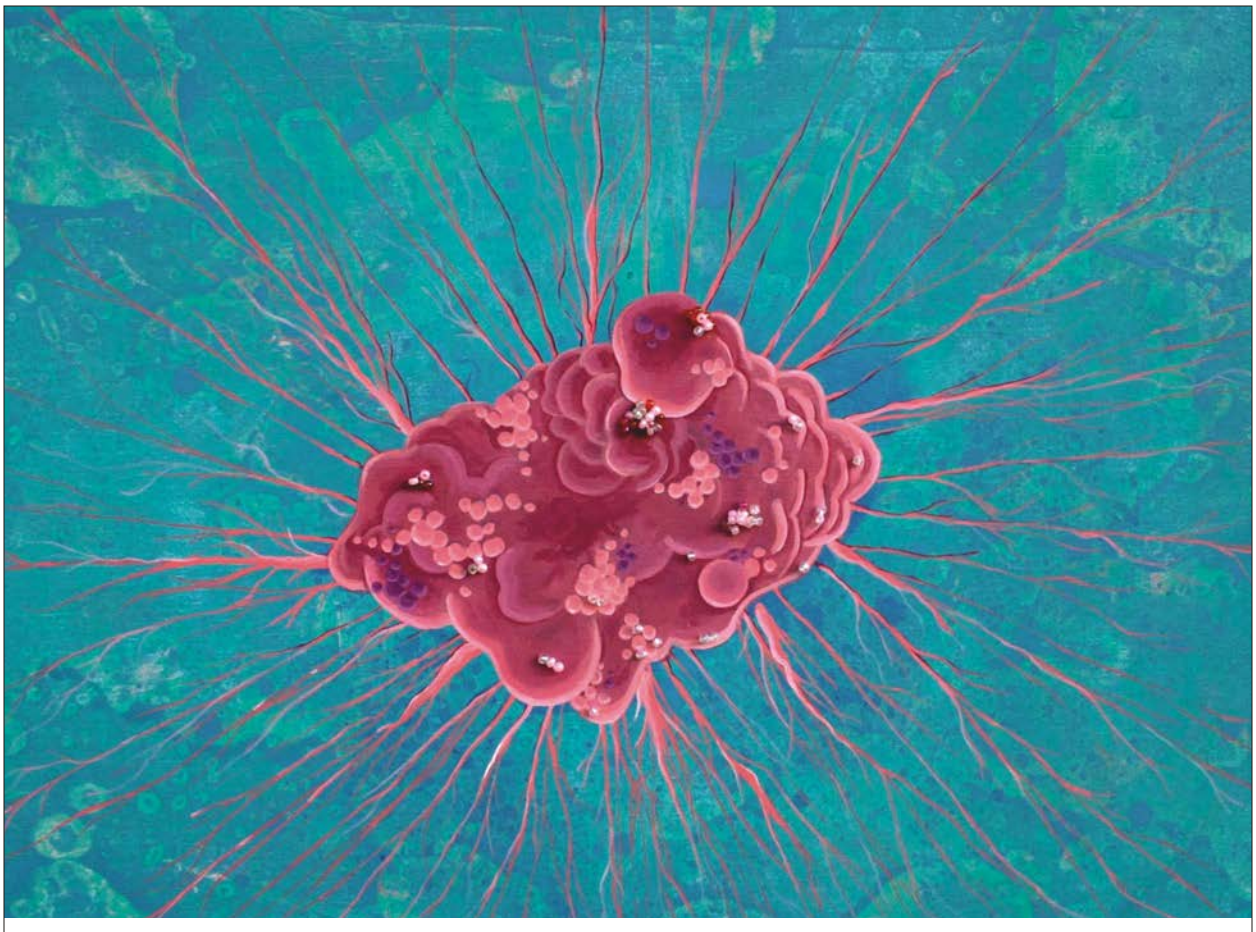

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The Role of *Phaseolus vulgaris* in DMBA-Induced Sprague-Dawley Rat Mammary Carcinomas

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ABSTRACT

The present study intended to study the influence of *Phaseolus vulgaris* beans in mammary carcinogenesis especially considering the main malignancy indicators through a histopathology study. A total of 72 female rats 50 days old were divided in three groups and hold in constant conditions temperature, light and humidity. Group B and C received 20 mg of 7, 12 dimethyl benzanthracene by gavages. All the animals were fed with the same standard food, group C were supplemented with Phaseolus vulgaris beans. The study had last for 150 days. Tumors in group C animals had lower pattern grades and lower mitotic counts despite the higher weight gain in this group. These results suggest a protective role of bean consumption in mammary carcinogenesis possibly due to fiber and oligossacharides content, especially present in *Phaseolus vulgaris* from "padrão tarrestre" species.

Key words: Legumes, histopathology, chemical carcinogenesis, fiber, amylose

INTRODUCTION

Cancer incidence had not decreased in the last three decades despite the extensive research done on the disease causes, risk factors and therapeutic approaches. According to American Cancer Society, Breast cancer is the leading cause within cancer deaths and it is the most frequent form of female cancers (Jemal *et al*., 2010). Family history had been considered an important risk factor (Makarian *et al*., 2007), however, genetics itself had been implicated in less than 25% of the cases (Cebrian *et al*., 2006).

The role of diet and nutrition in disease burden is well accepted and had been proven, however, it is not yet fully understood. Data from several studies associated the excessive consumption of alcohol (Ellison *et al*., 2001; Willett, 2001; Michels *et al*., 2007; Tjonneland *et al*., 2007; Boyle and Boffetta, 2009), red meat (Taylor *et al*., 2007; Cho *et al*., 2006; Ferrucci *et al*., 2009), whole milk (Ronco *et al*., 2002; Moorman and Terry, 2004) and sugar rich foods (Tavani *et al*., 2006; Bradshaw *et al*., 2009) with breast cancer increased risk. On the other hand, a diet rich in fruits and vegetables (Gandini *et al*., 2000; La Vecchia *et al*., 2001; Gaudet *et al*., 2004; Zhang *et al*., 2009) as well as legumes (Mathers, 2002), with a moderate intake of tea (Wu *et al*., 2003;

Sun *et al.*, 2006; Zhang *et al.*, 2006; Kumar *et al.*, 2009), olive oil Garcia-Segovia *et al.*, 2006; Lopez-Miranda *et al.*, 2010) and fatty fish (Gago-Dominguez *et al.*, 2003; Terry *et al.*, 2003; Kim *et al.*, 2009) had been associated with lower risk.

Pulses are an important source of carbohydrates in human nutrition, being also particularly rich in fiber and a wide range of bioactive substances considering the also great diversity of species included in this food group.

In fact, the high diversity in nutritional composition of vegetable products is clearly associated with environmental factors including climate, soils, sun exposure, within others (Davis *et al.*, 2004; Tubiello *et al.*, 2007). Storage and specially cooking, as well as the natural complexity of human food habits, can also significantly affect nutritional richness and nutrient availability in foods which can justify the some controversial results on the association of these foods and breast cancer risk (Terry *et al.*, 2001).

Data from epidemiological studies also fail to identify the mechanisms through which foods and/or nutrients exert their protective or aversive role in disease; this could had motivated the development of experimental studies using animal models of breast cancer (Terry *et al.*, 2001; Willett, 2001). With this type of studies become possible to evaluate the effect of foods, nutrients and even meals in mammary carcinogenesis. Furthermore, biological materials from them can be used for further histolopathologic studies and immuno-histochemical techniques that could possibly identify the mechanisms and some indicators of malignancy and clinical prognosis.

Mammary carcinogenesis malignancy can be primarily evaluated through macroscopic features like tumor number and volume, a further histopathologic characterization adds important data about tumor malignancy (Russo and Russo, 2000). In fact, histologic grade given by pattern and nuclear grades as well as mitotic count, was previously associated with clinical prognosis (Elston and Ellis, 1991) and had been commonly used as malignancy criteria in several laboratory studies. Tumor architectural pattern is related to the loss of the normal alveolar structure of mammary gland while pattern and nuclear grade, as well as mitotic count refer to tumor differentiation. A higher histologic grade is associated with lower differentiation, thus a greater capacity of invasiveness and metastasis. Russo and Russo (2000) considered all these characteristics the main hallmarks of tumor malignancy.

The goal of the present study was to evaluate the effect of adding *Phaseolus vulgaris* as supplement in mammary carcinogenesis, using the DMBA-induced Sprague Dawley female rat as experimental mode.

MATERIALS AND METHODS

Animal care: The experimental procedure was approved by the Veterinary Advice Commission published in the Portuguese legislation (DL 129/92 and Dir 86/609/CEE) and it was conducted in strict adherence to animal care guidelines established by competent ethic commissions, the project started in January 2004 and ended in April 2007. Seventy-two female Sprague-Dawley rats (Charles River Laboratories, Barcelona) of 42 days old were randomly assigned to three groups, housed in plastic cages maintained at 22±2 °C, 55±10% humidity and with 12 h light/dark cycle.

Experimental diets: All the animals received a standard food formula ISO 9002 certified (Standard Panlab A04) *add libitum* and had free access to tap water. Group C food was supplemented with 4% of lyophilized "padrão tarreste" beans. The nutritional composition of Standard Panlab Diet and *Phaseolus vulgaris* beans are presented in Table 1 and 2, respectively.

Table 1: Composition of the standard diet

Standard diet	Nutritional composition (%)
Humidity	12
Protein	15.5
Fat	2.7
Glucides	58.5
Minerals	5. 5
Fibre	3.7
Metabolizable energy	3000 kcal kg^{-1}

Table 2: Nutritional composition of *Phaseolus vulgaris* , "Padrão Tarreste" (g/100 g)

Nutrient	Content (g)
Protein	12.2
Carbohydrates	50.2
Fat	2.6
Fiber	3
Minerals	
Calcium	0.00565
Magnesium	0.00221
Chlorine	0.00050
Vitamins	
Thiamine	0.00171
Pyridoxine	0.00018
Niacin	0.00173
Riboflavin	<0.00050
Di and oligossacharides	
Estachiose	1.15
Raffinose	<0.03
Saccharose	1.17
Fatty acids	
Butyric	0.0012
Myristic	0.0070
Palmitic	0.1380
Stearic	0.0600
Oleic	0.2280
Linoleic	0.1710
Linolenic	0.2180

The beans were added with water and stay for 12 h, then were boiled and finally smashed with a fork. After these it was cooled down and put in small bags of 250 g and then lyophilized.

Protocol for chemical tumor induction: Animals from groups B and C received 20 mg DMBA (Sigma-Aldrich, Lisbon, Portugal) solved in 1 mL olive oil by gavages after one week of quarantine, 50 days old.

Other procedures: Animal's body weight was recorded every two weeks. The weight homogeneity index (HW) was calculated at the beginning of the study, according to the Eq. 1, being Ws is the lowest weight and Wg is the highest weight found in this group of rats. The body weight gain (WG) was monitored for a stipulated period of time, two weeks, considering the weight recorded in the beginning (Win) and the end (Wfin) of the considered period, according to the following Eq. 2:

$$HW = Ws / [(Ws + Wg) / 2] \quad (1)$$

$$WG = [(Wfin - Win] \times 100 \quad (2)$$

Necropsy: All the surviving animals were humanly sacrificed after 150 days through inhalation of carbon dioxide and they were all submitted to necropsy process during which neoplastic lesions were collected and prepared for histological studies.

Collection and evaluation of tissue: The rate of neoplastic lesions in this experimental model was described through the ratio between the number of rats that revealed neoplasms and the number of rats still alive at the end of experiment.

For each group the occurrence (Oc) of mammary lesions was determined according to the different types of volume using the formula Oc (%) = No tumors nx /No. of total mammary tumors found in each group at the end of the experiment, where x = tumors volume. The number of neoplastic lesions in each animal was divided into four classes, categorized as 0, 1-2, 3-5, 6-8, >9 tumors rat^{-1}.

Tumor size was evaluated according to their volume ($V = 4/3\ \pi r^3$) where r is the average radius of several tumors in the same group) and then classified in the following categories: categorized as type A (volume$\leq$0.033 cm^3); type B (0.033 cm^3<volume$\leq$0.267 cm^3); type C (0.267 cm^3 <volume$\leq$0.904 cm^3); type D (0.904 cm^3 <volume$\leq$2.143 cm^3); or type E (volume>2.143 cm^3).

Histopathologic study: The tumors were dissected and the fragments collected for histology were fixed in neutral buffered formaldehyde, processed and embedded in paraffin, cut in microtome and stained with hematoxylin and eosin.

Data from the present study refers exclusively to mammary neoplastic lesions. The histological classification done considered the following elements: type of lesion (benign or malignant, invasive or in situ), architectural pattern, cribriform areas and histological grade based on three parameters: tubular formation or Pattern Grade, nuclear pleomorphism or Nuclear Grade and Mitotic count (Russo and Russo, 2000; Costa *et al.*, 2002).

Statistical analysis: All statistical tests were two-tailed and conducted at 95% confidence level. Significance tests for all pair wise comparisons were adjusted for multiple comparisons by multiplying the actual p value by number of comparisons made for the evaluation of statistical significance. The software package used was SPSS 17 (SPSS Inc., Chicago, Ill).

The purpose of the analysis was to test whether the null hypothesis of distribution was equal in all groups. In addition to overall test of significance, pair wise comparisons between groups were also made through Mann-Whitney tests (Fagerland and Sandvik, 2009). The overall weight gain of the animals of all groups was compared by use of single classification variance analysis ANOVA with repeated measures. The interest of this test was to verify the difference between weight gain over time among the groups.

RESULTS

All the animals gained weight during the experiment, however, it was not possible to compare absolute values because initial average weight differed between groups, this is an important

methodological condition to apply ANOVA test. Considering this, the statistical test was done comparing weight gain values from each group. There were significant statistical differences between groups A and C, as well as B and C ($p<0.05$), however, the same did not happen between A and B ($p>0.05$) as presented in Table 3.

Like expected there were not found any neoplastic lesions in animals from group A but the tumor number did not differ significantly between groups B and C ($p>0.05$) and the average tumor volume from group C animals was higher than in group B ($p<0.05$) as presented in Table 3.

In what concerns to the number of tumors found in each animal, most animals from Group C (70.59%) animals had 1-2 tumors while in Group B (40.9%) most had 3-5 tumors like shown in Table 4. There was not a significant difference also in tumor volume distribution within groups, most tumors in Group C were considered large tumors but this group had less Type E lesions than Group B as presented in Table 5. However tumor number and size data had not been clearly associated with malignancy.

There was not found any comedocarcinoma in group C, half presented a pure cribriform pattern (53%) and 40.6% had shown papillary and cribriform areas. There was statistically significant difference within groups in each tumor architectural pattern frequency ($p<0.05$) as presented in Table 6.

The main histological parameters (Table 7) from tumors in the three groups differed significantly ($p<0.05$), most tumors in group C had lower pattern grade levels than group B as well as less mitoses, but the same did not happen with nuclear grade that was similar in both groups ($p>0.05$).

Table 3: Weight-gain and tumor number comparison between groups

*Group	Caloric value (kcal 100 g)	Weight gain (Wp = %)	Surviving animals	Mammary neoplastic lesions	Average tumor value (cm^3)
A	300	67.1±17.8	22	0	0
B	341	56.3±12.1	22	33	2.258±2.456
C	341	100.3±27.2	17	32*	4.359±6.919

*Mann-Whitney test $p < 0.05$ within groups. Comparison between Groups A and B weight gain: $p = 0.04599$; within Groups B and C $p = 0.0289$; in tumor volume comparison between Groups B and C, $p = 0.0545$

Table 4: Group distribution according to number of tumors per animal

*Group	0 tumors (%)	1-2 tumors (%)	3-5 tumors (%)	6-8 tumors (%)	≥9 tumors (%)
A	100.00	0.00	0.00	0.00	0.0
B	9.10	31.80	40.90	0.00	0.0
C	11.76	70.59	11.76	5.88	0.0

*Mann-Whitney Test $p>0.05$ when comparing groups B and C

Table 5: Tumor size distribution per group

	Small tumors		Medium tumors	Large tumors	
*Groups	Type A (%)	Type B (%)	Type C (%)	Type D (%)	Type E (%)
A	0.00	0.00	0.00	0.00	0.00
B	0.00	18.90	18.90	21.60	40.50
C	6.25	15.63	6.25	37.50	34.38

*Mann-Whitney Test $p>0.05$ when comparing groups B and C

Table 6: Characterization of tumor architectural patterns

Patterens	Group B (%)	Group C (%)
Papillary	2.63	6.3
Cribriform	36.84	53.0
Papillary and cribriform	42.11	40.6
Papillary, cribriform and comedo	5.26	0.0
Papillary and comedo	2.63	0.0
Cribriform and comedo	10.53	0.0

Mann-Whitney test at 95% confidence level comparing frequencies between groups ($p<0.05$), there was found a statistical significant difference within both groups tumor architectural patterns frequency. Papillary pattern $p = 0.0459$; Cribriform $p = 0.0480$; Papillary and cribriform $p>0.05$

Table 7: Main histopathologic parameters comparison between groups

	Pattern grade (%)			Nuclear Grade (%)			Mitotic counts [a] (%)				
*Group	I	II	III	I	II	III	= 3	4-6	7-9	10-19	= 20
B	15.8	52.6	31.6	13.2	81.6	5.3	26.3	21.1	5.3	31.6	15.8
C	46.9	34.4	18.8	12.5	81.3	6.3	59.4	3.1	12.5	25.0	0.0

*Mann Whitney Test comparing groups B and C frequency $p>0.05$, [a]Mitosis in 10 high power fields

When correlating Pattern and Nuclear Grade, a significant difference was found between group B and group C ($p<0.05$). In group B, half scored intermediate levels of both parameters while in Group C 37.5% scored Pattern Grade I and Nuclear Grade II and 28.1% with intermediate scores in both. The higher score in both Pattern and Nuclear Grade was present in 5.26% of group B and 3.1% in group C.

Lower mitotic counts were mostly present in lower pattern grades in group C while in group B even intermediate levels of pattern grade were associated with few mitosis, 21.1% had pattern grade III and more than 10 mitosis and 10.5% more or equal to 20 mitosis, with statistical difference between groups ($p <0.05$). Higher nuclear pleomorphism is normally associated with higher mitotic counts, in Group B 5.2% had nuclear grade III and more than 10 mitosis, however none of the Group C tumors achieved the same and half had nuclear grade II and 3 or less mitosis. These results suggested that tumors from group C were better differentiated, due to lower mitotic counts, which may be a lower malignancy indicator as suggested by Elston and Ellis (1991).

DISCUSSION

Animal studies of carcinogenesis chemically induced had been a valuable resource to study the carcinogenesis process, especially to study the influence of diet in breast cancer. The present study was conducted accordingly to previously validated conditions (Costa *et al*., 2002, 2004).

Data from present study had shown a possible positive effect of *Phaseolus vulgaris* beans on several aspects of neoplastic lesions. Although animals from group C gained more weight ($p<0.05$) and had larger tumors ($p<0.05$), their histopathologic grade was lower. Neoplastic lesions in group C presented less extent solid areas and lower mitotic counts when compared to group B ($p<0.05$). Pulses like *Phaseolus vulgaris* beans are an important dietary source of protein, carbohydrate and energy, besides several minerals (Acar, 2001) . Within the carbohydrate fraction, the vast majority is starch packed in starch granules with a high content of amylose. Amylose that as double helices with near six glucose molecules each side which allows it to support the hydrophobic bridge of polar lipids, forming enclosed complexes that reduce the ability for enzymatic hydrolyses. This property is responsible for the low glicemic index of amylose rich foods (Foster-Powell *et al*., 2002).

Beans are also rich in fiber and oligossacharides which is not completely digested (Caroline *et al*., 2003) so may suffer fermentation in the colon producing short chain fatty acids like propionate, acetate and butyrate, as well as gases like carbon dioxide and methane. Fiber consumption itself is inversely associated with several cancer sites (Moore *et al*., 1998; Pierce *et al*., 2007; Park *et al*., 2009). In one hand, insoluble fiber decreases transit time reducing carcinogen exposure, on the other hand soluble fiber and oligossacharides are highly fermentable by colonic flora producing short chain the previously referred fatty acids.

Butyrate could be responsible for an anti-neoplastic action due to the prevention of DNA damage through histone deacetylase inhibition (Wu *et al*., 2001; Hinnebusch *et al*., 2002). This short chain fatty acid is very important in healthy colonic function and is inversely associated with colon cancer (Hamer *et al*., 2008; Scharlau *et al*., 2009). Propionate seems to reduce cholesterol synthesis because of its competitive action against HMG CoReductase (Hara *et al*., 1999; Beylot, 2005). Hypercholesterolemia seems to be also positively associated with breast cancer risk (Vatten and Foss, 1990; Owiredu *et al*., 2009).

Considering this, *Phaseolus vulgaris* nutritional composition includes substances that can exert several anticarcinogenic such as the prevention of DNA damage, promoting apoptosis of damaged cells, ending or decreasing tumor growth, angiogenesis impairment and prevention of metastasis (Mathers, 2002).

Within the results, it seemed important to note that nuclear pleomorphism was not significantly lower in animals receiving the supplemented diet when compared to group B. Animals from this group gained more weight and weight gain happened to be an important risk factor for breast cancer (Huang *et al*., 1997; Ahn *et al*., 2007; Montazeri *et al*., 2008). It could be also relevant to note the complex composition of these beans, with nutrients and anti-nutrients that can exert a synergic or antagonist effect as well as the possible effect of cooking and soaking in potential anti-carcinogenic substances bioavailability (Aguilera *et al*., 2009; Wang *et al*., 2010).

Despite the extension research on benefits of beans, pulses and specifically *Phaseolus vulgaris* in colon cancer, however previous data did not always revealed a significant influence of beans in breast cancer (Aune *et al*., 2009).

In conclusion, the present work revealed a protective effect of *Phaseolus vulgaris* beans consumption, specifically from padrão tarrestre species, possibly due to their richness in fiber which may be responsible for several anti-carcinogenic effects.

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