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Evaluating Mutagenicity of Burned Arginine-based Heterocyclic Amines and Anti-mutagenicity Effect of Chinese Medicinal Herbs

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ABSTRACT

From the unexpected finding that cooked grains and meat substitutes elicit a mutagenic response in *Salmonella typhimurium* TA98, our work has been aimed at deconstructing this finding via a survey of heated binary amino acid combinations, involving arginine, a plant-based amino acid. Secondly, our work has looked towards using phytochemical extracts from traditional Chinese medicinal herbs to inhibit the mutagenic activity of heterocyclic amines (main culprit for mutagenicity in meat). Two fractions from the burned products of arginine and phenylalanine (RF-HCA-06 and -07) were statistically significant inducers of mutagenesis; *Scutellaria barbata* and *Oldenlandia diffusa*, both separately and together, were statistically significant inhibitors of PhIP-induced mutagenesis. Further fractionation of the compounds and herbal extracts may lead to the discovery of the compound(s) responsible for inducing or inhibiting mutagenicity, respectively.

INTRODUCTION

Cancer is no trivial concern in the United States. Reacting to the growing cancer problem, the American people demanded the government do something, which had been, and still is, the number two cause of death each year in the United States since 1938 (NCHS 2009, 2018). This national concern led to Congress passing the National Cancer Act of 1971 on December 23 of that same year. Earlier that year, President Nixon stated his plan to ask Congress to set aside $100 million for a program aimed at finding a cure for cancer (NCI [date unknown]). This large sum of money was not simply a one-time investment — from 1974 to 2017, about $125 million have been appropriated to the National Cancer Institute by Congress (NCI 2018). These figures do not account for money spent on cancer research by private organizations like the American Cancer Society, which recently approved funding for cancer research totaling nearly $40 million for 2018 (ACS 2017). Despite this mass effort to deal with cancer, cancer death rates per 100,000 U. S. population have increased from 162.8 in 1970 to 185.4 in 2015. When accounting for the percentage of cancer deaths relative to total deaths, the percent increase appears less drastic — from 17.2% to 22.0%. Nevertheless, there is a statistically significant difference in the percentage of deaths from cancer during the last five decades (1970s, 1980s, 1990s, 2000s, 2010s) as determined by one-way ANOVA ($F(4, 41) = 53.8$, $p = 9 \times 10^{-16}$, $\eta^2 = 0.840$). Figure 1 summarizes the overall trend by displaying the percentage of total deaths by cancer across the last five decades. Despite legislation and increased spending on research, we do not seem any closer to eliminating cancer than when we began the "war" against cancer. Preventing cancer, rather than searching for a cure, may be a more fruitful approach to combating the cancer issue.

Estimates regarding the sources of cancers state that 25% to 33% of all cancers are caused by "smoking, dietary factors and inflammation/infection" — environmental or lifestyle factors. The study of cancers caused by environmental factors may take two routes — studying chemical agents *in vitro* that induce mutations in DNA (mutagens) and *in vivo* to produce tumors in live tissue (carcinogens); or studying chemical agents that prevent mutations from occurring and/or prevent cancer from developing (Sugimura *et al.* 2004). Heterocyclic amines, a class of mutagenic/carcinogenic compounds produced as byproducts from the cooking of meats and fish, will be the focus of the causative portion of this study. Two Chinese medicinal herbs, barbed skullcap (*Scutellaria barbata*) and spreading hedyotis (formerly *Oldenlandia diffusa*, now *Hedyotis diffusa*), will be the focus of the preventative portion of this study. The Ames/*Salmonella* mutagenicity test, or simply the Ames test, is named after a bacterial mutagenicity assay developed and revised by Bruce N. Ames and his colleagues in the mid-1960s to early 1980s. In this particular study, these two perspectives (causation and prevention) will be adopted and considered at the mutation level, not the cancer level. While mutations do not necessarily correlate with the initiation of a cancer in every instance, the Ames test is a standard method used when beginning preliminary evaluation of the effects of a chemical compounds on human health (Ames 1971; Ames *et al.* 1975; Maron and Ames 1983).

Heterocyclic amines (HCAs) owe their notoriety to the discovery of mutagenic byproducts found when cooking a variety of meats in the late-1970s to mid-1980s. Initial testing was done to determine their mutagenicity in *Salmonella typhimurium* TA98 (TA98) and *S. typhimurium* TA100 (TA100) strains, which detect frameshift and base-pair change mutations, respectively. In the majority of cases, HCAs induce mutations more often in TA98 than in the

TA100 strain. Additionally, the majority of HCAs require metabolic activation by rat liver S9 enzymatic fraction. One HCA known as 2-amino-1-methyl-6-phenlyimidazo(4,5-b)pyridine (PhIP) was found to induce mutations in Chinese hamster and human fibroblast cells. The carcinogenicity of PhIP and many other HCAs has been established in numerous long-term animal studies involving mice or rats. Notably, certain HCAs produced tumors in sites that are commonly for tumors in Western countries — colon, breast, prostate cancers (Sugimura *et al.* 2004). A population-based study including over 1500 participants looked at the association of meat intake, meat cooking method, and HCA intake, with incidence of colon cancer and found that the associations found between red meat intake and incidence of colon cancer supported the hypothesis that HCAs are relevant compounds associated with the preparation of red meats (Butler *et al.* 2003). However, it may the case that the cooking foods of plant-based origin also present a risk to human health. In studies done by Knize and his colleagues, the possibility arose that HCAs may also be responsible for the mutagenic activity observed in heated foods of plant-based origin (bread and grain products, a variety of flours, and meat-substitutes), as well as binary combinations of free amino acids (1994a, b). It is known that PhIP forms with phenylalanine and creatine as precursors, and it may be the case that PhIP forms when creatine is heated with leucine, isoleucine, and tyrosine (Skog *et al.* 1998). If free amino acids might produce mutagenic compounds (presumably HCAs), it could be predicted that plant proteins with a high concentration of those amino acids might also produce mutagenic compounds when cooked. Furthermore, whole or processed foods high in those plant proteins may give similar products when heated. With the eventual long-term goal of testing whole and/or processed foods of plant origin to characterize the mutagenic compound(s) that might be produced after heating, we will characterize the mutagenic activity of the burned products of arginine (R) and phenylalanine (F). Should any mutagenic compounds be found, we will seek to confirm whether the mutagenicity of these compounds is S9-dependent, as is the case with PhIP.

Scutellaria barbata (SB) and *Oldenlandia diffusa* (OD), two herbs commonly used in Traditional Chinese Medicine, have a wide and varied history of use when it comes to treating human maladies. Herbal extracts of these two plants have separately been used to treat hepatitis, snake bites, and malignancies/neoplasms (Chong and Lee 1988). These two herbs were chosen because of their history of use in the treatment of cancers (separately and together) (Shao *et al.* 2011; Wong and Wong 2011). Additionally, and most relevant to this study, these herbs have been used to inhibit the mutagenic effects of mutagens/carcinogens aflatoxin B_1 and benzo[a]pyrene in TA100 (Wong 1992). In order to establish a broader range of use for these herbs, especially to combat mutagenic compounds commonly produced and consumed in a Western diet, we will evaluate the inhibitory capabilities of SB and OD on PhIP in TA98.

MATERIALS AND METHODS

Prior to experimentation, it was determined that our bacterial tester strain (TA98) included the necessary mutations that would allow for the mutagenicity/anti-mutagenicity testing to be reliable. Notable mutations in TA98 include an *rfa* mutation, Δ*uvrB*, and hisD3052, as well as ampicillin resistance. As a histidine auxotroph (His^-), TA98 requires a frameshift mutation to revert back to His^+ to form visible colonies (Ames *et al.* 1975; Maron and Ames 1983). In short TA98, is modified in such a way that test chemicals can easily enter the bacterial cells and mutate bacterial DNA. With a deleted DNA repair mechanism, mutations are propagated in

subsequent generations. Eventually, bacterial replication produces visible colonies (called "revertants"), which are the product of nanoscale mutation events now observable at the macroscopic level.

To evaluate the mutagenicity of heated arginine/phenylalanine (RF) HCA-like byproducts and the anti-mutagenicity of SB and OD on PhIP, we followed the mutagenicity protocol and used the same materials outlined by Ames and his colleagues. Specifically, we followed the procedure known as the "plate incorporation test" (Ames *et al.* 1975; Maron and Ames 1983). Testing was done in triplicate. Data was collected over the course of eight separate experiments. A few modifications were made to the protocol to better fit our laboratory conditions. Those modifications included a change in how the S9 mix was prepared and the order in which plate incorporation reagents (bacterial culture, test compound, herbal extract, S9 mix [includes S9 enzymatic fraction and associated cofactors], top agar) were combined. The S9 mix (excluding the S9 enzymatic fraction) was divided into two groups — the "cofactor" portion (0.4 M $MgCl_2$-1.65 M KCl solution, 0.2 M pH 7.4 phosphate buffer, and distilled water [dH_2O]) and the "energy" portion (0.1M nicotinamide adenine dinucleotide phosphate [NADP], 1 M glucose-6-phosphate [G6P]). The "cofactor" components were combined and stored at -20˚C prior to use, while the "energy" components were kept separate at -20˚C until addition to the "cofactor" mix at the time of each experiment, now dubbed the "S9 cofactor mix" because it only lacked the S9 enzymatic fraction itself. The particular S9 fraction was Aroclor-1254-induced male Sprague Dawley rat liver in 0.15 M KCl, manufactured by Molecular Toxicology, Inc. (40 mg/mL protein content). With regards to the order in which the plate incorporation reagents were combined, instead of adding the reagents to enriched top agar at 45˚C, the reagents were added to test tubes kept at room temperature in the following order: bacterial culture, S9 cofactor mix (exlcuding S9 enzyme fraction) test compound or vehicle solvent (as appropriate), herbal extract (as appropriate), and S9 enzyme fraction. The herbal extracts were activated prior to incorporation by being kept 45˚C for at least 15 min. Once all other plate incorporation reagents had been added to test tubes, enriched top agar (also at 45˚C) was added, and the remainder of the protocol followed the steps prescribed by Ames and his colleagues.

Prepared by fellow undergraduate researchers in the Department of Chemistry and Biochemistry, we tested 15 total different chemical fractions derived from the burned products of arginine and phenylalanine. Amino acids were refluxed in diethylene glycol for 2 hr at 200˚C with the two amino acids at a 1:1 mass ratio. RF fractions were sequential and number 1 through 11 (RF-01 through RF-11) and dissolved in methanol (MeOH). Three other fractions were isolated by fractionating fraction 6 (RF-06) — fractions RF-06a, RF-06b, and RF-06c. A final fraction (RF-03) would not dissolve in MeOH, but did dissolve in dimethyl sulfoxide (DMSO). The separate RF-03 fractions were named "RF-03 (MeOH)" and "RF-03 (DMSO)," respectively. These different fractions included replicates from different students who burned these two amino acids and ran an extraction procedure on these burned products to isolate compounds with HCA characterstics. PhIP was commericially obtained from Toronto Research Chemicals (Toronto, Ontario, Canada) and dissolved in MeOH. Negative controls included were either with no chemical (NC) added, MeOH alone, and DMSO alone. To determine the S9-dependence of certain chemicals/fractions, experimental conditions were manipulated so that only the S9 enzymatic fraction was excluded. A graduate student from the Department of Biology was responsible for creating the SB and OD extracts, which were dissolved in dH_2O. Following

previous mutagenicity work done with these herbs, herbal concentrations used in experiments were 1.5, 3.0, and 6.0 mg/plate (Wong 1992). Three cases of herbal treatment were tested in the presence of PhIP: SB, OD, or SB with OD (SB + OD), where the herbal conentrations (1.5, 3, 6 mg/plate) were a 50:50 split of each herb — 0.75 + 0.75, 1.5 + 1.5, and 3.0 + 3.0 mg/plate. The negative control for all cases was simply the appropriate herbal extract at the appropriate concentration. Tables 1 (Negative Controls), 2 (HCA), and 3 (Herbal) list all the test chemicals and the volumes (for solvents) and concentrations (μg/plate) at which they were tested; herbal concentrations (mg/plate) and the concentration of PhIP (either 0.1 μg or 1 μg/plate) against which they were tested. All experimental conditions in the Herbal data set included S9 enzymat fraction. Inclusion in the data sets depended on both personal confidence in methodology and results, as well as statistical determinance using Grubbs' test for outliers. Appropriate statistical analyses (with post hoc tests) were conducted on each data set (HCAs, Herbs) with necessary negative and positive controls included.

RESULTS

Over the course of eight independent experiments, 280 and 146 plates (after subtracting 5 outliers and 1 outlier) were obtained for each data set, HCAs and Herbs, respectively. The average revertants per plate (RPP) for each unique experimental condition can be found in Tables 1, 2, and 3. One-way ANOVA tests (with Bonferroni post hoc tests) were run on each data set. In the HCA and Herbal data sets, a statistically significant F statistic was obtained — $F(56, 223) = 22.04$, $p = 6 \times 10^{-65}$, $\eta^2 = 0.847$ and $F(29, 175) = 38.01$, $p = 8 \times 10^{-61}$, $\eta^2 = 0.863$, respectively. In the HCA data set, Bonferroni analysis (post hoc) revealed that the only significant ($p < 0.05$) comparisons were between particular doses of PhIP and controls. PhIP at 0.1 μg/plate (364 ± 144 RPP) was significantly different from MeOH (with or without S9), DMSO, and NC (only when compared to with S9), but not different from the same dose without S9. PhIP at 1 μg/plate (2114 ± 877 RPP) was significantly different from all other experimental conditions, including its without S9 counterpart.

No RF byproduct induced RPP counts significantly different from any of the controls. However, when the data set was grouped so that any particular RF fraction (disregarding μg/plate) was compared to all HCA controls (NC, MeOH, DMSO, disregarding with or without S9) collectively, significant differences were detected. Most notably, fractions RF-06 and -07 were both significantly different from all fractions, ($p < 1 \times 10^{-16}$). Among the most potent mutagens (PhIP, RF-06, RF-07), dose-responses were observed in PhIP (5.8× between and 0.1 and 1 μg/plate) and RF-07 (1.4× between 180 and 360 μg/plate), but not in RF-06 (Figure 2). Additionally, mutagen S9-dependence was tested and established ($p < 0.05$) for PhIP (0.1, 1 μg/plate), RF-06 (114 μg/plate), RF-07 (180 μg/plate), as shown in Figure 3.

Looking at the pairwise comparisons from the Herbal data set, there were no significant difference in average RPP between NC (37 ± 114) and each herb alone treatment (SB, OD, SB + OD) at all concentrations (1.5, 3, 6 mg/plate). All RPP values relevant to the Herbal data set may be found in Tables 1 and 3. There were significant differences ($p < 0.01$) between NC and PhIP alone at 0.1 and 1 μg/plate. While PhIP alone at 1 μg was significantly different ($p < 0.01$) from all other experimental conditions, the same was not true of PhIP alone at 0.1 μg/plate. This lower concentration of PhIP was only significantly different against NC, and certain cases of PhIP at 1

µg/plate with herbal extract — SB 1.5 and 3.0 mg, OD 1.5 mg, and SB + OD 1.5 and 3.0 mg. Figure 4 outlines the relationship between RPP and the various herbal experimental conditions. In addition to comparing RPP between experimental conditions, an analysis comparing percent inhibitions was conducted.

All herbal treatments with PhIP at 0.1 µg/plate, regardless of herb concentration, were significantly different ($p < 1 \times 10^{-9}$) from PhIP alone at 0.1 µg/plate. Within a particular herb treatment, increased herbal concentrations produced increased percent inhibition values. When treated with SB, 3.0 (72 ± 12) and 6.0 mg (79 ± 8.8), percent inhibition was significantly higher ($p < 0.05$) than percent inhibition with 1.5 mg (51 ± 25), but significantly different from each other ($p > 0.05$). SB and SB + OD (60 ± 16) at 1.5 mg were significantly higher ($p < 0.01$) than OD (24 ± 15) at the same concentration. Percent inhibition of OD at 3.0 (69 ± 6.2) and 6.0 mg (82 ± 3.1) were significantly greater than 1.5 mg, but not significantly different from each other. There was no significant difference among herbal treatments (SB, OD, or SB + OD) at 3.0 or 6.0 mg. Percent inhibition trends for all three herbal treatments across concentrations may be found in Figure 5A.

All herbal treatments with PhIP at 1 µg/plate, regardless of herb concentration, were significantly different ($p < 1 \times 10^{-4}$) from PhIP alone 1 µg/plate. Within a particular herb treatment, increased herbal concentrations produced increased percent inhibition values. When treated with SB, 6.0 mg (88 ± 0.91) produced a significantly higher ($p < 1 \times 10^{-4}$) percent inhibition relative to 1.5 mg (39 ± 21) but was not significantly different from 3.0 mg (63 ± 20). When treated with OD, each herbal concentration was significantly different ($p < 0.05$) from the other — 1.5 (44 ± 16), 3.0 (82 ± 5.6), and 6.0 mg (93 ± 3.8). The same pattern of statistical differences observed across concentrations of SB was observed in treatments of SB + OD — 6.0 mg (93 ± 0.13) was different from 1.5 mg (51 ± 11), but not different from 3.0 mg (77 ± 7.8). No herbal treatment was significantly better than others at 1.5, 3.0, or 6.0 mg. Percent inhibition trends for all three herbal treatments across concentrations may be found in Figure 5B.

DISCUSSION

Aside from the outright findings from this particular study, one of the most important developments associated with this study was optimizing and standardizing the protocol for conducting the Ames test at Andrews University, which saw an expansion from duplicate testing of experimental conditions and a maximum n per experiment of 16 to triplicate testing and a maximum number of 84. With enhanced consistency and the ability to collect more data per experiment, we were able to collect more Ames test data than had been previously collected in our lab. Standardization of the protocol not only allowed for a good deal of consistency in this study but will facilitate future collection of data by this lab.

Regarding our protocol and the outcomes of post hoc results in preliminary statistical tests, it would be recommended that any experimental condition be tested on at least 3 separate occasions and have an n no less than 9 (preferably more), to increase power. One example of how a low n prevented a significant p-value between RPP values in experimental conditions one might expect to observe a significant p-value was in the comparison between PhIP at 0.1 µg/plate with S9 compared to either NC with or without S9. The RPP values for each

condition were 364 ± 144, 37 ± 14, and 36 ± 11, with n of 30, 18, and 3, respectively (Tables 1, 2). Although the RPP values for NC with S9 and NC without S9 were very similar, post hoc analysis revealed a significant difference between PhIP and NC with S9, not between PhIP and NC without S9. Presumably, this was due to the very low n (3) associated with NC without S9. Observing a similar trend with RF-06 and RF-07 (maximum n = 6) leads to the recommendation for increased observations of induced mutagenesis in these and future mutagenic fractions and/or compounds.

Our findings concerning the mutagenicity of RF-06 and RF-07 encourages us to do further work with these fractions (i.e. further fractionation) to isolate the compound(s) responsible for inducing mutagenesis. Preliminary work has been done on RF-06, but to no avail. RF-07 may be the more fruitful fraction to mine for mutagenic compound(s), especially considering that it showed a dose-response when varying compound concentration. Further testing should be done on both these fractions across several concentrations to better characterize the dose-response nature of either fraction, but the ultimate goal would be to conduct compound concentration dose-response testing. Similarly, in these mutagenic fractions (RF-06 and -07) or in mutagens isolated from these fractions, S9 concentration dose-response testing may be done. This would be necessary because certain mutagens (2-aminofluorene and benzo[a]pyrene) differ in their optimum S9 concentration (10 and 100μL/plate, respectively) needed for inducing maximum mutagenicity (Maron and Ames 1983). Finally, our findings regarding mutagenicity inhibition of a potent mutagenic/carcinogenic compound (PhIP) encourages further and derivative work using these particular herbal extracts in relation to inhibiting mutagenicity of this particular HCA. Further work may be done on any forthcoming mutagenic compound discovered by our lab.

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Figures and Tables:

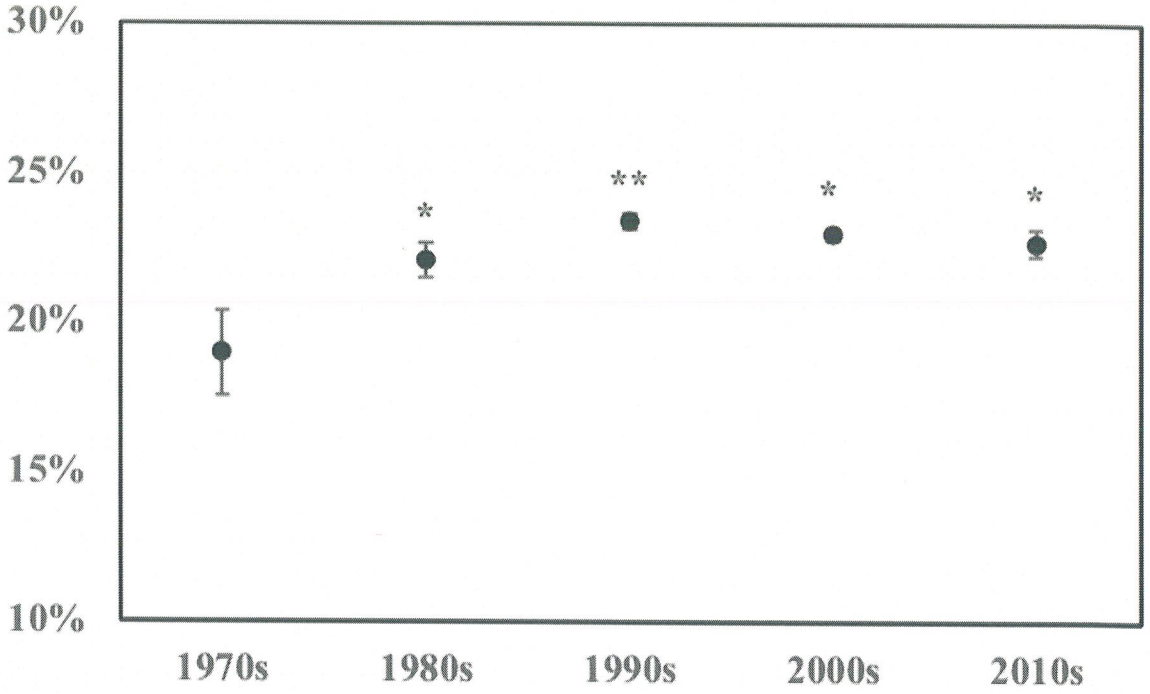

Figure 1: *Percentage of total deaths due to cancer between 1970 and 2015, by decade.* Data based on death rates per 100,000 U. S. population (NCHS 2009, 2018). The percentages for each decade from 1970 onward were 19.0%, 22.1%, 23.4%, 23.0%, and 22.7%, respectively. Single asterisks represent a significant difference ($p < 0.05$) between percent deaths from cancer in a given decade relative to 1970s, as determined by Bonferroni pairwise analysis. A double asterisk represents the same, but between that decade (1990s) and the two decades prior (1970s and 1980s).

Table 1: Descriptive statistics of negative control data

Treatment	n	Volume (μL)	± S9	Average Revertants/plate
No Chemical	3	-	-	36 ± 11
	18		+	37 ± 14
Methanol (MeOH)	6	10	-	28 ± 8
	3	30		33 ± 9
	20	10	+	39 ± 8
	6	20		43 ± 7
	8	30		33 ± 6
	3	60		42 ± 6
Dimethyl Sulfoxide (DMSO)	3	10	+	30 ± 5
	5	30		37 ± 9
	3	60		37 ± 8

Table 2: Descriptive statistics of HCA data

Chemical	n	Chemical Concentration (μg/plate)	± S9	Average Revertants/plate
RF-01	3	0.01	-	28 ± 4
		0.1		27 ± 5
		1		28 ± 5
		0.01	+	32 ± 9
		0.1		33 ± 4
		1		31 ± 5
RF-02	3	0.01	-	22 ± 3
		0.1		22 ± 8
		1		25 ± 2
		0.01	+	30 ± 5
		0.1		26 ± 1
		1		35 ± 5
RF-03 (MeOH)	3	0.1	+	28 ± 6
	5	1		35 ± 8
	2	3		49 ± 11
	3	6		49 ± 5
	3	9		60 ± 8
RF-03 (DMSO)	3	0.1	+	24 ± 2
		0.3		31 ± 8
		0.6		28 ± 3
RF-04	2	0.1	+	35 ± 7
	5	1		36 ± 6
	3	44		48 ± 8
	3	88		51 ± 3
	3	132		49 ± 9
RF-05	3	0.1	+	22 ± 2
	5	1		26 ± 3
	3	292		71 ± 18
	5	584		70 ± 7
	4	876		91 ± 2
RF-06	3	114	-	22 ± 5
	6	114	+	174 ± 24
	3	228		174 ± 4
RF-06a	2	36	+	31 ± 6
	3	100		42 ± 5
RF-06b	2	12	+	29 ± 6
	3	100		37 ± 8
RF-06c	2	19.2	+	34 ± 11
	3	50		31 ± 6
RF-07	3	180	-	27 ± 4
	6	180	+	164 ± 23
	3	360		237 ± 37

Table 2 (Continued)

Chemical	n	Chemical Concentration (μg/plate)	± S9	Average Revertants/plate
RF-08	3	159	+	85 ± 18
RF-09	3	225	+	80 ± 9
RF-10	3	180	+	67 ± 3
RF-11	3	90	+	64 ± 9
PhIP	3	0.01	-	31 ± 6
	3		+	58 ± 6
	3	0.1	-	29 ± 12
	30		+	364 ± 144
	3	1	-	23 ± 2
	12		+	2114 ± 877

Table 3: Descriptive statistics of Herbal data

Herbal Extract	n	Herb Concentration[a] (mg/plate)	Average Revertants/plate	% Inhibition
SB Alone	3	1.5	38 ± 4	-
	9	3.0	30 ± 9	
	3	6.0	41 ± 6	
OD Alone	3	1.5	41 ± 9	-
	9	3.0	44 ± 11	
	3	6.0	40 ± 7	
SB + OD Alone	3	1.5	35 ± 4	-
	3	3.0	40 ± 8	
	9	6.0	34 ± 8	
SB + MP[b]	9	0	324 ± 93	-
	8	1.5	160 ± 81	51 ± 25
	9	3.0	86 ± 37	72 ± 12
	9	6.0	66 ± 31	79 ± 8.8
OD + MP	9	0	324 ± 93	-
	9	1.5	237 ± 24	24 ± 15
	9	3.0	96 ± 15	69 ± 6.2
	8	6.0	58 ± 9	82 ± 3.1
SB + OD + MP	9	0	324 ± 93	-
	3	1.5	164 ± 64	60 ± 16
		3.0	74 ± 24	82 ± 6.0
		6.0	57 ± 18	86 ± 4.4
SB + HP[c]	9	0	2422 ± 708	-
	5	1.5	1480 ± 759	39 ± 21
		3.0	911 ± 752	63 ± 20
		6.0	385 ± 250	88 ± 0.91

Table 3 (Continued)

Herbal Extract	n	Herb Concentration (mg/plate)	Average Revertants/plate	% Inhibition
OD + HP	9	0	2422 ± 708	-
	5	1.5	1194 ± 477	44 ± 16
		3.0	378 ± 74	81 ± 5.6
		6.0	139 ± 40	93 ± 3.8
SB + OD + HP	9	0	2422 ± 708	-
	3	1.5	1307± 288	51 ± 11
	4	3.0	636 ± 252	77 ± 7.8
	3	6.0	193 ± 4	93 ± 0.13

[a] Average revertants/plate for PhIP alone (0 mg/plate of a given herbal treatment) calculated from experiments were herbal anti-mutagenicity was tested. [b] "MP" represents 0.1 µg/plate of PhIP. [c] "HP" represents 1 µg/plate of PhIP.

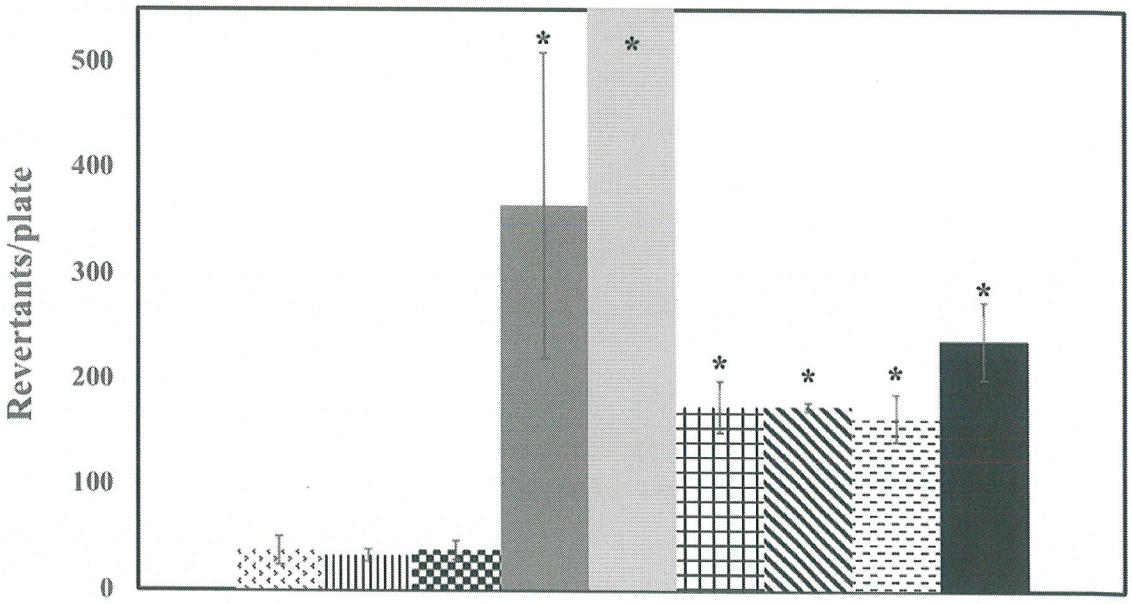

Figure 2: *Mutagenicity of negative controls, PhIP, and selected RF fractions*. Left to right: NC, MeOH, DMSO, PhIP 0.1 µg/plate, PhIP 1 µg/plate, RF-06 114 µg/plate, RF-06 228 µg/plate, RF-07 180 µg/plate, and RF-07 360 µg/plate. Asterisks represent a significant difference between chemical and all controls ($p < 0.05$).

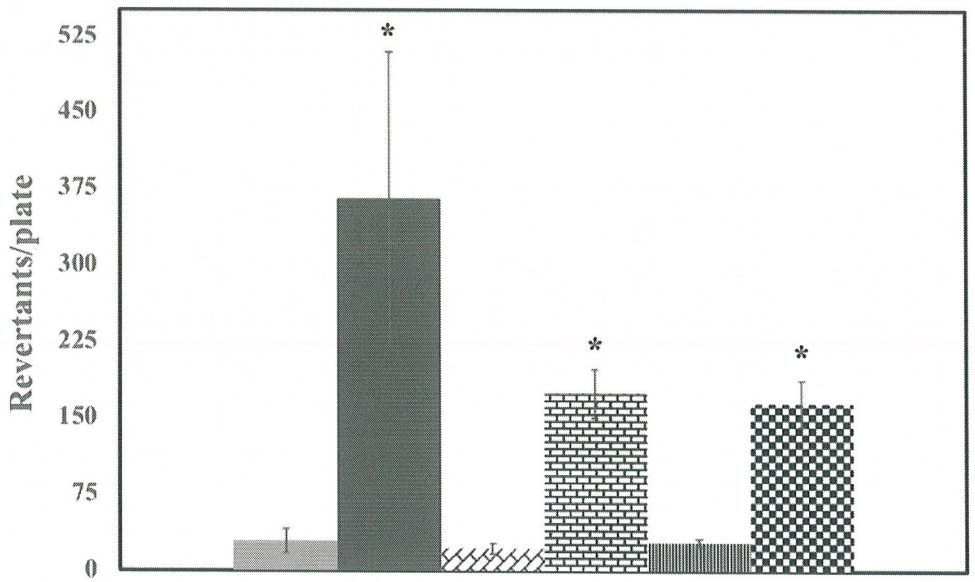

Figure 3: *S9-dependent mutagenicity of PhIP, RF-06 and RF-07.* Left to right: Data is paired — without S9 followed by S9 for PhIP, RF-06, and RF-07 at 0.1 μg, 114 μg, and 180 μg/plate, respectively. Asterisks represent a significant difference between chemical and corresponding "without S9" experimental condition ($p < 0.05$).

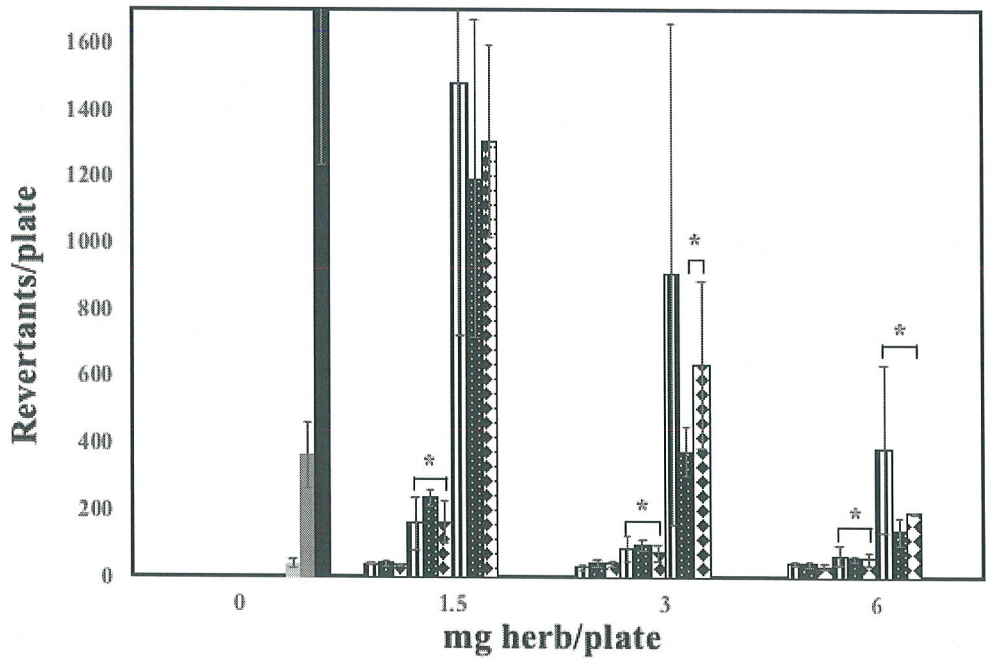

Figure 4: *Herbal data with relevant controls.* Left to right: Data is organized by herb concentration. At 0 mg/plate, the three test conditions are NC, PhIP at 0.1, and 1 μg/plate. At all other concentrations, this pattern is repeated, but in triplicate for each separate herb treatment — SB, OD, and SB + OD. Asterisks represent no significant difference in RPP between herbal treatment with PhIP and corresponding herb alone concentration ($p > 0.05$).

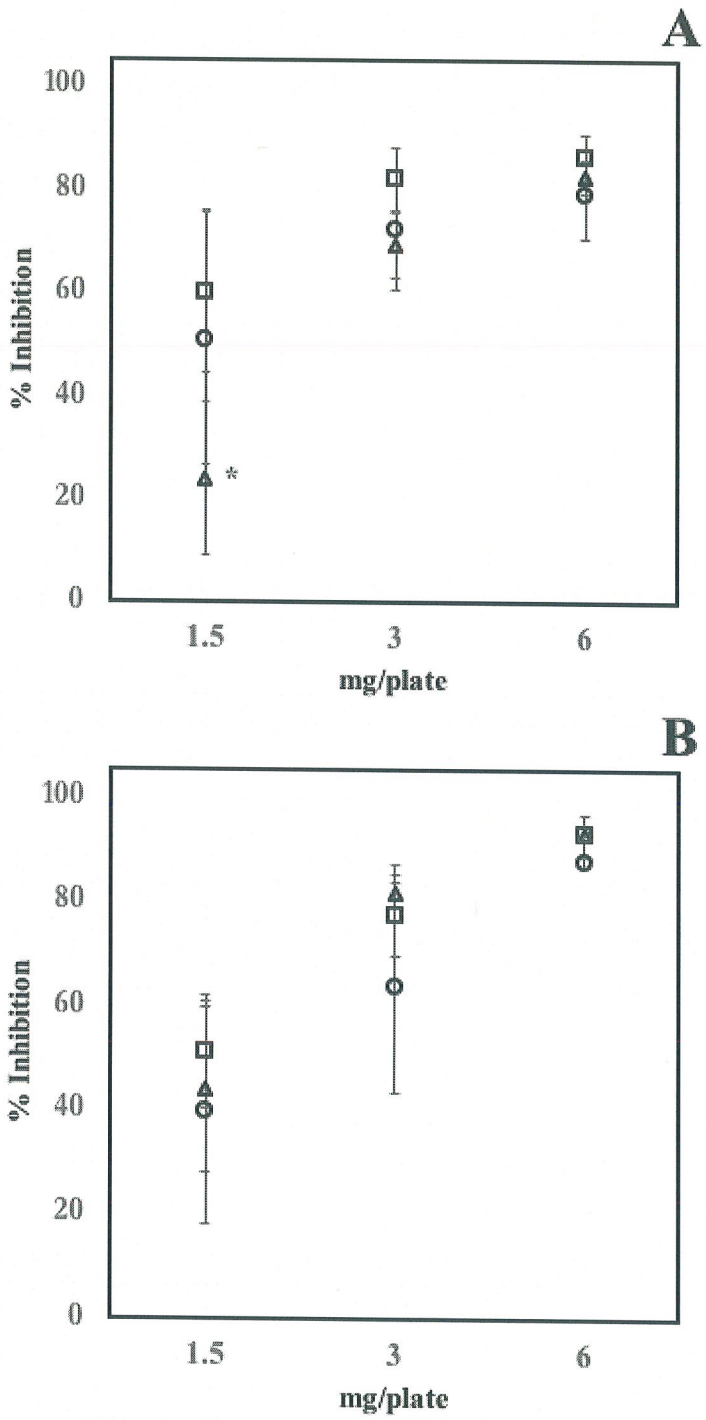

Figure 5: *Percent inhibition trends of herbs with PhIP at* **A.** *0.1* µg *and* **B.** *1* µg*/plate.* Symbols represent particular herbs treatments: The circle (○), triangle (△), and square (□) represent treatments SB, OD, and SB + OD respectively. Asterisks represent a significant difference in percent inhibition between different herbal treatments with PhIP at the same herbal concentration ($p < 0.05$).