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EXAMINATION OF RAW AND ROASTED *Mucuna pruriens* FOR TUMEROGENIC SUBSTANCES

S. Burgess, A. Hemmer and R. Myhrman*

World Hunger Research Center,

Judson College, Elgin, IL 60123-1404, USA

E-mail: rmyhrman@judsoncollege.edu

**Corresponding author*

SUMMARY

The seeds of *Mucuna pruriens* represent an important potential source of food and feed for undernourished people in many parts of the world. In order to evaluate whether *Mucuna* is free of substances that may cause cancer, a threefold approach was employed: 1) A literature search was conducted; 2) Samples of *Mucuna pruriens* were tested for the presence of mutagens using the Ames test; and 3) A chromatographic study of the effect of heating on *Mucuna* seeds was begun.

None of the known components of *Mucuna* appear on frequently consulted lists of known and suspected carcinogens published in Europe and the United States. Likewise, searches of the medical literature for coincidences of "*Mucuna*" and terms related to tumors returned no citations.

Ground samples from raw and gently roasted *Mucuna* seeds were examined for the presence of mutagens using the Ames *Salmonella* test. In no case did the rate of mutations in test samples exhibit a three-fold increase over controls, the criterion for a positive result.

Using gas chromatography in tandem with mass spectroscopy, the same *Mucuna* samples were examined for the presence of benzo[a]pyrene, a representative of the class of potentially carcinogenic compounds known as polycyclic aromatic hydrocarbons. Again, the results were negative at the sensitivity of the instrument.

While the study should be expanded to include samples that have been subjected to more intense heat, the present results are consistent with the assumption that *Mucuna* seeds are not a source of tumerogenic substances.

Key words: *Mucuna*, velvet bean, Ames salmonella mutagenicity test, gas chromatography, mass spectrometry, carcinogen

INTRODUCTION

One of the priorities of the project "Increasing *Mucuna*'s Potential as a Food and Feed Crop" is to ascertain the potential impact on human health of the "secondary" compounds of *Mucuna,* i.e. those that are present at low concentration. This report concerns substances that are potentially mutagenic or carcinogenic.

The following questions were addressed: 1) Do any of the substances currently known to be present in *Mucuna* appear on official lists of carcinogens, or does the word "*Mucuna*" appear in the medical literature in combinations with words such as "tumor?" 2) Will the standard assay for mutagenic activity, namely the Ames test, yield a positive result for either raw or roasted *Mucuna* seeds? 3) Will analysis of flour from raw or roasted *Mucuna* seeds reveal the presence of potentially carcinogenic compounds?

Accordingly, this report consists of three parts:
1) A comparison of the known components of *Mucuna pruriens* with the lists of known and suspected carcinogens maintained by the International Agency for Research on Cancer (IARC, Switzerland), the National Toxicology Program (NTP, USA), and the National Institute for Occupational Safety and Health (NIOSH, USA).

2) The application of the Ames test for mutagens to samples of flour from raw and toasted *Mucuna*. The Ames test is a powerful tool, since it can detect the presence of a wide range of mutagenic substances and yet does not require the experimenter to anticipate which specific ones might be present. In addition, there is a high degree of correlation between mutagenic and carcinogenic activity.

3) Analysis by gas chromatography and mass spectrometry for the presence of benzo[a]pyrene, which is found in a variety of foods that have been cooked under high heat. This compound was chosen as a representative of the class of substances known as polyaromatic hydrocarbons, many of which have produced tumors in animal studies.

MATERIALS AND METHODS

Supplies

Benzo[a]pyrene (B1,008-0), dimethylsulfoxide (D 8418), L-histidine (H 8125), 9-aminoacridine (A 7295), 2-nitrofluorene (N1,675-4), 9,10-dimethyl-anthracene (D14,670-6), sodium azide (19,993-1), D-biotin (B 4501), cumene hydroperoxide (C 0524), and dichloromethane (D6,510-0) were from Sigma-Aldrich (St. Louis, MO 63178, USA). Bacto-Agar (0140-01) was from Difco Labs (Detroit, MI 48201, USA). Ultrapure helium (BIPGC grade) was from Air Products (now Airgas, Radnor, PA 19087, USA). Nutrient Broth #2" (CM67) was from Oxoid LTD., Basingstoke, Hampshire, England. Rat liver extract S9 was from MOLTOX Inc., Boone, NC 28607, USA. *Salmonella typhimurium* strains (TA 97a, TA 98, TA 100, TA 102, and TA 1535) were from Xenometrics (San Diego, CA 92121, USA). Nalgene syringe filter units (176-0020) were from VWR Scientific Products (So. Plainfield, NJ 07080, USA), and Cameo 25AS syringe filter units were from Osmonics (Minnetonka, MN 55343, USA).

Preparation of *Mucuna* samples

To make "raw flour," the coats from ten *Mucuna* seeds were removed with a knife, and the seeds were ground in a Wiley laboratory mill ("intermediate model"). The material that passed through the 40-mesh screen was collected for analysis.

Roasted samples were prepared at a home in Morocelí in Central Honduras. Following traditional practice, a dome-shaped oven was heated to a temperature such that green leaves burned slowly rather than immediately. Dry *Mucuna* beans were washed with water and placed in the oven for six hours (during the roasting process, the seed coat fell off, after which the seeds themselves were roasted until they developed a brown color). The seeds were then ground into flour.

Literature searches

To determine whether any of the substances in *Mucuna* are suspected of producing tumors, the PhytochemDB list for *Mucuna pruriens*, as listed at: http://ukcrop.net/cgi.bin/WebAce/webace?db=PhytochemDB, and transcribed as Table 1, was compared to the following lists as they appeared in June, 2002:

1) International Agency for Research on Cancer (IARC): "Overall Evaluations of Carcinogenicity to Humans" (http://193.51.164.11/monoeval/crthall.html)

2) U.S. Department of Health and Human Services, Public Health Service, National Toxicology Program (NTP): "9th Report on Carcinogens" (http://ehp.niehs.nih.gov/roc)

3) U.S. Centers for Disease Control and Prevention (CDC), National Institute for Occupational Safety and Health (NIOSH): "NIOSH Carcinogen List" (http://www.cdc.gov/niosh/npotocca.html)

In addition, the PubMed search engine at (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi) was queried for combinations of the term "*Mucuna*" and each of the following: "cancer", "carcinogen*", "mutation*", "mutagen*", and "tumor*", where the "*" denotes any combination of characters.

PubMed is a service of the US National Library of Medicine and provides access to over 12 million citations in the MEDLINE database dating back to the mid-1960's.

Ames test

Description

The Ames test detects the presence of substances which can cause genetic mutations, and which therefore have a high probability of being carcinogenic. The test utilizes five strains of the bacterium *Salmonella typhimurium* that have been rendered histidine-dependent through the introduction of different types of point mutations. Each of the strains can regain its ability to grow without added histidine if exposed to a mutagenic test substance that produces a reversion of the original mutation.

To conduct the test, each of the bacterial strains is mixed with the substance that is being evaluated. These mixtures are plated onto media and then incubated for 48 hours, after which the number of (histidine-independent) bacterial colonies on each plate is counted. The numbers of colonies on the test plates are compared to the numbers that have appeared spontaneously on control plates made from an incubation mixture that contains bacteria but no test substance. The test is considered positive if the number of colonies on the test plates is at least three times the number on the control plates.

Table 1. Phytochemicals of *Mucuna pruriens* as listed June 3, 2002 At: http://ukcrop.net/cgi.bin/WebAce/webace?db=PhytochemDB.

Arachidic acid	Fat
Arginine	Fiber
Ash	Gallic acid
Aspartic acid	Glutamic acid
Behenic acid	Glutathione
Beta carboline	Glycine
Beta sitosterol	Histidine
Bufotenine	5-Hydroxytryptamine
Calcium	Indole-3-alkylamine
Carbohydrates	Iron
Choline	Isoleucine
Cystine	Lecithin
N,N-Dimethyltryptamine	Leucine
N,N-Dimethyltryptamine-N-oxide	Linoleic acid
L-Dopa	Linolenic acid
Cis-12,13-epoxyoctadec-trans-9-cis-acid	Lysine
Cis-12,13-epoxyoctadec-trans-9-enoic-acid	Methionine
5-Methoxy-N,N-dimethyltrytamine-N-Oxide	6-Methoxyharman
1-Methyl-3-carboxyl-6,7-dihydroxy-1,2,3,4-tetrahydroisoquinolone	
Mucunadine	Prurienidine
Mucunain	Prurienine
Mucunine	Riboflavin
Myristic acid	Saponins
Niacin	SD
Nicotine	Serine
Oleic acid	Serotonin
5-Oxyindole-3-alkylamine	Stearic acid
Palmitic acid	Thiamin
Palmitoleic acid	Threonine
Phenylalanine	Tryptamine
Phosphorus	Tyrosine
Proline	Valine
Protein	Vernolic acid

To verify that the test system is working properly, each strain is also incubated with a substance that is known to produce the type of mutation associated with that strain. In addition, each combination of test substance and bacterial strain is incubated with a liver extract in order to detect the presence of substances that are not mutagenic themselves, but can be converted to mutagens by enzymes in the liver.

Two *Mucuna* pruriens samples were tested: 1) Flour made in our laboratory from uncooked seeds; 2) Flour made in Honduras from roasted seeds using traditional methods.

Details

The test was performed as described by Maron and Ames (1983), with adjustments in the amounts of agar and culture broth as required to achieve proper hardening of the top agar, and an appropriate rate of spontaneous reversion in negative controls.

Salmonella strains TA 97a, TA 98, TA 100, TA 102, and TA 1535 were employed. Single colonies were removed from master plates of each strain and used to inoculate 50 mL of Oxoid #2 nutrient broth. The resulting culture broth was incubated in a 37°C shaking water bath set at 50 rpm to provide proper aeration. Growth times ranged from 3.5 to 8 hours.

The top agar was prepared by combining 2 mL of warm top agar mixture, 0.1 mL of test sample, and culture broth (0.1 mL for strains TA 97a and TA 100, 0.065 mL for strain TA 102, 0.4 mL for strain TA 98, and 0.5 mL for strain TA 1535). If applicable, 0.5 mL of S9 liver extract solution was added. The contents of the tube were vortexed for 3 seconds and then poured onto minimal glucose agar plates, which were tilted and swirled to distribute the contents, and

then placed on a flat surface at room temperature to harden. The plates were covered and placed inverted in an incubator for 48 hours at 37°C. At least five plates were used for each combination of test substance, bacterial strain, and presence or absence of S9.

Negative control plates contained top agar and culture broth only. Positive control plates had 0.1 mL of the following mutagenic chemical solutions added in place of the test substance, as indicated for each strain: TA 97a, 0.0075 g 9-aminoacridine in 10.0 mL DMSO; TA 98, 0.0110 g 2-nitrofluorene in 37.0 mL DMSO; TA 100, 0.0032 g sodium azide in 64 mL distilled H_2O; TA 102, 0.0075 mL cumene hydroperoxide in 20 mL DMSO; TA 1535, 0.0032 g sodium azide in 64 mL distilled H_2O.

The S9 negative control plates used top agar containing culture broth and 0.5 mL S9 solution. The S9 positive control plates also contained 0.1 mL of a mutagenic solution, which contained 2 mg 9,10-dimethylanthracene and 2 mg benzo[a]pyrene in 10 mL DMSO.

To verify that the dimethylanthracene and benzo[a]pyrene would induce substantial mutagenic activity only in the presence of liver enzymes, plates were prepared as described above for the positive controls, but with the S9 extract omitted.

In addition, plates containing bacteria, culture broth and 0.5 mL dimethylsulfoxide (DMSO) were used to test for the toxicity of that solvent.

To prepare solutions from *Mucuna* flour that could be subjected to the Ames test, it was necessary that the samples be extracted using solvents that are compatible with the test conditions. In order to maximize the possibility of extracting any mutagens that might be present, we performed separate extractions into water, 0.1 M hydrochloric acid (HCl), and DMSO. In each case, 2 g of flour was mixed with 25 mL of solvent and ultrasonicated for 10 minutes at room temperature using a Branson model 2510 (Branson Ultrasonics, Danbury, CT 06813, USA) followed by 30 minutes of refluxing (distillation with recycling of solvent) with magnetic stirring. After cooling, the mixture was centrifuged at 10,000 rpm (13,000g) for 15 minutes in the Hermle 220.78 V02 rotor of the Marathon 22KBR centrifuge (Fisher Scientific, Pittsburgh, PA 15275, USA) and the supernatant liquid was sterilized with an 0.22 micron acetate filter (25 mm diameter, Osmonics).

Each of the resulting solutions was tested by incorporation into the top agar as described above, and the number of (histidine-independent) bacterial colonies was measured.

Analysis for benzo[a]pyrene

Extracts for chromatographic analysis were prepared by combining 0.3 grams of flour and 3 mL of dichloromethane in a 10 mL round-bottom flask. The flask was loosely capped, and the mixture subjected to ultrasonication for 10 minutes in the Branson 2510. A magnetic stirring bar was added to the flask, and the mixture was refluxed over a heater/stirrer for one hour. Prior to injection of a 1.0 microliter aliquot into the gas chromatograph, samples were passed through an 0.2 micron nylon filter (4 mm diameter, Nalgene).

The chromatographic method was adapted from EPA method 8100 (http://www.epa.gov/epaoswer/hazwaste/test/pdfs/8100.pdf) and Chromatograph #191 from Restek Corporation (2000).

The analysis for benzo[a]pyrene was performed on a Varian GC-MS system consisting of a model 3800 gas chromatograph with model 1079 programmable injector, and a Saturn 2000 mass spectrometer in electron ionization (EI) mode. The column was a Chrompack CP-SIL 8CB-MS (5% diphenyl) 30 m x 0.25 mm, film thickness 0.25 micron. The mobile phase was helium at a constant flow rate of 1 mL min^{-1}. A 1.0 µL sample was injected in splitless mode at 280°C with a one minute delay prior to flushing the injection port with helium. The column temperature was held at 40°C for 30 seconds, then increased at 10°C min^{-1} to 280°C, then held constant for 15 minutes. The trap of the mass spectrometer was set at 200°C, the manifold at 40°C, and the transferline at 250°C. The total ion chromatogram was collected.

RESULTS

Literature

None of the substances listed in the PhytochemDB list for *Mucuna pruriens* appears on the IARC, NTP or NIOSH lists of known or suspected carcinogens.

Ames test

As indicated in Materials and Methods, two *Mucuna* samples were tested: 1) Flour made in our laboratory from uncooked seeds (a mixture of four varieties in equal parts); 2) Flour made in Honduras from roasted seeds using traditional methods.

Table 2 presents the results as percentages of the corresponding control, with values of 300% or greater interpreted as evidence of mutagenic activity. With the exception of the TA98, samples that were incubated with liver extract, sonicated and refluxed in DMSOdid not have values above 200%. Because the refluxed DMSO solutions were dark and foul smelling, we suspect that the observed results might

be an artifact of the high temperature (about 200 °C) that was necessary to achieve reflux. To test this hypothesis, we re-extracted TA98 and two other *Salmonella* strains using 20 minutes of sonication instead of 10, and omitting the heating/refluxing step. None of these values exceeded 110%.

Table 3 summarizes the results of experiments in which each bacterial strain was challenged with a known mutagen as a positive control.

Table 2. Percentage of spontaneous-reversion control with five strains, different extraction media, and with or without the addition of liver extract. The study used both raw and toasted *Mucuna* bean samples.

Strain	Sample	Addition	Percentage of spontaneous-reversion control			
			Extraction medium			
			Water	HCl	DMSO, sonicated and refluxed	DMSO, sonicated only
TA97a	Raw	None	114	130	195	
		Liver extract	118	122	172	103
	Toasted	None	154	161	202	
		Liver extract	140	145	161	100
TA98	Raw	None	117	110	112	
		Liver extract	162	136	631	102
	Toasted	None	134	129	141	
		Liver extract	142	142	437	110
TA100	Raw	None	92	120	115	
		Liver extract	159	165	197	
	Toasted	None	81	116	130	
		Liver extract	188	154	177	
TA102	Raw	None	97	107	100	
		Liver extract	123	96	105	
	Toasted	None	100	104	92	
		Liver extract	104	91	89	
TA1535	Raw	None	72	81	102	
		Liver extract	105	102	141	102
	Toasted	None	184	89	136	
		Liver extract	129	126	198	99

Table 3. Percentage of spontaneous-reversion control when the bacterial strains used in this study were challenged with a known mutagen as a positive control.

Strain	Percentage of spontaneous-reversion control				
	TA97a	TA98	TA100	TA102	TA1535
No addition	719	2073	409	244	859
Liver extract	236	545	267	108	139

For the liver-free incubations, the control values for all strains except TA102 exceed 300%. Although the value for TA102 is only 244%, none of the TA102 values for the *Mucuna* samples in Table 2 exceeds 123%, so it appears that the TA102 system can in fact recognize a mutagen, but that the *Mucuna* data for TA102 suggest the absence of mutagenic activity.

For the liver-extract system, the same combination of mutagens (9,10-dimethylanthracene and benzo [a]-pyrene) was used as a positive control for all strains. Since each strain reverts to histidine independence by a different type of mutation, it is not expected that all strains will revert in the presence of these two mutagens. One positive result is sufficient, and the 545% value for TA98 confirms that the liver extract is functioning properly.

The data in Tables 2 and 3 are consistent with the assumption that no mutagens are present in *Mucuna* flour prepared from either raw or roasted seeds as described.

Analysis for benzo[a]pyrene by GC-MS

In order to test for the presence of benzo[a]pyrene ("BaP"), flour samples were extracted with dichloromethane as described in Materials and Methods, and the extracts were injected into the gas chromatograph, which separates the various substances in such a manner that they emerge from the column into the mass spectrometer at different times. The mass spectrometer then measures the amount of each substance present, and also generates a "mass spectrum" for each substance. Substances can be identified by analyzing the details of the spectrum, or by comparing the spectrum either to a library of spectra stored in the computer, or to a spectrum obtained on the same instrument using an authentic sample of the substance.

Under the conditions of the analysis, BaP emerges from the column at 27.8 minutes. The mass spectrum of this compound has a distinct appearance, with a base (most intense) peak at 252 m/z, and it is therefore relatively easy to ascertain the presence or absence of BaP in a sample of material.[1] The system is also very sensitive: when authentic BaP is injected, as little as 5 nanograms can be detected by the mass spectrometer and correctly identified by automated comparison with library spectra.

We compared the flour made in Honduras from roasted beans with flour made in our laboratory from raw beans of the same variety. In each case, we extracted the material by ultrasonication and reflux in dichloromethane as described in Materials and Methods.

The chromatograms of the two extracts were very similar. In both cases, there were peaks in the region of the chromatogram where BaP emerges, but in no case did the corresponding mass spectrum exhibit the distinctive peak at 252 m/z that we associate with BaP. In addition, we observed no difference between the raw and roasted material that could be attributed to the generation of BaP during the heating process.

The maximum amount of BaP that could be present in the samples can be estimated as follows: One microliter of extract is injected into the GC-MS, and this corresponds to 0.1 milligrams (100,000 nanograms) of flour. Since we can positively identify as little as 5 nanograms of BaP, the absence of a recognizable BaP peak in the *Mucuna* samples allows us to conclude that if BaP is present at all, the concentration can be no higher than 5 parts in 100,000. Further studies should allow us to refine this estimate to even lower numbers.

[1] "m/z" denotes the mass-to-charge ratio of the molecular fragment which gave rise to a peak in a mass spectrum. The value of m/z is almost always equal to the mass of the fragment in atomic mass units (amu).

DISCUSSION

Because of the growing interest in *Mucuna* as food and feed, it is important to demonstrate that no substances are present which are likely to be mutagenic or carcinogenic in humans or animals at expected levels of ingestion. Although this concern has been noted previously (Versteeg *et al.*, 1998), we have seen no reports of work in this direction.

In the present study, the primary tool is the Ames test, which was designed to recognize the presence of any substance that can produce any of several types of mutations that might result in tumors in humans or animals. The strength of the test is twofold: first, it can screen for the presence of a wide variety of substances; second, it does not require us to identify in advance what substances we are looking for. Since our present understanding of the relationship between the incidence of tumors and the ingestion of particular substances is far from complete (Ames and Gold, 1998), the second feature makes the test especially powerful.

As reported above, our application of the Ames test to both raw and roasted *Mucuna* provides no evidence to suggest the presence of mutagenic compounds. We had expected that the roasting process might generate substances that would cause a mutation in at least one of the strains, but the numbers for the roasted samples are all well below the threshold value for a positive result. It should be noted, however, that the description of the roasting conditions suggests a low to moderate temperature in the oven.

Likewise, our examination of the literature reveals no coincidences between the known compounds of *Mucuna* and the generally accepted lists of known or potential carcinogens. We also find no articles in the MEDLINE database linking the word *Mucuna* to terms such as cancer, tumor, or mutagen.

We undertook the search for benzo[a]pyrene by GC-MS as the first step in a chromatographic search for known or suspected carcinogens in *Mucuna* samples that have been subjected to heat. This also prepared us to analyze for the presence of specific compounds should the Ames test reveal the presence of mutagenic activity. Benzo[a]pyrene is known to be produced during the combustion of plant material, and was the positive control for one of the strains in the Ames test. We could therefore expect that the test would detect it if it were present in sufficient concentration in *Mucuna*. The negative result of the Ames test, combined with the results of the GC-MS analysis, suggests that benzo[a]pyrene is indeed not

present in *Mucuna* at a level which need be of concern.

In summary, the results presented in this paper are consistent with the assumption that neither raw nor gently roasted *Mucuna* seeds contain substances that are mutagenic, or substances that can be converted to mutagens by metabolism in the liver.

Further work should explore variables such as roasting temperature and time, and the concentration of *Mucuna* sample utilized in the Ames test. The effects of extremely high temperatures, such as those encountered when seeds are toasted in a pan directly over a fire, should also be examined. Both gas and liquid chromatography, in conjunction with mass spectroscopy, are powerful tools for the detection of potentially carcinogenic compounds in strongly heated samples.

While such experiments may be expected to increase our confidence with respect to the absence of mutagens and carcinogens in *Mucuna,* the results of this study suggest that, at the present time, the primary focus in preparing *Mucuna* for use as food and feed should be on L-dopa and other potentially psychoactive compounds, as well as on the substances that are traditionally of concern in legumes.

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