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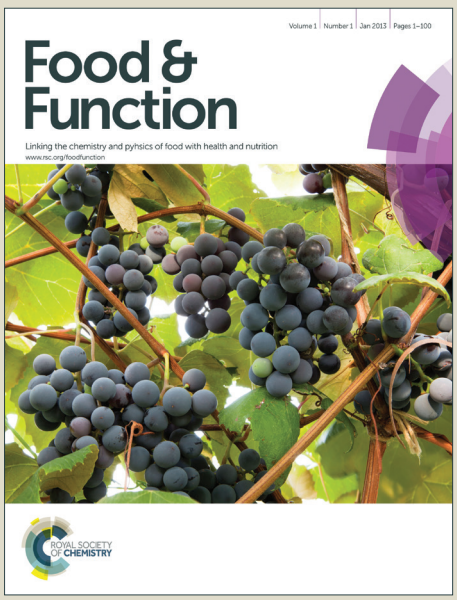

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Enhanced action of apigenin and naringenin combination on estrogen receptor activation in non-malignant colonocytes: Implications on sorghum-derived phytoestrogens

Running title: Enhanced actions of sorghum-derived phytoestrogens

Liyi Yang[a], Kimberly F. Allred[b], Linda Dykes[a], Clinton D. Allred[b], Joseph M. Awika[a,b]*

[a]Cereal Quality Laboratory, Soil & Crop Science Department, Texas A&M University, College Station, Texas, USA

[b]Nutrition and Food Science Department, Texas A&M University, College Station, Texas, USA

Cereal Quality Laboratory, Soil & Crop Science Department, Texas A&M University, College Station, Texas, USA

***Correspondence to:** Joseph Awika, 2474 TAMU, Texas A&M University, College Station, Texas 77843-2474. Phone 979-845-2985; Fax 979-845-0456; Email: jawika@ag.tamu.edu

Abstract

Activation of estrogen receptor-β (ERβ) is an important mechanism for colon cancer prevention. Specific sorghum varieties that contain flavones were shown to activate ER in non-malignant colonocytes at low concentrations. This study aimed to determine positive interactions among estrogenic flavonoids most relevant in sorghum. Apigenin and naringenin were tested separately and in combination for their ability to influence ER-mediated cell growth in non-malignant young adult mouse colonocytes (YAMC). Sorghum extracts high in specific flavanones and flavones were also tested. Apigenin reduced ER-mediated YAMC cell growth comparable to physiological levels of estradiol (E_2, 1 nM) at 1 μM; naringenin had similar effect at10 μM. However, when combined, 0.1 μM apigenin plus 0.05 μM naringenin produced similar effect as 1 nM E_2; these concentrations represented $1/10^{th}$ and $1/200^{th}$, respectively, of the active concentrations of apigenin and naringenin, demonstrating a strong enhanced action. A sorghum extract higher in flavones (apigenin and luteolin) (4.8 mg/g) was more effective (5 μg/mL) at activating ER in YAMC than a higher flavanone (naringenin and eriodictyol) (28.1 mg/g) sorghum extract (10 μg/mL). Enhanced actions observed for apigenin and naringenin were adequate to explain the level of effects produced by the high flavone and flavanone sorghum extracts. Strong positive interactions among sorghum flavonoids may enhance their ability to contribute to colon cancer prevention beyond what can be modeled using target compounds in isolation.

Keywords: Estrogenic activity, apigenin, naringenin, sorghum, colon cancer prevention

Introduction

Consumption of whole grains is linked to reduced risk of colon cancer. Given the prevalence of cereal grains as staples in the human diet, understanding the mechanisms by which they contribute to cancer prevention is important in order to maximize their impact on human health. Among the components that contribute the chemopreventive benefits of whole grain consumption are polyphenols. Polyphenols are capable of contributing to cancer prevention via various mechanisms depending on their structure, estrogenic activity being among the well-recognized mechanisms [1, 2]. Phytoestrogens reduce the risk of colon cancer by mimicking estrogen activity; the protective effects of estrogen against colon cancer are largely mediated via estrogen receptor-β (ERβ) [3-5], the predominant ER in the colon. Among the important mechanisms by which ERβ activation contributes to colon cancer prevention are induction of apoptosis [6] and tumor suppressor genes [7] in colonocytes *in vitro* and *in vivo*. Given the central role ERβ activation plays in colon cancer prevention, a clear understanding of the relative contribution of whole grain phytoestrogens in colon cancer prevention is warranted.

Sorghum consumption has been linked to a significant reduction in risk of various gastrointestinal cancers when compared to other cereal grains [8-10]. Limited laboratory evidence using animal models and/or cell culture suggest the chemoprotective properties of sorghum is attributable to its unique polyphenol composition [11-13]. For example, sorghum is unusual among cereal grains in that it contains high levels of specific flavonoids with known estrogenic activity, such as flavones and flavanones [14, 15]. Of particular interest among these compounds are apigenin and naringenin, the two most abundant flavone and flavanone, respectively, in sorghum with a lone para-hydroxyl group in the B-ring (Fig 1). The structural conformation of these two molecules makes them significantly more estrogenic than other respective flavones and

flavanones with catechol moieties on the B-ring [16-19], e.g., luteolin and eriodictyol, also abundant in sorghum [14, 15]. In addition, apigenin has been demonstrated to be a more potent ERβ activator than naringenin [19]. Sorghums are genetically diverse with distinct flavonoid compositional differences. Therefore an understanding of how the composition of estrogenic flavonoids in sorghum influence colon cancer prevention is important.

We recently demonstrated that sorghum varieties that contain flavones were capable of activating ERα and ERβ in MCF-7 breast cancer cells and non-malignant young adult mouse colonocytes (YAMC), respectively, at relatively low concentrations [20]. In the same study, a sorghum that did not contain any flavones did not show estrogenic activity. Polyphenol content and composition of other components did not correlate with ER activation. Of particular interest was the fact that both sorghum samples that activated ER had high levels of apigenin (>1,000 μg/g extract). These findings are interesting because they demonstrate that ER activation is a potential mechanism by which sorghum contributes to cancer prevention, and that composition of sorghum has a major impact on its ability to influence ER activation. However, an anomaly observed in this study was the fact that the concentrations at which the sorghum extracts activated ER were much lower than could be explained by their content of estrogenic flavonoids. Additionally, a sorghum extract that was high in flavanones did not activate ER. We hypothesize that synergistic interactions of flavones in sorghum with other less potent ER agonists accounts for the high ER activation potential of sorghum extracts in non-malignant colonocytes. In this study, we use apigenin and naringenin, as well as extracts from sorghums with different flavone and flavanone profiles to demonstrate the interactive effect of estrogenic flavonoids on possible ER activation using YAMC cells as a model.

Materials and Methods

Materials

Commercially available apigenin (> 97% pure) was obtained from Indofine Chemical Company, Inc., Hillsborough, NJ; naringenin (> 99% pure) was purchased from MP Biomedicals, Solon, OH). Two sorghum varieties were selected for this study: A red pericarp variety with tan secondary plant color (99LGWO50), previously shown to be high in flavones, mainly apigenin and luteolin; and a lemon-yellow pericarp variety with purple secondary plant color (SC748), which is high in flavanones, mainly glycosides of eriodictyol and naringenin. The sorghum grains were kindly provided by Dr. W. L. Rooney, Department of Soil & Crop Sciences of Texas A&M University. The sorghum grains were harvested in 2008 at College Station, TX and were stored at -20 °C until use.

Young Adult Mouse Colonocytes (YAMC) cells were supplied by Dr. Robert Chapkin (Department of Nutrition and Food Science, Texas A&M University). These cells are a well-characterized non-malignant cell line derived from the Immortomouse, and are morphologically primitive epithelial cells with no evidence of differentiation. We have used the YAMC cells as a model to study the role of estrogen and estrogenic compounds in mediating cellular changes related to colon cancer prevention and the results correlated with *in vivo* studies well [6, 7, 21]. The YAMC cells predominantly express ERβ. Estradiol, administered at non-permissive conditions, inhibits the growth of YAMC cells [6, 7].

Sorghum extraction

Whole kernels of sorghum grain were ground by a cyclone mill (UDY, Boulder, CO) to pass through 0.1 mm screen before extraction. Ground samples were defatted using hexane at a ratio of 1:2 (w:v) by stirring for 2 h. The matrix was then centrifuged at 3100 ×g and the residue was

dried inside a fume hood overnight at room temperature. Defatted samples were extracted with 70% (v/v) aqueous acetone with stirring for 30 min. Supernatant was collected by centrifuging (3100 × g) for 15 min at 4 °C. Acetone was immediately removed from the supernatant under vacuum at 40 °C. The aqueous phase was freeze-dried and used as a crude extract. Extracts were kept at -20°C till use.

Acid hydrolysis of sorghum extract. Preliminary analysis confirmed that the lemon-yellow sorghum extract contained mostly flavonoid glycosides which are known to be easily hydrolyzed by digestive and microbial enzymes in the GI tract to their more biologically active aglycones [22, 23]. The lemon-yellow sorghum extract was hydrolyzed in acidified aqueous methanol (HCl : water : methanol = 0.1 : 49.9 : 50, v/v/v), at 60 °C for 27 h in a water bath to obtain flavanone aglycones. Based on quantitative HPLC data, 90% of the flavanone glycosides were hydrolyzed to their corresponding aglycones. Methanol was rotary evaporated and the aqueous fraction was freeze-dried.

LC-MS analysis of sorghum extracts

The phenolic profile of the sorghum extracts were characterized using protocols recently detailed [24, 25]. A Waters-ACQUITY UPLC/MS system (Waters Corp., Milford, MA) was used to structurally identify polyphenols as previously described [26] with a modified gradient as follows: solvent A (0.05% formic acid in water), solvent B (acetonitrile), and the percentage of solvent B was 12-41% from 0-23.5 min, 41-75% from 23.5-25.5 min, 75% isocratic from 25.5-28.5 min, then 75-12% from 28.5-29.5 min, and 12% isocratic for 5 minutes. The monitoring wavelength for 3-deoxyanthocyanidins and derivatives was 485 nm; for phenolic acids and flavones was 340 nm; for flavanones and other polyphenols was 280 nm. Mass spectrometric (MS) data were acquired in positive mode for 3-deoxyanthocyanidins as well as its derivatives, and in negative

mode for all the rest of compounds. The MS scan was recorded in the range of 100−1200 Da. Mass parameters were optimized as follows: capillary voltage, 3.5/3.0 kV; and cone voltage, 60/30 V for positive/negative ionization, respectively. The MS/MS scan was optimized as follows: cone voltage of 60/(30−50) V and collision energy of (35-45)/(15−40) V. Compound identification was based on matching UPLC retention profile, UV-*vis* spectra and MS data with authentic standards. Where standards were not available, compounds were identified based on the fragment patterns compared with reports in the literature.

An Agilent 1200 series HPLC system (Agilent Technologies, Santa Clara, CA) was used for quantitative analysis of polyphenols as previously described [20] with minor modifications. Solvent A was 1% formic acid in water and solvent B was 1% formic acid in acetonitrile. The gradient based on solvent B was as follows: 0-3 min, 10%; 5 min, 18%; 10 min, 20%; 23 min, 26%; 25 min, 28%; 28 min, 40%; 30 min, 60%; 30-32 min, 60%; 34-40 min 10%.

Effect of apigenin, naringenin, and sorghum extracts on YAMC cell growth

YAMC cells were maintained and cultured as previously described [20]. The cells were maintained at permissive conditions (33 °C with 10 units of interferon gamma) and cultured at non-permissive conditions (39 °C without interferon gamma) for all treatments as previously described [20]. Cells were cultured in charcoal-dextrose treated FBS (CDFBS) supplemented media 48 hr before plating and thereafter in order to deplete the estrogen present in culture media. Cells were then plated at 25,000 cells per well, after attachment to the bottom of plate (24 hr later), cells were treated with control (0.1 % DMSO), positive control (1 nM estradiol), and treatments (sorghum extract, 1, 5, 10, and 50 μg/mL; apigenin and naringenin 0.01-10 μM) for 96 hr under non-permissive conditions (39 °C, without IFNγ). At the end of 96 hr incubation, cells were harvested with trypsin and cell numbers of each treatment were counted with a

Beckman Coulter particle counter (> 20 μm, Beckman Coulter, Brea, CA). Relative growth of each treatment was calculated by comparing the cell numbers of each treatment with that of the control. Each experiment was repeated 3 times.

ER antagonist assay

In order to confirm the effect of sorghum extracts on YAMC cell growth was mediated through ER, ER antagonist ICI (ICI 182, 780; Fulvestrant, Sigma-Aldrich, St. Louis, MO) was used. All the handling and plating procedures were the same as in determining YAMC cell growth. The only difference was that in this set of experiments, each treatment and control was additionally co-treated with 1 μM ICI, each to 3 wells, respectively. Cell number and relative cell growth were determined as previously described by a Beckman Coulter particle counter. Each experiment was repeated 3 times.

Statistical analysis

The data were analyzed with one way analysis of variance (ANOVA). Means were compared by Dunnett's t-test as a multiple comparison technique to determine the difference between treatments and their corresponding control. Paired comparisons between treatments was analyzed by two-tailed t-test. All statistical analysis was performed using SAS 9.2 (Cary, NC).

Results and Discussion

Interactive effects of apigenin and naringenin on ER activation

Interactive effects of flavonoids on ER activation have been shown to be mostly additive or mildly antagonistic [27]. Apigenin and naringenin, being the most abundant and estrogenic flavonoids in sorghum, were used to determine the potential nature of the interactive effect of

sorghum-derived phytoestrogens using the YAMC cells as the model. This helped explain why sorghum extracts were able to show estrogenic activity in the YAMC at such low concentrations [20]. Apigenin inhibited YAMC cell growth similar to 1 nM E_2 at 1 μM; naringenin produced a similar effect at 10 μM (Fig 2A). Administration of the ER antagonist ICI reversed the cell growth inhibitory effect of apigenin (1 μM) and naringenin (10 μM) to the level of control (Fig 2B), which suggested the growth inhibition effect was likely mediated through ER. The difference in relative estrogenic potency of apigenin and naringenin on the ER in YAMC cells was consistent with other reporter assays targeting ERβ signaling [28, 29]. The YAMC cell model, under the non-permissive culturing conditions, has clear physiological relevance to the cellular characteristics of colonic epithelia *in vivo* [30] and we have effectively used the model in the past to predict how colonic epithelia will respond to compounds that elicit protective effects in the colon [6, 7].

The molar concentration at which apigenin activated ER in the YAMC cells was about 15 – 30 times higher than the total content of the putative estrogenic flavones and flavanones in the sorghum extracts that activated ER in the previous study [20]. A likely explanation is that in a natural mixture, small quantities of phytoestrogenic compounds may favorably interact to induce physiologically relevant estrogenic response. Because it is practically impossible to truly isolate and determine the absolute contribution of an individual compound in a complex mixture characteristic of most natural food matrices, we proceeded to use apigenin and naringenin to gain insight on the possible interactive effects of sorghum phytoestrogens.

The combination of sub-optimal concentrations of apigenin (0.1 μM) and naringenin (0.05, 0.1, and 1 μM) significantly inhibited the growth of YAMC cells, suggesting ER activation (Fig 3). For example, apigenin at 0.1 μM co-treated with naringenin at 0.05 μM

showed activity similar to 1 nM E_2 (Figure 3A); these concentrations corresponded to 1/10 and 1/200, respectively, of their optimal concentrations (producing effect similar to 1 nM E_2) for ER activation in the YAMC cells (Fig 2). Such strong enhanced action was somewhat unexpected and has not been previously reported. Wong et al, [31] observed mostly additive effects when they tested various mixtures of flavones (apigenin and luteolin) and flavonols (kaempferol and quercetin) in ERα and ERβ models. The fact that increasing concentrations of naringenin, 20-fold, (from 0.05 μM to 1 μM) while holding apigenin level constant at 0.1 μM did not change the YAMC response may suggest different activation mechanisms for the two molecules. Apigenin, a flavone, like the more widely studied phytoestrogen, genistein (an isoflavone), is achiral due to the presence of a double bond between C2 and C3 of the heterocyclic ring (Fig 1), and thus has a 3-dimensional conformation very similar to genistein. The two molecules may thus bind to ER in a similar manner. In fact apigenin and genistein appear to have similar estrogenic activity in YAMC cells under similar experimental conditions [21]. Naringenin (a flavanone), on the other hand, has a chiral center at C2 which makes its 3-D conformation markedly different from apigenin (or genistein), and perhaps accounts for its lower estrogenic activity, despite apparent structural similarity to apigenin.

The above observation is interesting because it indicates that the potential bioactivity of a given compound in a complex natural mixture typical of most foods may be much higher than what can be predicted or modeled using isolated/pure compounds. Thus using purified compounds to infer magnitude of bioactivity of polyphenols in foods can be misleading.

Interestingly, we observed that sub-optimal concentrations of naringenin (0.1 and 1 μM) did not affect the YAMC cell response to optimal concentration of apigenin; the extent of growth inhibition was the same as apigenin alone, when we combined optimal levels of apigenin (1 μM)

with naringenin (0.1-1.0 μM) (Fig 3). However, at higher concentrations of 5 and 10 μM, naringenin surprisingly eliminated the YAMC response to 1 μM apigenin (Fig 3), where the growth inhibition was reversed. This apparent antagonistic effect was unexpected. Naringenin has been reported as both a weak ER agonist or antagonist depending on concentration [17]. Given that both apigenin and naringenin, like most estrogenic flavonoids, are only partial ER agonists, the data suggests that co-interference at high concentrations may be at play. The possible loss of estrogenic activity after combining optimal levels of both apigenin and naringenin potentially suggests that more is not always better when it comes to use of phytochemicals to prevent disease, and the moderating effect of foods may perhaps be more beneficial than, for example, concentrated supplements.

Estorgenic activity of the red and lemon-yellow sorghum extracts in young adult mouse colonocytes (YAMC)

These sorghum varieties were selected based on their distinctly different profiles of putative estrogenic flavonoids, flavones and flavanones, and low levels of other flavonoids with unknown estrogenic properties, e.g., 3-deoxyanthocyanins [14, 15]. The composition of the major flavonoids in the two sorghum varieties was confirmed using LC-MS and is summarized in Table 1. The lemon-yellow sorghum had much higher levels of flavanones (eriodictyol and naringenin), primarily as glycosides, than the red sorghum. The red sorghum on the other hand had higher levels of flavones, in particular apigenin (Table 1). The lemon-yellow sorghum extract was further hydrolyzed to release flavanone aglycones and better reflect the fate of these compounds in the GI tract [22, 23]; high amounts of flavanones (28.1 mg/g total), with almost equal

amounts of naringenin and eriodictyol, were obtained (Table 1) and relatively low levels of flavones (1.12 mg/g total, mainly as luteolin and its glycosides) were detected.

Among the two sorghum varieties tested, the red sorghum extract showed a higher inhibitory effect on YAMC cell growth than the unhydrolyzed lemon-yellow one (Fig 4A). The red sorghum extract (5 µg/mL) inhibited YAMC growth (20.5% reduction) similar to 1 nM E_2 ,positive control, (19.1% growth reduction). The lemon-yellow sorghum extract, on the other hand, showed similar ($p > 0.05$) YAMC growth inhibition effects (23.6% inhibition) at 50 µg/mL; it showed a smaller but significant growth inhibition (9.6%) at 10 µg/mL (Fig 4A). Use of the ER antagonist, ICI, reversed the growth inhibition by the sorghum extracts, indicating the effect is likely mediated via ER (Fig 4B). The major compositional differences between these two sorghum extracts are flavone and flavanone types and content (Table 1). The red sorghum had apigenin as the major flavone at 4.1 mg/g extract, and naringenin glycosides (2.6 mg/g) as the major flavanones. On a molar basis, these two compounds were present in the 5 µg/mL treatment, which was equivalent in effect to the E_2 positive control, at 76.5 nM and 29.4 nM, respectively. These values were within order of magnitude of the 100 nM and 50 nM mixture of apigenin and naringenin, respectively, which showed significant ER activation in YAMC cells (Fig 3). Thus considering the enhanced effect demonstrated by the combination of the pure compounds, it is reasonable to assume that the levels of flavones and flavanones in the red sorghum were adequate to account for its estrogenic effect in YAMC cells. Even though the red sorghum also contained small amounts of 3-deoxyanthocyanins that were not present in the lemon-yellow sorghum, these compounds likely had no contribution to ER activation. This is because apigeninidin and its *O*-methyl derivatives, the major 3-deoxyanthocyanins in the red

sorghum, had no effect on YAMC cell growth when tested individually at up to 50 μM (data not shown).

In the unhydrolyzed lemon-yellow sorghum extract, relatively high levels of flavanones were present, primarily as glycosides of naringenin (42.7 mg/g) and eriodictyol (43.6 mg/g). Among flavones, luteolin glucoside was the dominant compound (3.9 mg/g), while apigenin and luteolin aglycones were only present in very small amounts (Table 1). Glycosides of estrogenic flavonoids have markedly reduced activity than their aglycones [32, 33], thus the limited content of estrogenic aglycones may have accounted for the lower apparent ER activating properties of the unhydrolyzed lemon-yellow sorghum extract in YAMC cells compared to the red sorghum extract. The 50 μg/mL lemon-yellow sorghum extract treatment (that produced an effect equivalent to E_2) contained 5 μM each, of naringenin and eriodictyol glycosides, and 689 nM of luteolin glucoside.

Testing the estrogenic flavonoid glycosides *in vitro* may underestimate their potential effects *in vivo* given that flavonoid glycosides are easily hydrolyzed in the digestive tract by native or microbial enzymes into the more active aglycones. This enzyme hydrolysis has been shown to result in near identical estrogenic activity of estrogenic aglycones and their glycosides *in vivo* [22]. For this reason, we were interested in demonstrating how the high levels of flavanones in the lemon-yellow sorghum may impact ER activation when deglycosylated. As expected, the hydrolytic release of flavanone aglycones resulted in a significant increase in estrogenic activity of the lemon-yellow sorghum extract in YAMC cells (Fig 4), with a significant ($p < 0.05$) 12% growth inhibition at 5 μg/mL and 25.2% inhibition at 10 μg/mL. Thus, though not as effective as the higher flavone red sorghum extract at estrogen-mediated YAMC growth inhibition at low concentrations, the high flavanone hydrolyzed lemon-yellow extract was more effective than the

non-hydrolyzed version. The 10 µg/mL lemon-yellow extract contained approx. 450 nM naringenin and 382 nM eriodictyol, within the range that would likely show estrogenic activity in YAMC cells in a synergistic environment (Fig 3). The overall data suggests higher estrogenic potency of high flavone sorghum compared to high flavanone sorghum.

Evidence indicates that significant absorption and metabolism of dietary flavonoids by phase II enzymes occurs in intestinal epithelial cells, and that the ABC (ATP-binding cassette) transporter proteins play an important role in effective bioavailability (and by extension bioactivity) of the flavonoids and their metabolites [34, 35]. The ABC transporters are generally reported to reduce bioavailability of flavonoids by primarily increasing the efflux of their conjugated (mainly glucuronides/sulfates) metabolites into the apical side of epithelial cells. Depending on their structure, the flavonoids themselves have been reported to either inhibit [36], or induce [37] the ABC transporters in the intestinal epithelial cells, with most estrogenic flavonoids (including apigenin and naringenin used in this study) generally acting as inhibitors [38, 39]. This suggests that the estrogenic effects we observed for the flavonoids in the YAMC model may be partly dependent on their interaction with the ABC transporters. In fact, apigenin is a stronger inhibitor of ABC transporters than naringenin [40], which may partly account for its higher estrogenic potency. Furthemore, Brand et al [39], recently demonstrated that co-administration of hesperetin with other flavonoids that inhibit the ABC transporter, BCRP (breast cancer resistance protein), significantly diminished hesperetin metabolism and apical efflux, and enhance its excretion on basolateral side of Caco-2 cells, signifying increased bioavailability [39]. The enhanced estrogenic activity of apigenin-naringenin combination, and sorghum flavonoids at low concentrations in YAMC is thus possibly contributed by complex interactions involving different mechanisms that may involves the ABC transporters.

Conclusion

We have demonstrated that combination of apigenin and naringenin significantly enhances their action on estrogen receptor activation in YAMC cells. This enhanced action is adequate to explain the relatively low concentrations of sorghum extracts containing flavones and flavanones needed to significantly influence ER-mediated cell response in the non-malignant colonocytes. Thus our findings suggest that ER activation in colonocytes is an important mechanism by which sorghum may contribute to colon cancer prevention. The evidence also indicates that the composition of flavonoids in sorghum, rather than the content of the polyphenolics, is the critical factor in determining potential ER activity. More importantly, it is apparent that modeling bioactivity with pure/isolated compounds may lead to misleading conclusions regarding potential health benefits by not accounting for potentially strong synergistic effects among different compounds typical of natural food components.

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414 **Tables:**

415 Table 1. Composition of the major flavonoids in the red, lemon yellow and hydrolyzed lemon

416 yellow sorghum extracts used in this study[a].

Compound	Red sorghum	Lemon-yellow sorghum	Hydrolyzed lemon-yellow sorghum
Total phenols[b]	103 ± 8.7	58.5± 3.3	187.6 ± 2.6
Luteolin glycosides[c]	ND	3,940 ± 64	592 ± 4
Luteolin	712 ± 89	379 ± 16	444 ± 18
Apigenin glycosides[d]	100 ± 15	ND	ND
Apigenin	4,130 ± 170	224 ± 35	Trace
Tricin[e]	ND	126 ± 21	81 ± 3
Total flavones	**4,840 ± 260**	**4,670 ± 0.09**	**1,120 ± 18**
Eriodictyol glycosides[f]	ND	43,600 ± 1460	1,910 ± 01
Eriodictyol	ND	217 ± 05	11,000 ± 660
Naringenin glycosides[g]	2,550 ± 29	42,700 ± 1180	2,770 ± 14
Naringenin	62 ± 14	2,170 ± 60	12,200 ± 20
Total flavanones	**2,610 ± 300**	**88,600 ± 2700**	**28,100 ± 670**
Total 3-deoxyanthocyanins	**231 ± 76**	**ND**	**ND**

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419 [a]All values are expressed as μg/g extract (mean ± SD) of two separate HPLC runs. [b]Determined

420 by Folin-Ciocalteu method, expressed as mg gallic acid equivalent / g extract. [c]Expressed as

421 luteolin-7-*O*-glucoside. [d]Expressed as apigenin-7-*O*-glucoside. [e]Expressed as luteolin. [f]Expressed

422 as eriodictyol-7-*O*-glucoside. [g]Expressed as naringin (naringenin-7-*O*-rutinoside). Total flavones,

423 flavanones, and 3-deoxyanthocyanins were sum of major HPLC peaks. ND = not detected.

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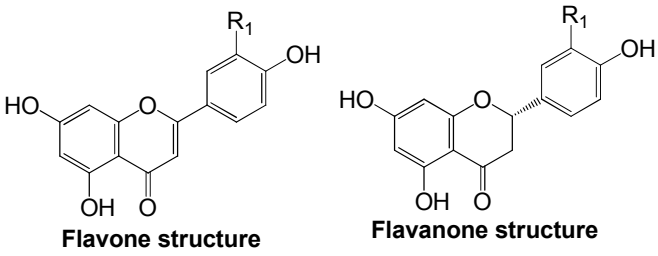


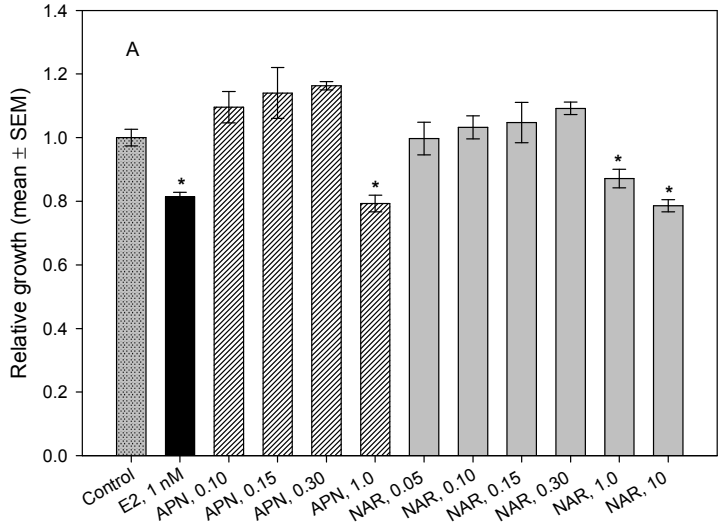
Flavone structure

Apigenin: R1 = H
Luteolin: R1 = OH

Flavanone structure

Naringenin: R1 = H
Eriodictyol: R1 = OH

Figure 1. Skeletal structure of the primary flavones and flavanones found in sorghum.



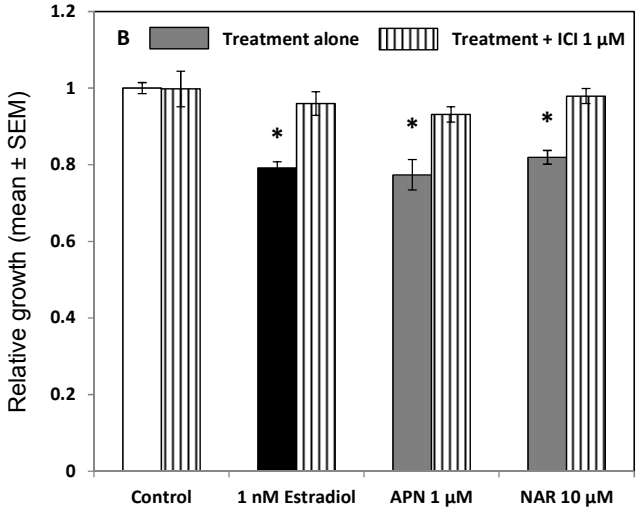
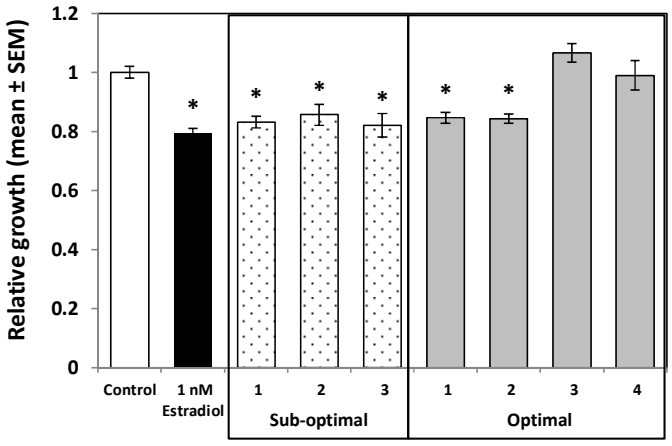


Figure 2. Effect of apigenin (APN) and naringenin (NAR) (µM) on the growth of non-malignant young adult mouse colonocytes (YAMC) (A); Effect of co-treatment of 1 µM ICI 182, 780 (estrogen receptor antagonist) with apigenin (1 µM) and naringenin (10 µM) on the growth YAMC (B). Data expressed as mean ± SEM from three separate experiments. Dunnett's t test was used to compare the least squares means of each treatment with corresponding control post a one-way ANOVA analysis. Treatments with an asterisk are significantly lower than control ($p < 0.05$).



	Sub-optimal levels			Optimal levels			
	1	2	3	1	2	3	4
Apigenin, μM	0.1	0.1	0.1	1	1	1	1
Naringenin, μM	0.05	0.1	1	0.1	1	5	10

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456 **Figure 3.** Effect of sub-optimal (0.1 μM) and optimal (1.0 μM) levels of apigenin co-treated
457 with naringenin (0.05-10 μM) on the growth of non-malignant young adult mouse colonocytes
458 (YAMC). Data expressed as mean ± SEM from three separate experiments. Dunnett's t test was
459 used to compare the least squares means of each treatment with corresponding control post a
460 one-way ANOVA analysis. Treatments with an asterisk indicated significant difference from the
461 control ($p < 0.05$).

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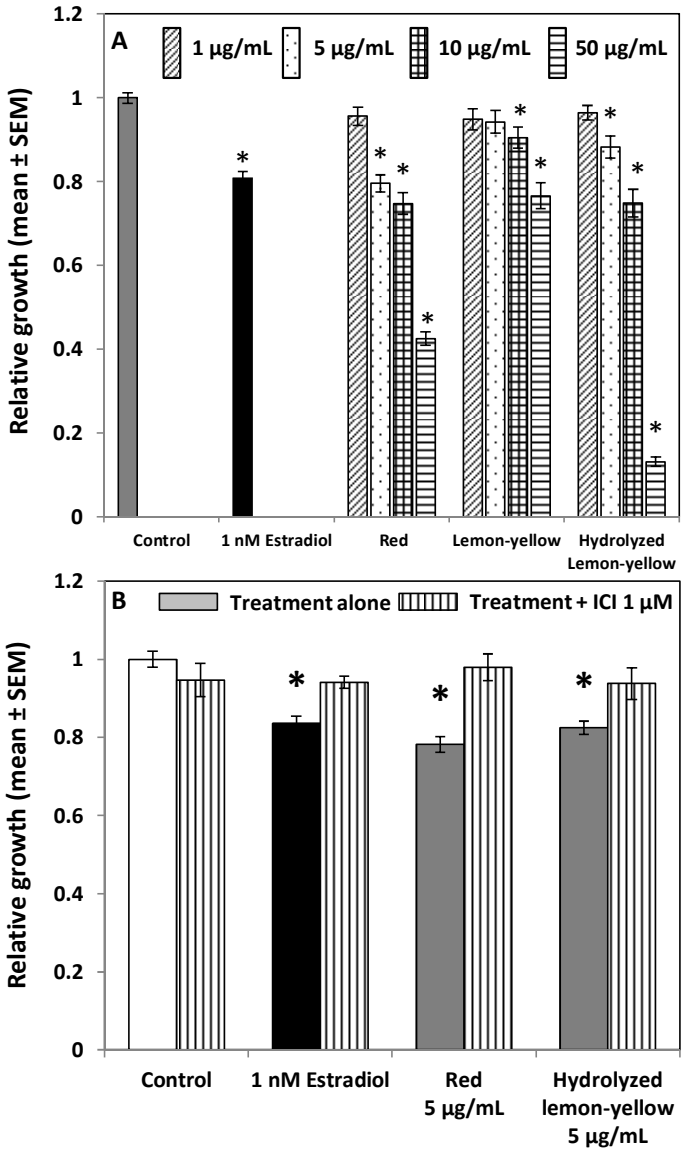

Figure 4. The effect of red, lemon-yellow, and hydrolyzed lemon-yellow sorghum extracts on the growth of non-malignant young adult mouse colonocytes (YAMC) (A); effect of co-treatment of 1 µM ICI 182, 780 (estrogen receptor antagonist) with red and hydrolyzed lemon-yellow sorghum extracts (5 µg/mL) on YAMC growth (B). Data expressed as mean ± SEM from three separate experiments. Dunnett's t test was used to compare the least squares means of each treatment with control post a one-way ANOVA analysis. Treatments with an asterisk indicated significant difference from the control ($p < 0.05$).