used in combination to treat a variety of diseases, among them cancer. Cancer has been explained as a combination of damp-heat and blood stasis [2, 3] where the anti-cancer effects of Chinese goldthread and turmeric can be explained as damp-heat removing [2, 13] and blood stasis improving [13], respectively. The use of Chinese goldthread and turmeric complement our previous studies involving a polyherbal mixture containing these two herbal extracts [7-9].

While Chinese goldthread (Coptis chinensis) has been tested in a number of cancers [14-17], very little is known about its impact on PCa. Instead, berberine, the major bioactive phytochemical in Chinese goldthread (estimated to be ~6% [18, 19]) has been preferentially

studied and has been found to inhibit cell growth at concentrations of 30 μM to 100 μM in androgen-dependent and androgen-independent PCa cells [17, 20, 21].

Table 2. Effect of oral dosing of curcumin from a single dose (g) to 24 weeks treatment (g/d) on maximum serum/plasma concentrations (nM) of curcumin and its metabolic derivatives

Reference	Dose (g)	Curcumin (underivatized) (nM)	Total Curcumoids (curcumin + derivatives) (nM)
LC or GC Mass Spectral Analysis			
[54]	0.45	nd	
	1.8	nd	
	3.6	nd-11	35.8
[55]	4	21	149
[56]	0.03	4.9	
HPLC-UV Detection			
[57]	2	nd	
	4	nd	
	6	nd	
	8	nd	
	10	137	
	12	157	
[58]	4	510	
	6	630	
	8	1770	
[59]	0.45	<3	<3
	1.8	<3	<3
	3.6	<3	<3
[60]	0.036	nd	nd
	0.072	nd	nd
	0.108	nd	nd
	0.144	nd	nd
	0.18	nd	nd
[61]	2	nd	
[62]	12	nd	

nd: not detectable.

A minor constituent in this herb is coptisine (ratio of berberine to coptisine is approximately 5:1) [18]; however, this compound and the potential synergy between these compounds has yet to be tested. Turmeric (*Curcuma longa*) is a plant commonly found in Asia from the ginger

family. Like Chinese goldthread, extracts of turmeric are not typically used in studies; instead curcumin, one of its primary ingredients known for its anticancer properties, is the primary target of investigation [22, 23]. Curcumin is the major curcuminoid in turmeric at a level of ~5-6% [24, 25]. The molecular targets of curcumin are extensive (as reviewed elsewhere [22, 26]). For example, curcumin has been shown to modify those targets involved in inflammation, apoptosis, angiogenesis, metastasis and the expression of transcription factors, various receptors, growth factors and a variety of protein kinases. However, curcumin inhibits androgen-dependent and androgen-independent PCa cell growth at concentrations of 40 μM to 100 μM [27]. While the diversity of compounds in turmeric is extensive [28, 29], a less commonly studied one is *ar*-turmerone from the sesquiterpenes (ratio of curcumin to *ar*-turmerone in turmeric is approximately 4:1) [30, 31], with reported IC_{50} values in a variety of cancer cell lines of >243 μM [32-36]; however, *ar*-turmerone has not been studied in PCa. Similarly, the potential synergy between these compounds has yet to be tested. The objective of this study was to build upon the concept that the combination of bioactives in botanicals is far more effective than an individual bioactive in isolation. While this seems intuitive, a systematic approach yielding solid experimental evidence has yet to be fully explored. To address this issue, we determined if synergy could explain the enhanced effect of *(a)* the combination of herbs over isolated bioactives derived from those herbs, *(b)* the combination of bioactives over isolated bioactives derived from the same herb (companion compounds), *(c)* the combination of herbs over individual herbs, *(d)* the combination of bioactives from different herbs over the isolated bioactives, and *(e)* whether these synergistic effects on proliferation can apply at the molecular level. NF-κB transcriptional activity was chosen as the molecular target because of its link in castrate-resistant PCa [37, 38] and because it has been previously identified as a target of bioactive phytonutrients and natural products [39, 40].

MATERIALS AND METHODS:

Experimental design: The experimental design for testing synergistic interactions is presented in Figure 1. The comparisons made in this study included the combinations of herbs (inter-interactions), comparison of the herb to its major bioactive (i.e., Chinese goldthread versus berberine and turmeric versus curcumin), combinations of bioactives from the same herb (intra-interaction) and combinations of bioactives from different herbs (inter-interactions).

Chemicals: The herbal extracts of Chinese goldthread (*Coptis chinensis*) and turmeric (*Curcuma longa*) were provided by New Chapter (Brattleboro, VT). Fourier transform infrared spectroscopy was used to independently identify the raw materials and they were further verified by independent laboratories using a variety of analytical approaches (i.e., high performance and thin layer chromatographies and mass spectrometry) (as described previously) [63]. Quality control was determined by ranges of select bioactives, such as curcuminoids in turmeric and berberine in Chinese goldthread [63]. Berberine (98% purity) was purchased from MP Biomedicals (Solon, OH), curcumin (98% purity) from Fischer Scientific (Pittsburgh, PA), coptisine (98% purity) from Quality Phytochemicals (Edison, NJ), and *ar*-turmerone (90% purity) from Sigma-Aldrich (St. Louis, MO). All substances were dissolved in dimethyl sulfoxide (DMSO) (Sigma-Aldrich, St. Louis, MO) to varied stock concentrations. RPMI 1640

cell culture media (Invitrogen, Grand Island, NY), supplemented with 0.5% fetal bovine serum (FBS, Invitrogen, Grand Island, NY) was used to dilute each stock solution to the required final concentrations.

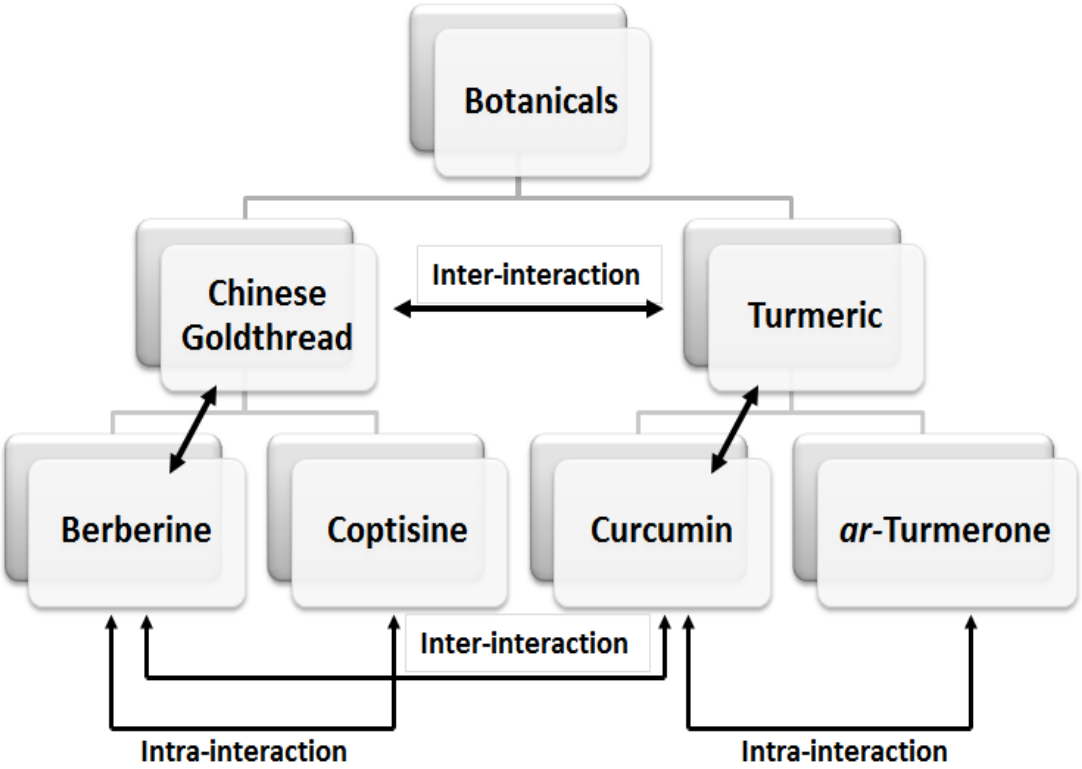

Figure 1. Experimental design. Comparisons were made between herbs and their major bioactives, among combinations of herbs (inter-interaction of herbs) and combinations of bioactives from the same herb (intra-interaction of isolated bioactives) or different herbs (inter-interactions of isolated bioactives).

Cell Culture: For the experimental treatments, CWR22Rv1 cells (American Type Culture Collection, Rockville, MD), a human-derived castrate-resistant PCa line, were grown in RPMI 1640 media supplemented with 10% FBS under an atmosphere of 5% CO_2 at 37°C. Prior to the experiments, the level of FBS was reduced to 0.5%. HEK293 cells (ATCC, Rockville, MD), a human embryonic kidney cell line, were grown in DMEM media supplemented with 10% FBS under an atmosphere of 5% CO_2 at 37°C.

Cell Proliferation: Cell growth and proliferation inhibition studies were performed using standard 96 well plates and analyzed with the MTT [3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide] (Chalbiochem, Darmstadt, Germany) assay following the manufacturer's instructions. CW22Rv1 cells were plated at a density of 2×10^4 cells per well. After 24 hours, the FBS concentration in the cell culture media was reduced to 0.5% FBS and the cells were incubated overnight prior to the addition of the herbal extracts or phytochemicals.

HEK293 cells were plated at a density of 1×10^4 cells per well. After 24 hours, the cells were incubated with herbal extracts or phytochemicals. After 48 hr incubation, cell viability and proliferation were measured via the MTT assay. Briefly, medium was replaced by 100 µl of 0.5 mg/ml MTT and cells were incubated for 4 hr at 37°C. Intracellular formazan crystals were solubilized with 100 µl isopropanol/0.04 N HCl. Absorbance was read at 540 nm on a SpectraCount microplate photometer (Perkin Elmer Inc, Waltham, MA).

Transfection and luciferase assay: The multimerized NF-κB promoter luciferase vector has been described elsewhere [11]. Transient transfection of luciferase reporter constructs into HEK293 cells was achieved using Lipofectamine® 2000 (Life technologies, Grand Island, NY) following manufacturer's instructions. Briefly, HEK293 cells were plated at a density of 1.5×10^4 cells per well in 96 well plates. After 24 hours, 4 ng/well plasmid was transfected with 0.32 µl/well Lipofectamine® 2000. After incubating overnight, transfection medium was removed and cells were incubated with herbal extracts or phytochemicals in 0.5% FBS supplemented DMEM medium for 1 hr. After incubation, HEK293 cells with luciferase reporter were stimulated by 5 ng/ml TNF-α for 4 hr. Whole cell lysate was achieved by 20 µl passive lysis buffer (Promega, Madison, WI) and 10 µl was used for luciferase activity measurement by using the Luciferase Assay Reagent (Promega, Madison, WI). Luciferase activity was detected using the GloMax plate reading luminometer and normalized to total cellular protein concentration per well using the BCA assay (Thermo Scientific, Rockford, IL) following manufacturer's instruction.

Assessment of Synergistic, Additive or Antagonistic Effects of Combinations of Herbal Extracts and/or Phytochemicals: To calculate the effects of compounds or herbs in combination, IC_{50} values were determined for each ingredient. For the experiments with the combination of herbal extracts, turmeric and Chinese goldthread were combined at a concentration equivalent to their IC_{50} values followed by serial dilutions to generate a dose response. For the experiments that combined individual bioactive phytochemicals from different herbal extracts (berberine and curcumin), they were combined at concentrations equivalent to their IC_{50} values, followed by serial dilutions to generate a dose response. For the experiments that combined individual bioactive phytochemicals from the same herbal extract (i.e., berberine and coptisine, curcumin and *ar*-turmerone), they were combined at concentrations equivalent to their IC_{50} values or based on their relative ratios in their respective herbal extracts followed by serial dilutions to generate dose responses.

Assessment of synergistic, additive or antagonistic effects of combinations was determined using the Chou-Talalay method [64]. This method is derived from mass-action law principles as described in detail elsewhere [65]. The combination index (CI) and dose-reduction index (DRI) were used to differentiate synergy, antagonism and additive effects for combinations of components. CI and DRI were generated for all treatments with CompuSyn® 1.0 (Combo Syn, Paramus, NJ) according to the manufacturer's instructions. CompuSyn® 1.0 software uses an algorithm based on the Chou-Talalay method to simulate the interaction of 2 or more compounds. Briefly, concentrations and corresponding effect levels for all data points were input to generate a complete report of analytical results. CI is a parameter that indicates whether the interaction of two compounds is synergistic, additive, or antagonistic [additive (CI = 1);

synergistic (CI < 1); antagonistic (CI > 1)]. DRI is a parameter that indicates the degree to which the concentration of a compound can be reduced when used in combination with another compound to maintain an equivalent effect. The use of CI and DRI provide numerical assessments of not only whether there is synergy, but the extent of that synergy and synergy at particular doses [64].

Statistical Analysis: The results were presented as mean ± SEM and were analyzed by two-tailed student's T-test. Differences were considered significant at $p<0.05$.

RESULTS:

Cell proliferation:

Dose response curves were generated for all bioactives tested in each cell type. All the bioactives and their extracts of origin reduced cell proliferation in a dose dependent manner (Fig. 2). IC_{50} values of bioactives were used, in part, to determine the synergistic effect between different bioactives.

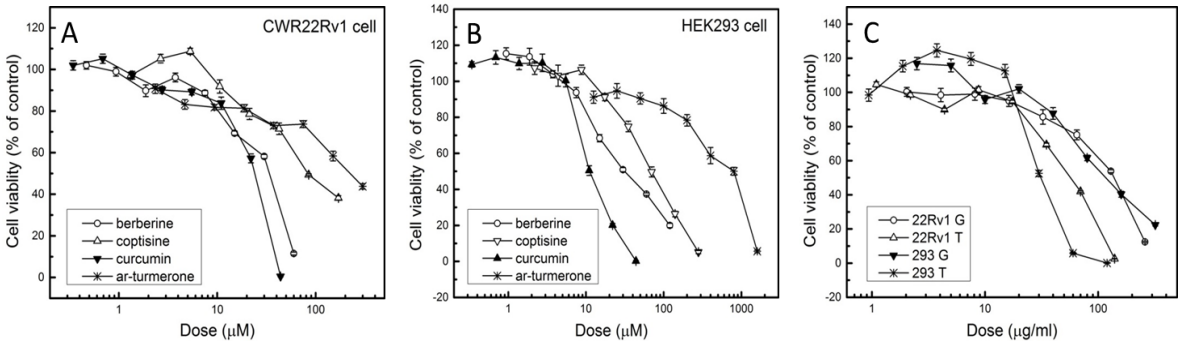

Figure 2. The effects of berberine, coptisine, curcumin, *ar*-turmerone, Chinese goldthread and turmeric on cell proliferation. Dose-response curves for cell proliferation were generated with berberine, coptisine, curcumin, *ar*-turmerone using (A) CWR22Rv1 cells and (B) HEK293 cells. (C) Dose-response curves for cell proliferation were also generated with the herbal extracts of Chinese goldthread and turmeric for both cell lines. IC_{50} values of bioactives and herb extracts were generated from these curves.

Comparisons of Chinese goldthread with berberine, and turmeric with curcumin on cell proliferation: Dose-response curves were generated for Chinese goldthread, berberine, turmeric and curcumin and their effectiveness inhibiting cell proliferation were compared based on the relative amounts of the phytonutrients in the herbal extract (i.e., the amount of berberine in Chinese goldthread and the amount of curcumin in turmeric). Proliferation of CWR22Rv1cells and HEK293 cells was reduced in dose-dependent manners with Chinese goldthread and berberine (Fig. 2). Following normalization based on the relative amount of berberine in Chinese goldthread, there was a shift to the left in the IC_{50} value, from 30 μM to 19 μM in CWR22Rv1 cells (Fig. 3A) and from 30 μM to 11 μM in HEK293 cells (Fig. 3C).

Cell proliferation was also reduced in dose-dependent manners with turmeric and curcumin (Fig. 2). Following normalization based on the relative amount of curcumin in turmeric, there was a shift to the left of the IC_{50} value from 22 μM to 9.5 μM in CWR22Rv1 cells (Fig. 3B) and from 11 μM to 4 μM in HEK293 cells (Fig. 3D). These results collectively infer that other

compounds within the original herbal extracts (companion compounds) may be acting in concert (i.e., synergism) with each of the bioactive phytonutrients. To explore this possibility, we investigated the effects of combining companion phytonutrients from Chinese goldthread (i.e., berberine with coptisine) and turmeric (i.e., curcumin with *ar*-turmerone).

Intra-herbal comparisons of companion phytonutrients (berberine with coptisine, curcumin with *ar*-turmerone) on cell proliferation: Berberine and coptisine are two bioactive phytonutrients found in Chinese goldthread (companion phytonutrients) and their synergy could help explain, in part, the enhanced effectiveness of Chinese goldthread when compared with berberine in isolation. Berberine and coptisine reduced the proliferation of CW22Rv1 cells in dose-dependent manners with IC_{50} values of 30 μM and 85 μM, respectively (Fig. 2). In a similar manner, the proliferation of HEK293 cells was reduced by berberine and coptisine with IC_{50} values of 30 μM and 70 μM, respectively (Fig. 2). To determine the effects on proliferation with the combination, cells were treated with berberine plus coptisine with serial dilutions starting with concentrations equivalent to their IC_{50} values (30 μM:85 μM for CWR22Rv1, 30

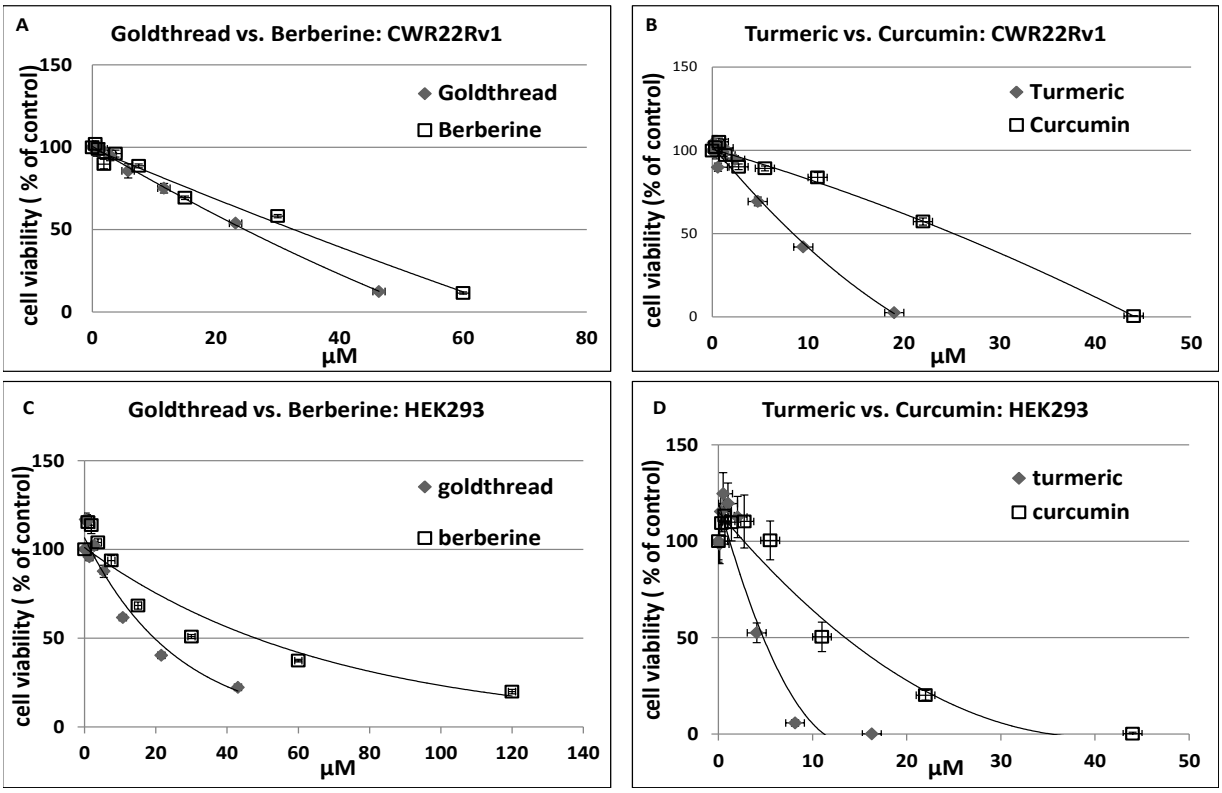

Figure 3. The comparison of berberine and curcumin on cell proliferation compared to their herbal extracts of origin, Chinese goldthread and turmeric, respectively, in CWR22Rv1 cells and HEK293 cells. Following normalization based on the relative amount of berberine in Chinese goldthread and the relative amount of curcumin in turmeric, dose-response curves were compared between Chinese goldthread and berberine in (A) CWR22Rv1 cells and (C) HEK293 cells, and between turmeric and curcumin in (B) CWR22Rv1 cells and (D) HEK293 cells. Compared to individual compounds, herbal extracts had a more pronounced effect on reducing cell proliferation compared to their isolated bioactives (shifts in IC_{50} values to the left).

μM:70 μM for HEK293) and reducing these to nanomolar concentrations while maintaining the same ratio. The combination of berberine plus coptisine reduced cell proliferation in a dose-dependent manner and the minimum effective doses were determined for both single phytochemicals and their combinations (Tables 3 and 4). CI and DRI values were generated for minimum effective doses by CompuSyn® 1.0, respectively. The combination of berberine plus coptisine reduced the minimum effective doses from μM level to nM level in CWR22Rv1 cells (7.5 μM for berberine, 21 μM for coptisine, and 0.24 μM+0.66 μM for their combination) and was highly synergistic in inhibiting cell growth at this level (CI equal to 0.05), with the DRIs being 52 and 37 when combined at the lowest concentrations (0.24 μM+0.66 μM). This means the addition of berberine to coptisine or coptisine to berberine reduced the effective doses 52 and 37 fold, respectively, compared with each alone. In a similar manner, the combination of berberine plus coptisine also reduced the minimum effective doses significantly in HEK293 cells (15 μM for berberine, 17.5 μM for coptisine, and 3.75 μM+8.75 μM for combination) and was highly synergistic in inhibiting cell growth at this level (CI equal to 0.42 with the DRIs being 3.2 and 32).

Curcumin and *ar*-turmerone (two bioactive phytonutrients found in turmeric) reduced the proliferation of CW22Rv1 cells in dose-dependent manners with IC_{50} values of 22 μM and 150 μM, respectively (Table 3). In a similar manner, the proliferation of HEK293 cells was reduced by curcumin and *ar*-turmerone with IC_{50} values of 11 μM and 200 μM, respectively (Table 4). To determine the effects on proliferation with the combination, cells were treated with curcumin plus *ar*-turmerone with serial dilutions starting with concentrations equivalent to their IC_{50} values (22 μM:150 μM for CWR22Rv1, 11 μM:200 μM for HEK293) and reducing these to nanomolar concentrations while maintaining the same ratio (Tables 3 and 4). The dose-response curves with the combination of curcumin plus *ar*-turmerone reduced cell proliferation in a dose-dependent manner. The combination of curcumin plus *ar*-turmerone reduced the minimum effective doses in CWR22Rv1 cells (5.5 μM for curcumin, 9.4 μM for *ar*-turmerone, and 0.17 μM +1.2 μM for their combination) and was highly synergistic in inhibiting cell growth at this level (CI equal to 0.09 with the DRIs being 37 and 16). In a similar manner, the combination of curcumin plus *ar*-turmerone also reduced the minimum effective doses significantly in HEK293 cells (11 μM for curcumin, 200 μM for *ar*-turmerone, 0.69 μM+25 μM for their combination) and was highly synergistic in inhibiting cell growth at this level (CI equal to 0.45 with the DRIs being 14 and 2.6).

While it is standard practice to use the ratio of the IC_{50} values to evaluate synergy, the ratio of these bioactive phytonutrients from their respective herbal extracts are different. For example, the ratio of berberine to coptisine in Chinese goldthread is ~5:1, and curcumin to *ar*-turmerone is ~4:1. As such, synergy was also assessed at more "natural" ratios (Tables 3 and 4). For CWR22Rv1 cells, the combination of berberine and coptisine was highly synergistic in inhibiting cell growth at minimum effective concentrations (0.469 μM+0.094 μM) where the CI was 0.16, respectively. The DRI values at these concentrations were 6.8 and 14.6 for berberine and coptisine, respectively. Similarly, using HEK293 cells, the combination of berberine and coptisine in a 5:1 ratio was highly synergistic in inhibiting cell growth in minimum effective concentrations (1.88 μM+0.38 μM) where the CI was 0.10, respectively. The DRI values at these concentrations were 11 and 109 for berberine and coptisine, respectively.

Using CWR22Rv1 cells, the combination of curcumin plus *ar*-turmerone were also highly synergistic in inhibiting cell growth at concentrations as low as 0.344 μM+0.086 μM, respectively (Table 3), where the CI was 0.06. The DRI values at these concentrations were 17.6 and 187 for curcumin and *ar*-turmerone, respectively. Using HEK293 cells, the combination of curcumin and *ar*-turmerone was synergistic in inhibiting cell growth at minimum effective concentrations of 11 μM+2.75 μM, respectively, where the CI was 0.64 (Table 4). The DRI values at these concentrations were 2.4 and 3.8 for curcumin and *ar*-turmerone, respectively.

Inter-herbal comparison of the extracts of Chinese goldthread with turmeric on cell proliferation: The extracts of Chinese goldthread and turmeric reduced the proliferation of CWR22Rv1 and HEK293 cells in dose-dependent manners (IC_{50} values for CWR22Rv1 cells were 130 μg/ml and 65 μg/ml, for HEK293 cells were 80 μg/ml and 30 μg/ml, respectively) (Fig. 2). To determine the effects on proliferation with the combination, cells were treated with Chinese goldthread plus turmeric with serial dilutions starting with combined concentrations equivalent to their IC_{50} values (130 μg/ml:65 μg/ml for CWR22Rv1 cells and 80 μg/ml:30 μg/ml for HEK293 cells) and going as low as ≤1 μg/ml (maintaining the same ratio). The combination of Chinese goldthread plus turmeric qualitatively reduced cell proliferation in a dose-dependent manner for both cell lines (Tables 3 and 4). CI and DRI values were generated at minimum effective concentrations. For CWR22Rv1 cells, the minimum effective concentration was more than 100 times lower than their respective IC_{50} values (1.103 μg/ml+0.508 μg/ml, respectively). The combination of Chinese goldthread and turmeric were highly synergistic in inhibiting cell growth at these doses, where the CI was 0.04, respectively. The DRI values at the minimum effective concentrations were 51.6 and 52.1 for Chinese goldthread and turmeric, respectively. Using HEK293 cells, the minimum effective concentrations were significantly reduced to 10 μg/ml+3.75 μg/ml. The combination was highly synergistic at these doses, where the CI was 0.29. The DRI values at the minimum effective concentrations were 6.8 and 6.8, respectively.

Inter-herbal comparison of phytonutrients (berberine with curcumin): To explain, in part, the synergism between Chinese goldthread and turmeric, isolated bioactive compounds from each herb (i.e., berberine and curcumin) were evaluated for synergy. To determine the effects on proliferation with the combination, cells were treated with berberine plus curcumin with serial dilutions starting with concentrations equivalent to their IC_{50} values (30 μM:22 μM for CWR22Rv1 cells and 30 μM:11 μM for HEK293 cells) and reducing these to nanomolar concentrations while maintaining the same ratio. The combination of berberine plus curcumin reduced cell proliferation in a dose-dependent manner for both cell lines (Tables 3 and 4). CI and DRI values were generated for minimum effective concentrations. For CWR22Rv1 cells, the combination of berberine and curcumin was highly synergistic in inhibiting cell growth at these levels (CI equal to 0.08, respectively), with the DRIs being 23.5 and 23.7 when combined at the minimum effective concentrations (0.235 μM+0.172 μM) (Table 3). Similar results were observed with HEK293 cells (Table 4). The combination of berberine and curcumin was highly synergistic in inhibiting cell growth at these levels (CI equal to 0.32), with the DRIs being 5.3 and 7.5 when combined at the minimum effective concentrations (3.75 μM+1.38 μM).

Table 3: Dose-effect relationships of berberine and coptisine (from Chinese goldthread), curcumin and *ar*-turmerone (from turmeric), Chinese goldthread and turmeric and their combinations using their IC_{50} values or using ratios corresponding to relative amounts in their native extracts on proliferation of CWR22Rv1 cells.

Isolated compounds and combinations	Minimum Effective Dose (μM)[a]	IC_{50} (μM)	Combination Index (CI)	Dose Reduction Index (DRI)
Berberine	7.5	30		
Coptisine	21	85		
Ber + Cop (ratio = 1 : 2.8)[b]	0.24 + 0.66		0.05	Berberine: 52 Coptisine: 37
Ber + Cop (ratio = 5 : 1)[c]	0.47 + 0.09		0.16	Berberine: 7 Coptisine: 15
Curcumin	5.5	22		
*a*r-turmerone	9.4	150		
Cur + *a*r-tur (ratio = 1 : 6.8)[b]	0.17 + 1.2		0.09	Curcumin: 37 *a*r-turmerone: 16
Cur + *a*r-tur (ratio = 4 : 1)[d]	0.34 + 0.09		0.06	Curcumin: 18 *a*r-turmerone: 87
Berberine	7.5	30		
Curcumin	5.5	22		
Ber + Cur (ratio = 1.4 : 1)[b]	0.24 + 0.17		0.16	Berberine: 24 Curcumin: 24

Isolated herbal extracts and combinations	Minimum Effective Dose (μg/ml)[a]	IC_{50} (μg/ml)	Combination Index (CI)	Dose Reduction Index (DRI)
Chinese Goldthread	65	130 μg/ml		
Turmeric	35	65 μg/ml		
Chinese GT + Tur (ratio = 2 : 1)[b]	1.10 + 0.51		0.14	Chinese GT: 52 Turmeric: 52

[a]Concentration derived from the dose-response curves where growth inhibition was significantly inhibited ($p<0.05$). [b]Ratio based on IC_{50} values. [c]Ratio based on relative amounts in Chinese goldthread. [d]Ratio based on relative amounts in turmeric

Table 4: Dose-effect relationships of berberine and coptisine (from Chinese goldthread), curcumin and *ar*-turmerone (from turmeric), Chinese goldthread and turmeric and their combinations using their IC_{50} values or using ratios corresponding to relative amounts in their native extracts on proliferation of HEK293 cells.

Isolated compounds and combinations	Minimum Effective Dose (μM)[a]	IC_{50} (μM)	Combination Index (CI)	Dose Reduction Index (DRI)
Berberine	15	30		
Coptisine	18	70		
Ber + Cop (ratio = 1 : 2.3)[b]	3.75 + 8.75		0.42	Berberine: 5.2 Coptisine: 4.5
Ber + Cop (ratio = 5 : 1)[c]	1.88 + 0.38		0.10	Berberine: 11 Coptisine: 109
Curcumin	11	11		
*a*r-turmerone	200	400		
Cur + *a*r-tur (ratio = 1 : 36)[b]	0.69 + 25		0.45	Curcumin: 15 *a*r-turmerone: 2.6
Cur + *a*r-tur (ratio = 4 : 1)[d]	11 + 2.75		0.64	Curcumin: 2.8 *a*r-turmerone: 1.4
Berberine	15	30		
Curcumin	11	11		

Ber + Cur (ratio = 2.7 : 1)[b]	3.75+1.83		0.32	Berberine: 5.3 Curcumin: 7.5

Isolated herbal extracts and combinations	Minimum Effective Dose (µg/ml)[a]	IC_{50} (µg/ml)	Combination Index (CI)	Dose Reduction Index (DRI)
Chinese Goldthread	40	80 µg/ml		
Turmeric	30	40 µg/ml		
Chinese GT + Tur (ratio = 2 : 1)[b]	10 + 3.75		0.29	Chinese GT: 6.8 Turmeric: 6.8

[a]Concentration derived from the dose-response curves where growth inhibition was significantly inhibited ($p<0.05$). [b]Ratio based on IC_{50} values. [c]Ratio based on relative amounts in Chinese goldthread. [d]Ratio based on relative amounts in turmeric.

TNFα-stimulated NF-κB transcriptional activity: Dose response curves were generated for all bioactives and extracts tested in HEK293 cells. All the bioactives and their extracts of origin, except berberine and Chinese goldthread, reduced NF-κB transcriptional activity in a dose dependent manner (Fig. 4). IC_{50} values of bioactives were used, in part, to determine the synergistic effect between different bioactives.

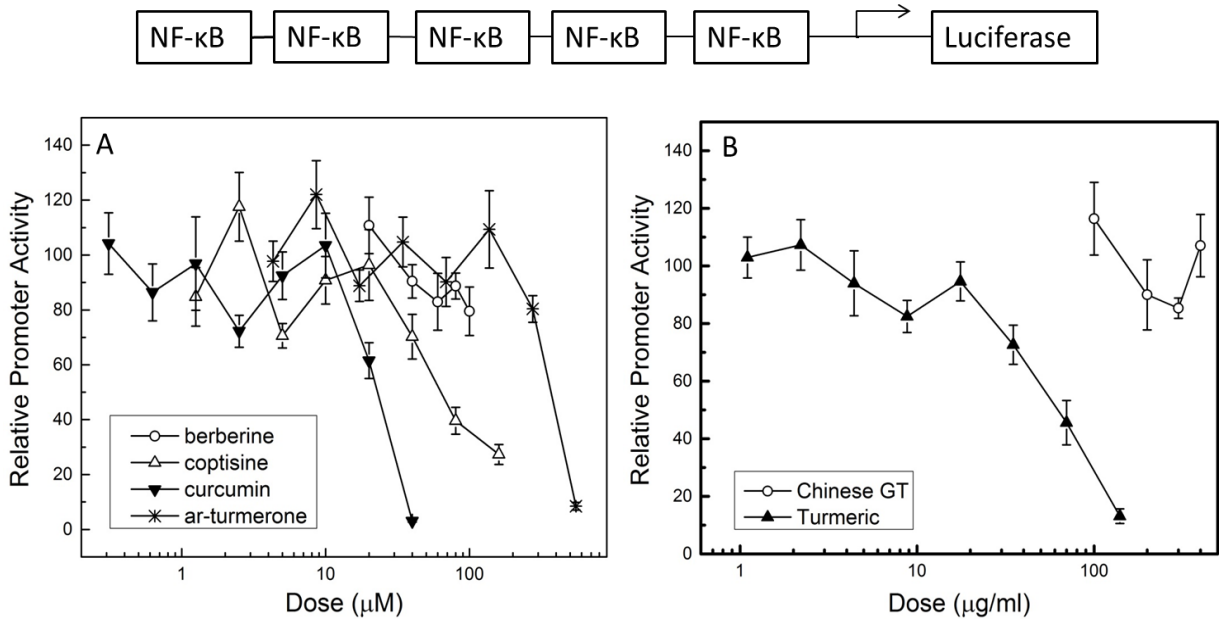

Figure 4. The effects of berberine, coptisine, curcumin, *ar*-turmerone, Chinese goldthread and turmeric on inhibition of TNFα-stimulated NF-κB transcriptional activity in HEK293 cells. Dose-response curves were generated with (A) berberine, coptisine, curcumin, *ar*-turmerone) and (B) Chinese goldthread and turmeric. IC_{50} values of the bioactives and turmeric were generated from these curves.

Comparisons of turmeric with curcumin on TNFα-stimulated NF-κB transcriptional activity: Dose-response curves were generated for turmeric and curcumin and their effectiveness inhibiting NF-κB transcriptional activity were compared based on the relative amounts of the phytonutrients in the herbal extract (i.e., the amount of curcumin in turmeric). NF-κB transcriptional activity was reduced in dose-dependent manners with turmeric and curcumin (Fig. 4). Following normalization based on the relative amount of curcumin in turmeric, there was a shift to the left in the IC_{50} value, from 20 µM to 10 µM (Fig. 5). These results collectively infer that companion compounds in turmeric may be acting synergistically with curcumin. To explore

this possibility, we investigated the effects of combining curcumin with one of its companion compounds *ar*-turmerone.

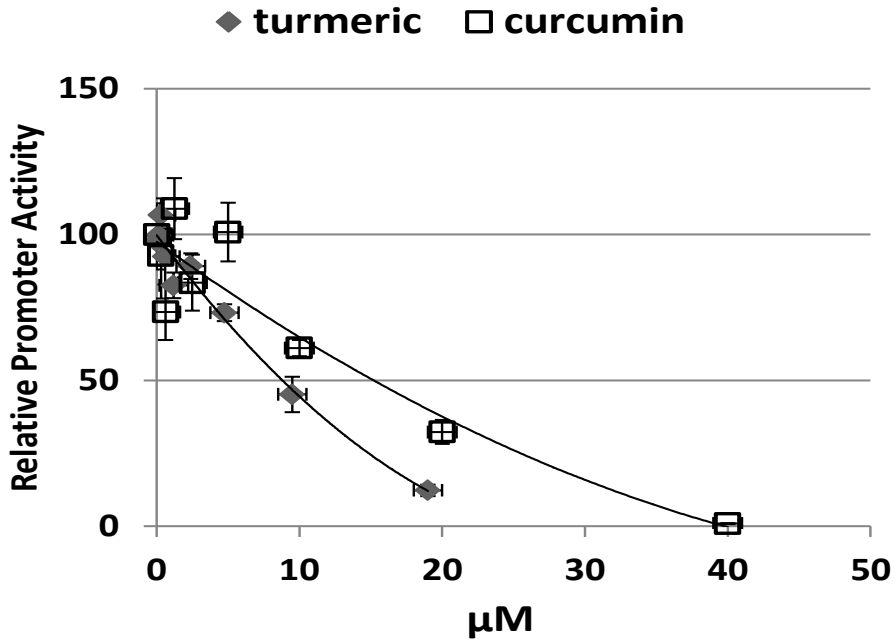

Figure 5. The effects of curcumin and turmeric on inhibition of TNFα-stimulated NF-κB transcriptional activity. HEK 293 cells were treated with serial dilutions of either curcumin or turmeric and inhibition of TNFα-stimulated NF-κB transcriptional activity was determined. Following normalization based on the relative amount of curcumin in turmeric, dose-response curves were compared between curcumin in isolation to turmeric.

Intra-herbal comparisons of companion phytonutrients (curcumin with *ar*-turmerone) on TNFα-stimulated NF-κB transcriptional activity: Curcumin and *ar*-turmerone (two bioactive phytonutrients found in turmeric) reduced the NF-κB transcriptional activity in dose-dependent manners with IC_{50} values of 20 µM and 275 µM, respectively (Fig. 4). To determine the effects on NF-κB transcriptional activity with the combination, cells were treated with curcumin plus *ar*-turmerone with serial dilutions starting with concentrations equivalent to their IC_{50} values (20 µM:275 µM) and reducing these to nanomolar concentrations while maintaining the same ratio (Table 5). The dose-response curves with the combination of curcumin plus *ar*-turmerone reduced NF-κB transcriptional activity in a dose-dependent manner. The combination of curcumin plus *ar*-turmerone reduced the minimum effective concentrations of 20 µM for curcumin and 275 µM for *ar*-turmerone to 0.16 µM+2.15 µM for their combination, respectively (Table 5). The combination was highly synergistic in inhibiting NF-κB transcriptional activity (CI equal to 0.01 with the DRIs being 109 and 311). In a similar manner, the combination of curcumin plus *ar*-turmerone in a ratio based on relative amounts in turmeric (ratio of 4:1; Table 5) also reduced the minimum effective doses significantly from 20 µM for curcumin and 275 µM for *ar*-turmerone to 0.63 µM+0.16 µM for their combination, respectively. This effect was also highly synergistic in inhibiting NF-κB transcriptional activity at these concentrations (CI equal to 0.04 with the DRIs being 23 and 3563).

Inter-herbal comparison of the extracts of Chinese goldthread with turmeric on TNFα-stimulated NF-κB transcriptional activity: The extract of Chinese goldthread did not reduce NF-κB transcriptional activity using concentrations up to 400 µg/ml (Fig. 4B). In contrast, turmeric reduced the NF-κB transcriptional activity in a dose-dependent manner (IC_{50} value was 70 µg/ml) (Fig. 4B). To determine the effects on NF-κB transcriptional activity with the combination, cells were treated with 200 µg/ml Chinese goldthread plus progressive dilutions of turmeric beginning at 70 µg/ml. The combination of Chinese goldthread plus turmeric reduced the minimum effective dose of turmeric from 70 µg/ml to 8.75 µg/ml with the combination (Table 5). CI and DRI values were not generated due to the lack of a dose-response curve for Chinese goldthread.

Table 5: Dose-effect relationships of curcumin and *ar*-turmerone (from turmeric), curcumin (from turmeric) and coptisine (from Chinese goldthread), and Chinese goldthread and turmeric and their combinations using their IC_{50} values or using ratios corresponding to relative amounts in their native extracts on NF-κB promoter activity using transfected HEK293 cells.

Isolated compounds and combinations	Minimum Effective Dose (µM)[a]	IC_{50} (µM)	Combination Index (CI)	Dose Reduction Index (DRI)
Curcumin	20	20		
ar-turmerone	275	275		
Cur + *ar*-tur (ratio = 1 : 14)[b]	0.16 + 2.15		0.01	Curcumin: 109 *ar*-turmerone: 311
Cur + *ar*-tur (ratio = 4 : 1)[c]	0.63 + 0.16		0.04	Curcumin: 23 *ar*-turmerone: 3563
Curcumin	20	20		
Coptisine	80	80		
Cur + Cop (ratio = 1 : 4)[b]	0.31+1.25		0.03	Curcumin: 70 Coptisine: 42

Isolated herbal extracts and combinations	Minimum Effective Dose (µg/ml)[a]	IC_{50} (µg/ml)	Combination Index (CI)	Dose Reduction Index (DRI)
Chinese Goldthread	-	-		-
Turmeric	70	70		-
Chinese GT + Tur	200 + 8.75		-	

[a]Concentration derived from the dose-response curves where NF-κB transcription was significantly inhibited ($p<0.05$). [b]Ratio based on IC_{50} values. [c]Ratio based on relative amounts in turmeric. Abbreviations: Cur, curcumin; *ar*-tur, *ar*-turmerone; Cop, coptisine; Chinese GT, Chinese Goldthread; Tur, turmeric.

Inter-herbal comparison of phytonutrients (coptisine with curcumin) on TNFα-stimulated NF-κB transcriptional activity: Berberine, just like Chinese goldthread, did not reduce NF-κB transcriptional activity using concentrations up to 100 µM (Fig. 4A). In an effort to explain, in part, the inter-herbal synergism between Chinese goldthread and turmeric, coptisine from Chinese goldthread and curcumin from turmeric were evaluated for synergy (Table 5). To determine the effects on NF-κB transcriptional activity with the combination, cells were treated with curcumin plus coptisine with serial dilutions from concentrations equivalent to their IC_{50} values (20 µM:80 µM) to nanomolar concentrations while maintaining the same ratio. The combination of curcumin plus coptisine reduced NF-κB transcriptional activity in a dose-dependent manner. CI and DRI values were generated for minimum effective concentrations.

The combination of curcumin and coptisine was highly synergistic in inhibiting NF-κB transcriptional activity at these levels, where the CI was equal to 0.03, and the DRIs being 70 and 42 when combined at the minimum effective concentrations (0.31 μM+1.25 μM) (Table 5).

DISCUSSION:

It has been proposed that traditional medicines from various cultures (i.e., Traditional Chinese Medicine) provide effective remedies due to their combinations of herbals, but there is a challenge in establishing a rationale for their superior therapeutic value compared with using isolated bioactives [66]. There is growing evidence that the benefits of combinations of essential and non-essential nutritives lie in their synergy and multiple targets of action [67]. This concept is presented in several reviews describing the lack of concordance between observational studies of bioactives compared with the foods from which the bioactives are derived (i.e., the impact of β-carotene versus foods that contain β-carotene on the risk of lung cancer) [3, 67].

The mechanisms of the anticancer effects of berberine, curcumin and their herbs from which they are derived have been extensively studied, where they have been shown to modulate cancer cells to induce cell-cycle arrest, cellular apoptosis and inhibition of cell invasion and metastasis. Multiple signaling pathways are known to be affected (including cyclin D, caspase-3, NF-κB, PI3 kinase, TNF-α, COX-2, MMP-9, etc.) and are well-documented [2, 13, 21-23, 68-71]. Thus, it is not necessary for us to duplicate these mechanistic studies. The aim of this paper was not to revisit the established mechanisms of the anticancer effects of these bioactives, but to assess the synergistic effect between bioactives and compare them to the herbs from which they are derived.

It has been suggested that synergy of polyherbals is related to their multiple targets of action, their coordinated impact on bioavailability, and their collective ability to minimize/neutralize adverse side effects [72]. Furthermore, while many bioactives from botanicals have the same targets [73], it is not always clear whether the mechanisms modifying these targets are similar. Because of our ability to demonstrate the combinations used in this study function synergistically (and not additively), these data suggest while some mechanisms may overlap, others involve biological convergence (modifying a biological outcome, such as cell proliferation, via several pathways) and biochemical convergence (modifying a biochemical pathway, such as NF-κB transcriptional activity, via several mechanisms).

Also important is the concept that the combination of compounds could sufficiently lower the effective dose/concentration when compared with the individual compounds, and similarly, the ability of the combination of herbs to lower the effective dose/concentration when compared with individual herbs or individual bioactives derived from the herbs. If this effective dose/concentration can be sufficiently lowered, there is a better chance that it could have biological meaning, i.e., shifting the effective levels from micromolar to nanomolar concentrations. For most underivatized bioactive phytonutrients, their effective concentrations on cells in culture are typically between 20-100 μM (see Table 1) [1], while the total plasma/serum concentrations (derivatized and underivatized) are typically two to three orders of magnitude lower following oral dosing (see Tables 1 and 2) [74-76].

Investigations in the chemopreventative effects of phytonutrients are not new, but confirming the concept that effectiveness of the botanicals from which these phytonutrients are

derived, particularly through dose-reduction, may be superior because the synergy of action has been poorly explored. To quantitate this kind of interaction, we used the Chou-Talalay method of analysis [65]. This method can differentiate between synergy, additive effects and antagonism of combinations of agents. It is based on the use of a constant ratio of components through a variety of concentrations (i.e., serial dilutions) while monitoring a single end point (in our case cell proliferation or promoter activation). This computerized model establishes the CI (combination index) to determine the type of interaction, and the DRI (dose-reduction index) represents the magnitude of the dose reduction as a result of the combination for a given dose.

These data are typically generated using ratios of the IC_{50} values from dose-response curves generated for each individual agent and are presented in each of the tables. Data can also be generated, and was presented in the tables, using concentrations that approach physiological relevance where cell growth was still significantly inhibited. The DRI is important at physiologically relevant concentrations (those plasma/serum concentrations following an oral dose) to establish potential therapeutic value. Because individual phytonutrients within the same botanical (what we refer to as "companion compounds") exist in ratios that may be different from the IC_{50} values, we also ran a parallel set of experiments using those ratios for biological relevance. For example, the IC_{50} values for berberine and coptisine are 30 μM and 85 μM, respectively, for a ratio of 1:2.8, but in Chinese goldthread these two compounds exist in a ratio closer to 5:1. The DRI can tell us to what extent one compound can enhance the effectiveness of another compound at a given ratio and dose compared with the compound alone [64].

This study systematically demonstrates that the enhanced effects of herbal extracts compared with individual bioactives found in those extracts may be explained by the synergy of action of the companion compounds found within that extract. When the extract of turmeric was compared with curcumin and the extract of Chinese goldthread compared with berberine, the dose response curves shifted to the left suggesting companion compounds within the extracts could be responsible for this shift by acting in synergy. This was confirmed by establishing the synergy (CI values <1) between companion bioactives from the same extract (i.e., curcumin plus *ar*-turmerone from turmeric, and berberine plus coptisine from Chinese goldthread).

In addition, this study further supports the concept that the synergy observed by combining two different herbal extracts could, in part, be explained by their synergistic action and due, in part, to the synergistic action of the combination of individual bioactives uniquely derived from these different sources. This is easily illustrated by comparing our previous results with our current results on the ability of Chinese goldthread and turmeric to inhibit proliferation of CWR22Rv1 cells (see Supplemental Figure 1). Chinese goldthread and turmeric, when isolated from a polyherbal mixture, were unable to inhibit cell proliferation at doses of 4.1 μg/ml and 11.3 μg/ml, respectively, while our current data demonstrates inhibition of proliferation was observed at doses as low as 1.1 μg/ml and 0.5 μg/ml, respectively, when used in combination (Table 3). It is believed that this synergistic effect is due, in part, to the combinations of bioactives unique to each of the extracts. When berberine (from Chinese goldthread) was combined with curcumin (from turmeric), the CI value demonstrated strong synergism. This supports the concept of improved effectiveness with targeted combinations of botanicals.

Our previous studies investigated the mechanisms of a polyherbal mixture containing Chinese goldthread and turmeric on the inhibition of castrate-resistant PCa *in vitro* and *in vivo*

[7-9]. These inhibitory effects also included mechanisms involving TNFα-induced cell proliferation (See Supplemental Figure 2). TNFα signals through NF-κB. As a follow-up, this study helps to better define the actions of two of the components in the polyherbal mixture, Chinese goldthread and turmeric, on this signaling pathway. Turmeric and curcumin inhibited NF-κB promoter activity, but Chinese goldthread and berberine were ineffective. Similar to our results, curcumin has been reported by others to inhibit NF-κB transcriptional activity at similar concentrations observed in this study (~20 μM) [77-82]. In contrast, Chinese goldthread and berberine did not inhibit NF-κB transcriptional activity in this study, and others report similar results with berberine [83, 84]. Interestingly, coptisine, a component of Chinese goldthread, inhibited NF-κB transcriptional activity, but these levels were much higher (IC_{50} =70 μM) than what could be achieved as a component of Chinese Goldthread, partially explaining why Chinese goldthread was ineffective. Interestingly, when curcumin was used in combination with the least active bioactives (i.e., coptisine and *ar*-turmerone), curcumin enhanced their effectiveness 32 and 3563 fold, respectively. In addition, an ineffective Chinese goldthread lowered the minimum effective dose of turmeric 8 fold (a CI value could not be determined due to the lack of a dose response with Chinese goldthread).

Importantly, these data suggest that even when bioactives appear to have little or no activity, they may still act synergistically with other bioactives when used in combination, underscoring the concepts of biological and biochemical convergence. In addition, our data demonstrate that these actions of synergy may be concentration dependent. The use of concentrations higher than the IC_{50} values (i.e., IC_{90}) for the combinations can act in a synergistic manner; however, many times this is not the case. In many instances, the CI values are >1 (antagonism) at concentrations higher than the IC_{50} value, but <1 (synergy) for concentrations lower than the IC_{50}, (see Supplemental Table 1), underscoring the importance of concentrations used in studies.

CONCLUSIONS:

In summary, most of today's cancers are influenced by our environment, particularly by the botanicals we eat (i.e., herbs, fruits, vegetables). The major source of bioactive phytonutrients is from these foods. To understand the impact of their consumption, our objective was to investigate the concept that the combination of bioactives in botanicals is potentially far more effective than an individual bioactive in isolation. Our data is proof-of-principle that combinations of companion bioactives from the same source and bioactives from different sources act synergistically, providing some evidence that combinations found in botanicals (and foods) have advantages over isolated bioactives. Even compounds with poor efficacy can become more biologically active in the presence of companion compounds. These advantages translate into effective doses that are more physiologic.

List of abbreviations: ber, berberine; cop, coptisine; CI, combination index; cur, curcumin; DRI, dose reduction index; GT, goldthread; tur, turmeric;

Competing interests: The following authors have no conflicts of interest: J. Whelan, Y. Zhao, J. Jason Collier and E-Chu Huang. No competing interests exist regarding interpretation of the data or presentation of information. There are no personal or financial relationships with other people

or organizations or any financial/non-financial competing interests that influenced any aspect of the research or the writing of the manuscript.

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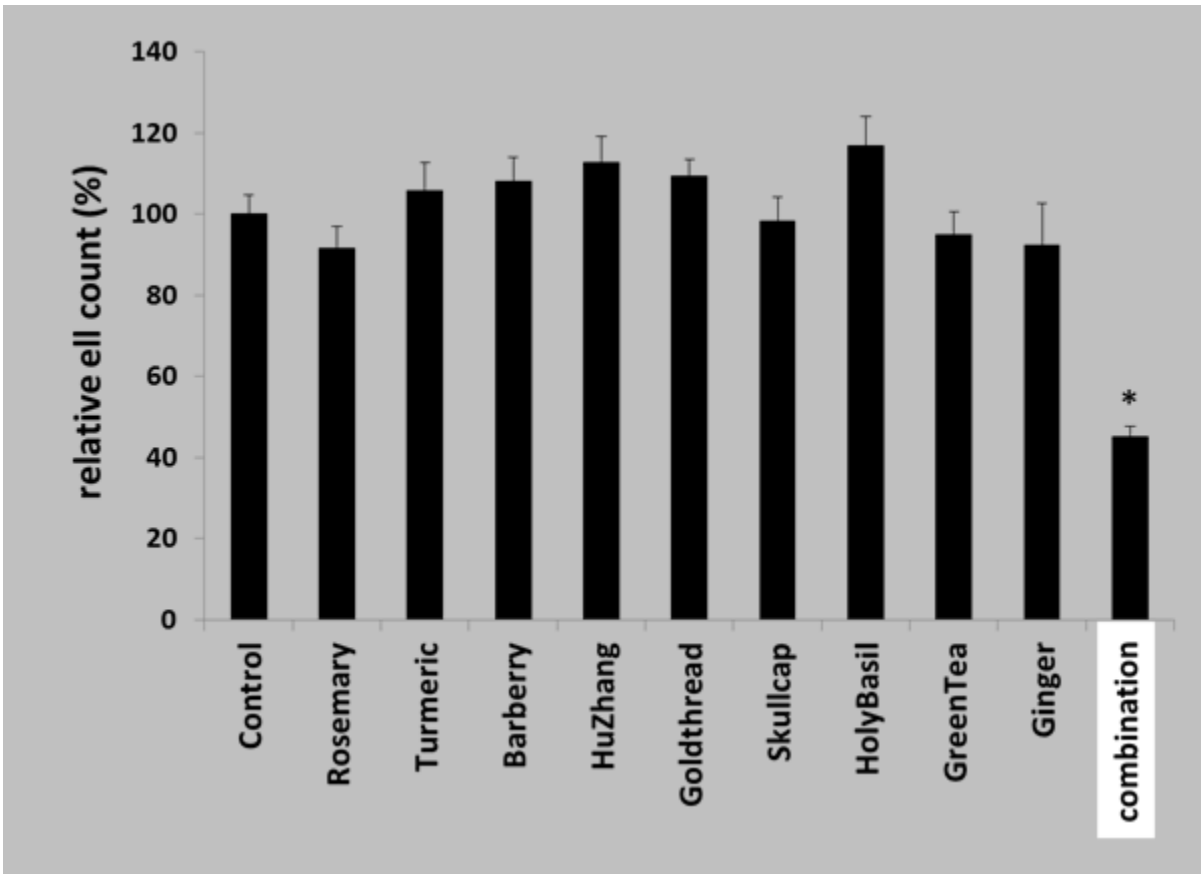
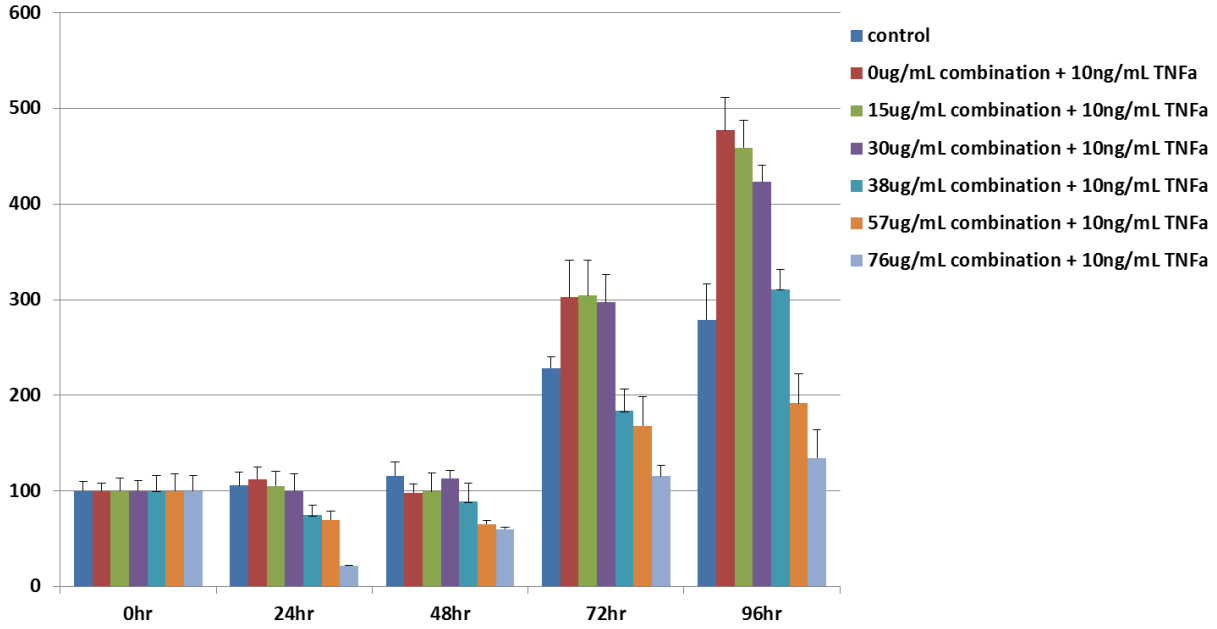
SUPPLEMENTAL FIGURES AND TABLES:



Supplement Figure 1: The effects of individual herbs versus their combination on cell proliferation. CWR22Rv1 cells were treated with individual herbal extracts (10.2 µg/ml ginger, 15.4 µg/ml rosemary, 11.3 µg/ml turmeric, 4.1 µg/ml Chinese goldthread, 10.2 µg/ml holy basil, 8.2 µg/ml hu zhang, 4.1 µg/ml barberry, 10.2 µg/ml green tea, 2.0 µg/ml Baikal skullcap) or in combination equivalent to amounts used individually (76 ug/ml) for 48 hr and cell viability was measured by MTT assay (see "Methods" section of the paper). For more details of the mixture see references 7-9. Treatment with individual herbal extracts did not significantly affect cell viability, whereas their combination significantly reduced cell viability. Data is presented as means±SD (n=8). Bars with an asterisk (*) are significantly different from the non-treated Control at $p<0.05$.



Supplemental Figure 2. CWR22Rv1 cell were treated with a combination of herbal extracts (composition: 10.2 µg/ml ginger, 15.4 µg/ml rosemary, 11.3 µg/ml turmeric, 4.1 µg/ml Chinese goldthread, 10.2 µg/ml holy basil, 8.2 µg/ml hu zhang, 4.1 µg/ml barberry, 10.2 µg/ml green tea, 2.0 µg/ml baikal skullcap) with increasing combined concentrations (0, 15, 30, 38, 57, 76 ug/ml) with or without TNF-α (10 ng/ml) for 0, 24, 48, 72, and 96 hr. For more details of the herbal mixture see references 7-9. Cell viability was measured by MTT assay (see "Methods" section of the paper). TNF-α had a progressive effect on cell proliferation over time and this effect was attenuated in the presence of herbal mixture in a dose-dependent manner. Data is presented as means±SD (n=8).

Supplemental Table 1: Dose-effect relationships of Chinese goldthread, turmeric, berberine, coptisine, curcumin and *ar*-turmerone and their combinations on proliferation of CWR22Rv1 and HEK293 cells and NF-κB promoter activity.

Compounds / combinations	Dm (μM)	m	r	CI IC_{25}	CI IC_{50}	CI IC_{75}	CI IC_{90}	DRI-IC_{25}	DRI-IC_{50}	DRI-IC_{75}	DRI-IC_{90}	DRI-IC_{25}	DRI-IC_{50}	DRI-IC_{75}	DRI-IC_{90}
Inhibition of Proliferation using CWR22Rv1 Cells								**Ber**	**Ber**	**Ber**	**Ber**	**Cop**	**Cop**	**Cop**	**Cop**
Berberine	23.1	1.7	0.9												
Coptisine	96.7	0.8	0.9												
Ber:Cop (1: 2.8)[a]	27.2	0.6	0.8	0.2	0.5	1.5	4.7	11.8	3.3	0.9	0.1	8.4	4.8	2.7	1.6
Ber:Cop (5: 1)[b]	25.3	1.2	0.9	0.8	0.9	1.2	1.6	1.4	1.1	0.8	0.6	14.5	22.9	36.3	57.3
Inhibition of Proliferation using CWR22Rv1 Cells								**Cur**	**Cur**	**Cur**	**Cur**	***ar*-T**	***ar*-T**	**ar-T**	**ar-T**
Curcumin	11.4	2.1	0.9												
ar-turmerone	328.3	0.4	0.9												
Cur:*ar*-T (1:6.8)[a]	13.8	1.2	0.7	0.3	0.2	0.2	0.4	10.0	6.4	4.1	2.7	6.0	27.3	124	564
Cur:*ar*-T (4: 1)[b]	4.4	1.0	0.8	0.2	0.3	0.6	1.1	6.1	3.2	1.7	0.9	99.4	371	1386	5173
Inhibition of Proliferation using CWR22Rv1 Cells								**Ber**	**Ber**	**Ber**	**Ber**	**Cur**	**Cur**	**Cur**	**Cur**
Berberine	23.1	1.7	0.9												
Curcumin	11.4	2.1	0.9												
Ber:Cur (1.4: 1)[a]	13.8	1.2	0.7	0.5	0.6	0.7	0.9	4.8	4.3	3.9	3.5	3.9	2.9	2.1	1.6
Inhibition of Proliferation using CWR22Rv1 Cells								**CG**	**CG**	**CG**	**CG**	**Tur**	**Tur**	**Tur**	**Tur**
Chinese goldthread	106	1.6	0.9												
Turmeric	49.6	1.8	0.8												
Chinese GT: Tur (2: 1)[a]	87.5	0.6	0.8	0.3	1.1	3.7	12	5.7	1.8	0.6	0.2	5.8	1.7	0.5	0.2
Inhibition of Proliferation using HEK293 Cells								**Ber**	**Ber**	**Ber**	**Ber**	**Cop**	**Cop**	**Cop**	**Cop**
Berberine	42.9	2.6	0.9												
Coptisine	81.9	2.8	0.9												
Ber:Cop (1:2.3)[a]	51.2	2.0	0.9	0.7	0.8	0.9	1.0	3.2	2.8	2.5	2.2	2.7	2.3	2.0	1.7
Ber:Cop (5: 1)[b]	33.3	1.0	0.9	0.3	0.7	1.5	3.0	3.2	1.5	0.8	0.4	31.3	14.8	7.0	3.3
Inhibition of Proliferation using HEK2931 Cells								**Cur**	**Cur**	**Cur**	**Cur**	***ar*-T**	***ar*-T**	**ar-T**	**ar-T**
Curcumin	17.3	3.2	0.9												
ar-turmerone	400.6	1.0	0.9												
Cur:*ar*-T (1:6.8)[a]	100.3	1.3	0.9	0.4	0.4	0.4	0.6	10.6	6.4	4.0	2.4	3.1	4.1	5.5	2.4
Cur:*ar*-T (4: 1)[b]	11.1	1.5	0.8	0.4	0.5	0.8	1.1	2.9	1.9	1.3	0.9	122	180	267	395
Inhibition of Proliferation using HEK2931 Cells								**Ber**	**Ber**	**Ber**	**Ber**	**Cur**	**Cur**	**Cur**	**Cur**
Berberine	37.8	2.6	0.9												
Curcumin	17.2	3.2	0.9												
Ber:Cur (1.4: 1)[a]	18.0	1.9	0.9	0.5	0.6	0.8	0.9	3.3	2.9	2.5	2.1	4.5	3.6	2.8	2.2
Inhibition of Proliferation using HEK2931 Cells								**Ber**	**Ber**	**Ber**	**Ber**	**Cur**	**Cur**	**Cur**	**Cur**
Chinese	152	2.3	0.9												

goldthread															
Turmeric	49.3	2.9	0.8												
Chinese GT: Tur (2: 1)[a]	73.7	2.6	0.9	0.8	0.8	0.8	0.8	2.7	2.8	3.0	3.1	2.6	2.5	2.4	2.3
NF-κB Promoter Activity using Transfected HEK293 Cells								**CG**	**CG**	**CG**	**CG**	**Tur**	**Tur**	**Tur**	**Tur**
Curcumin	35.0	1.3	0.5												
ar-turmerone	1418	1.3	0.5												
Cur:*ar*-T (1: 14)[a]	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Cur:*ar*-T (4: 1)[b]	43	0.8	0.4	0.6	0.9	1.7	2.9	1.8	1.0	0.6	0.3	273	166	101	61
NF-κB Promoter Activity using Transfected HEK293 Cells								**Cur**	**Cur**	**Cur**	**Cur**	**Cop**	**Cop**	**Cop**	**Cop**
Curcumin	35.0	1.3	0.5												
Coptisine	90.3	1.1	0.6												
Cur: Cop (1:4)[a]	16.2	0.7	0.8	0.1	0.2	0.4	0.7	20.5	10.8	5.7	3.0	11.6	7.0	4.2	2.5

D_m: median-effect dose (concentration which inhibits cell growth by 50%). m: shape of the dose-effect curves, where m=1, hyperbolic; m>1, sigmoidal; and m<1, flat sigmoidal, respectively. r: linear correlation coefficient of the median-effect plot (indicates conformity of data). [a]Ratio based on IC_{50} values. [b]Ratio based on relative amounts in Chinese goldthread.

Abbreviations: *ar*-T, *ar*-turmerone; Ber, berberine; CG, Chinese goldthread; CI, combination index; Cop, coptisine; Cur: curcumin; DRI, dose reduction index.