The Effect of Consuming a Cake Containing Propolis on Gut Micro flora and Toxicity.

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Abstract: This work was done to study the effect of crude propolis and its extracts (water and ethanol) on modulation of micro flora in the gut and protection against toxicity with aflatoxin. Experiment was done on Sprague Dawley white Albino rats that were divided into 9 groups. Group 1 was fed on basal diet, group 2 was fed on a cake, groups 3, 4 & 5 each was fed on either cake fortified with crude, water or ethanol extract of propolis. Group 6 was fed on the cake but contaminated with aflatoxins, then, groups 7, 8 &9 each was fed on cake contaminated with aflatoxins and either crude, water or ethanol extract of propolis. All groups continued for eight weeks. Feces were collected during the experiment and the secum was isolated at the end of the feeding period for assaying the pattern of micro flora either the beneficial bacteria or the harmful ones. The Results showed that the microbial count of the *Bifidobacterum* increased by addition of propolis to the cake. The value obtained in case of control rats was 50 x 10^4, this value was 77 x 10^4, in case of rats fed on the cake. Addition of propolis crude, the water or ethanol extract raised the count of bifidobacterium.The value obtained for ethanol extract was 30 x 10^6. Addition of aflatoxin to the cake markedly increased the count of coilform in feces. Adding propolis to aflatoxins contaminated cake caused a reduction in enumerated colony of coliform. The values obtained were 25 X 10^4, 48 x 10^4 and 33 x 10^4 for crude, water, and ethanol extract of propolis respectively. The activities of the liver enzymes, namely AST and ALT were markedly increased in rats fed on the aflatoxin contaminated cake. Adding propolis to the cake caused a return to normal values of the activities of these enzymes. Conclusion is that supplementation of food with propolis can promote growth of the beneficial bifidobacterium and inhibit that of the harmful coliorm type present in the gastrointestinal tract. In addition, it can protect against toxicity.
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1. Introduction

Propolis is a multifunctional material used by bees in the construction and maintenance of their hives. Propolis has been used in folk medicine and proved to have numerous biological activities including anti-oxidant, anti-microbial, anti-carcinogenic, anti-fungal, anti-viral, anti-ulcer, immunostimulatory and anti-inflammatory properties. (Barros et al., 2007; Atungulu et al., 2007). Use of propolis by humans has a long history, predated only by the discovery of honey. In addition, propolis was extensively used to improve health and prevent diseases such as diabetes, atherosclerosis, heart diseases and cancer. (Sforcin, 2007). Propolis contain over 300 constituents, some of which are nutrients as proteins, amino acids, vitamins, minerals and other natural compounds such as polyphenols, terpenoids, and steroids. (Buratti et al., 2007). Egyptian propolis became a subject of research by biologists and chemists (Hegazi et al., 2002). Some varieties may contain enzymes such as glucose oxidase, catalase and peroxidase. These presented compounds are in part responsible for the health benefits of propolis. Propolis is considered as a source of natural antioxidant and has a strong antioxidant activity. It contains large amounts of anti-oxidative compounds; the ethanol extract exerts an anti-lipid peroxidative action. (Ahn et al., 2007) Dietary propolis suppressed the lipoxygenase pathway of arachidonic acid, thus inhibit prostaglandin and leucotriene generation and in turn inflammation. (Borrelli et al., 2002).

The micro flora in the human gut exists in a dynamic state and ecologically diverse environment. This micro flora is made up of hundreds of microorganisms (Moore and Holdeman, 1974). Several types of these microorganisms present in the gut exert activities that have a direct impart on host health (Mitsuoka, 1992). It is thus clear that selective enumeration of bifidobacteria is of great interest because of the assumed health and nutritional benefits ascribed to these bacteria. There are several disorders that affect the different sectors of the population in Egypt and need to be avoided. Children usually suffer from diarrhea due to infection with pathogenic bacteria. Adults suffer from environmental pollution which causes several health disorders such as oxidative stress, atherosclerosis and liver toxicity. Exposure to

aflatoxin contamination in the diet is considered an important factor for the development of primary hepatocellular carcinoma (Bennett and Klich, 2003; Antonio et al.2008) showed that the liver is the main site of aflatoxin biotransformation with the mitochondrial cytochrome P450 oxidative system converting the AFB1 into AFM1, thus considered a detoxification process.

Based on these multiple health benefits of propolis, the aim of this study is to add it to some food items such as cake that is usually consumed by children, adolescent or adults in the breakfast. This will supply them with the adequate amount of propolis that realize all the fore mentioned health benefits. It is hopeful that such cake with propolis will participate or help to avoid these health complications and promote the growth of the beneficial bacteria in the gut.

2. Materials and Methods

Material:

Propolis: Propolis used in this study was obtained from the Ministry of Agricultural farms in El-fayome. *Aspergillus parasiticus* (NRRL 3145) was kindly provided from the Central laboratory of Mycotoxins, National Research Center.

Animals: Albino rats of Sprague-Dawley strain, body weight (70 ± 20 gm) were obtained from the animal house of the National Research Center.

Diet ingredients: The ingredients used for the preparation of the cake such as, wheat flour, fat, sugar, eggs, chickpea, whey protein concentrate and others were purchased from the local market.

Standard diet: The ingredients used for the preparation of the standard diet such as casein (85% protein), Cellulose, mineral or vitamin mixtures, Choline bitartrate and L-Cystine were obtained from National Research Center stores.

Media: Dehydrated Agar Medium (ready to use) was obtained From Fluka, sigma Switzerland. (Oxiod manual .1998).

Methods:

Aflatoxin Production and Assay: Potato dextrose agar medium was used to produce aflatoxins with *Aspergillus parasiticus* NRRL 3145 (highly and multi-toxin-producing strain). The medium was prepared according to Harrigan and Margaret (1966).

Extraction of Propolis: In this study we used propolis in three forms, Crude propolis, water extract and ethanol extract. Water extract of propolis was prepared according to Nagai *et al.* (2003). Ethanol extract of propolis according to Choi *et al.* (2006).

Preparation of cake: The Cake was prepared with some modification in the method given for cake preparation in AOAC (2000).

Animal experiment:

The experiment was done on 54 male Sprague Dawly rats, body weight (70+ 20g), housed individually in stainless steel cages. Rats were classified into 9 groups each of 6 .Each group was put on certain diet as shown in table(1). Food and water were allowed adlibitum. The consumed food was calculated and the body weight gain was recorded. Experiment lasted 8 weeks. The fresh feces were collected once a week from all groups in sterile plastic bags and transferred to the laboratory to be assayed for microbial load within 1 hour. At the end of the experiment, rats were fasted overnight and in the morning were again weighed, anesthetized with diethyl ether then blood was withdrawn by open heart puncture, part over heparin and the other without for separation of serum by centrifugation at 3500 rpm for 15 minutes. The liver kidney and spleen were separated, washed with saline, plotted between 2 sheets of filter paper and kept in plastic bags till needed. The secum was tied and isolated, washed with saline and put in a sterile plastic bag for microbiological assay. The endogenous populations of colonic pathogenic and prebiotic bacteria such as bifido and coliform had been counted according to Wehr and Frank (2004) ; Haddadin *et al.* (2004). Ten grams feces were homogenized diluted with buffered peptone water pH 7.0 supplemented with 0.5% L-cysteine for growth of bifidobacteria and Violet Read Bile Agar (VRBA) for growth of coilform. Total coliforms were determined by using a three-tube dilution series. Growth of bifidobacteria was determined by using a five-tube dilution series, MRS and VRBA Plates were incubated at 37oC for 24-48 h. The above procedure was repeated twice for each sample.

Statistical analysis:

Statistical analysis was carried out by using SPSS, PC Statistical Software the results were expressed as mean ± SD. Data were analyzed by one way analysis variance (ANOVA). The difference between means were tested for significance using least significant difference (LSD) test at ($p < 0.05$).

3. Results

Microbiology

The microbial count of samples obtained from secum of rats at the end of the experiment is shown in table (2). Rats fed on the cake showed an increase in number of *bifidobacterum* and a decrease in coliform, compared to those fed on the basal control diet. The value obtained for *bifidobacterum* in case of control was 50 x 10^4, this value was 77 x 10^4, in case of rats fed on the cake. Addition of crude propolis raised the value to 10 x 10^6 and to the same value for rats fed on cake + water extract. The value obtained for ethanol extract was 30 x10^6. When

aflatoxin was added to the cake, few *bifidobacteria* was detected in feces. However, addition of propolis to the aflatoxin contaminated cake caused reappearance of this type of bacteria. The values reported for the enumerated colony were $20x10^6$, $10x10^6$ and 30 $x10^6$ for crude propolis, water or ethanol extract respectively. Addition of aflatoxin to the cake markedly increased the count of coilform in feces. The value obtained was $62x10^4$. Adding propolis to aflatoxins contaminated cake caused a reduction in enumerated colony of coliform. The values obtained were 40 X 10^4, 48 x 10^4, 33 x 10^4 for crude, water and ethanol extract respectively.

Table (3) show the values reported for microbial count in samples of feces collected from animals during the experiment. As shown in the table, the number of *bifidobacterium* in feces of control rats was $24x10^5$ that for rats fed on the cake was $30x10^6$. Addition of crude propolis to the cake caused an appreciable increase in bifidobacterium $30x10^6$. When water extract of propolis was added to the cake, the value obtained was 27 x 10^6 , in case of ethanol extract the value was $33x10^7$.

The pattern obtained for coliform bacteria is different. Coliform was detected in feces of rats fed on the control diet 14 x 10^5, also found in feces of rats fed on the cake, however, it was more or less absent in rats fed on the cake to which propolis was added, (10 x 10^2, in case of crude propolis, $14x10^3$, in case of water extract, and $10x10^3$ in case of ethanol extract). Addition of aflatoxin to the cake caused an elevation in the enumeration colonies of coliform bacteria. The value reported was $62x10^5$. This value became much lower when propolis was added to the contaminated cake. The values reported were 25 $x10^4$, 84 $x10^3$ and 33 x 10^4.

Nutritional evaluation:

Body Weight gain and Food Efficiency Ratio:

The values for gain in body weight gain and the food efficiency ratio (FER) are given in Table (4), As shown in the table, rats fed on the standard diet consumed 668.2 ± 6.42g during the experimental period. The food intake of the other groups was more or less lower than that of group B which was fed on the basal diet. The food intake ranged between 540.0 ± 18.24 to 592.7 ± 2331 g.

As shown in the table, the highest gain in body weight was that of rats fed on the cake that contain water extract of propolis. The gain in body weight reached 276.2 ± 18.54 g. The lowest gain of body weight was reported for the group given the cake contaminated with aflatoxins.

The food efficiency ratio which is the relation between the gain in body weight and food intake was also highest for the group fed on the cake with water extract of propolis and lowest for the group given the cake with aflatoxin.

Weight of organs:

The mean values ± SE and significance of difference of weight of liver, kidney and spleen are shown in Tables (5), the ratio of liver to body weight is also given. As shown in the table, addition of propolis, crude or extracts to the cake caused a slight decrease in liver weight that was most marked in case of water extract. The weight of liver of rats fed on the AFT contaminated cake was lower than that of rats fed on that containing propolis or those fed on the control diet.

Addition of crude or extracts of propolis to the contaminated cake caused slight increase in liver weight (Table 6).The liver to body weight ratio also showed slight decrease in case of rats given propolis with either the contaminated or the non-contaminated cake, (Table 9).

Activities of transaminases

The activities of the two enzymes ALT and AST which are representative to liver function are shown in Table (6) the highest value reported for these two enzymes was found for group fed on the cake contaminated with aflatoxin. The values reported were 33.86 ± 4.2U/L for ALT and 141.46 ± 4.12 U/L for AST. These values for rats given different extracts of propolis with the contaminated cake were lower.

Gamma Glutamyl transferase (GT):

The activity of GT was remarkably high in case of rats belonging to the group which were fed on the AFT contaminated diet. Addition of either crude propolis of its extracts to the contaminated diet caused the activity of the GT to return back to near normal value, see Table (6).

4. Discussion

Propolis, the glue material collected by honey bee from plant buds and exudates (Marcucci, 1995), has several health benefits that encourage its use in food to give it an added health value. Propolis was proved to posses antioxidant properties (Guney et al. 2007; Ahn et al. 2007), anti-inflammatory (Ramos and Miranda, 2007), anti-bacterial (Alexandra *et al.,* 2004), anti-fungal (silic *et al.,* 2005), antiviral (Ramos and Miranda, 2007), in addition to its character as a prebiotic (Macfarlane et al., 2008). In the present study, propolis was added to cake, prepared in a traditional manner and contains whey to improve its nutritional and health value. Propolis was added in three forms; crude, water or alcohol extracts. The reason for this is that propolis contains variable biological compounds, some of which may be soluble in water, alcohol, others may

not be soluble in any and thus propolis has to be taken as it is.

The advantage of the cake is that most people at different age stages are used to eat. Children, elder and young are used to eat cakes. Besides, this is a type of food that can be prepared at home and in turn, most family members can benefit from its nutritional and health value.

Modulation of the human gut microflora towards improved health status using prebiotic, probiotic and synbiotics or other power will directly contribute towards human health (Tuohy et al., 2005).

In this study, the effect of adding propolis to the diet of rats either the normal or those challenged with myctoxin caused high enumeration of bifiedobacterium and a decrease in coliform. It was not possible to find any study about the effect of propolis ingestion on the gut microflora pattern in vivo, perhaps this is the 1st study in this area. However, Abd El-hady and Hegazi. (2002) showed that propolis possesses antibacterial activity against *staphyloccus aurus, Escherichia coli,* and *Candida albicans.*The antibacterial activity of the propolis was attributed to the presence in propolis of compound such as aliphatic and aromatic acids, caffeate esters, triterpenes and Flavonoids. The finding in this study is in agreement with that of those authors; however, the present study was done in vivo. The study also agrees with that of Mertzner (1979). The antibacterial activity of propolis against *Pseudomonas aeruginasl*, *Salmonella typhi*, *Esherichia coli*, *Staphylococcus aureuss* and *Bacillus subtilus* was also proved by Mulie and Maingi (2007). It is worth mentioning that the alcohol extract was found to be most active with regard to inhibition of coliform group of bacteria. This is also in agreement with Mulie and Maingi, (2007) who stated that the extraction procedures determine the antibacterial activity. Probably different extraction procedures lead to extraction of different compounds which alternately contribute to difference in the antibacterial activity. On the other hand, addition of propolis to the cake or that with aflatoxin caused an appreciable increase in the enumeration of bifidobacteria. In this case, it is assumed that propolis act as a prebiotic to the beneficial bacteria or it may synergize the action of the prebiotics present in the gut, such as oligosaccharides and other dietary fibers (Tuohy et al., 2005). Also, microbial populations present in the gut provide an efficient barrier to invading gastro intestinal pathogens (Hentges, 1992). Although little is known about the effect of a bacterial species on the other, yet it is thought that, these species compete for nutrients, attachment sites on intestinal mucosa, production of bacteria, and stimulation of immune system (Khalil, 2007). Most probably all these factors work together towards favoring beneficial bacteria.

Rats fed on the cake formula consumed less food than those fed on the standard diet; however, the gain in body weight and the food efficiency ratio were higher. This is an indication that the composition of the formulated cake is balanced and most nutrient content are bioavailable. Addition of propolis or its extract to the formula did not change food consumption of the animals. However, the gain in body weight of rats fed on the cake that contain propolis was higher particularly in case of water extract of propolis. This indicates that water is able to extract active compounds in propolis that can promote growth. Results obtained by Tatlı Seven *et. al (*2007) suggested that propolis supplements improved the growth and carcass yield in broilers under heat stress. This was attributed to the flavonoid content and palatable properties of propolis. Flavanoids can act as antioxidants by chelating with free radicals (Wang, 2004; Prytzyk *et al.,* 2003). It was reported that they protect unsaturated fatty acids against the oxidants in the cell membrane (Havsteen, 2002).This is again in support to our finding that the loss in body weight of rats fed on the cake that contain aflatoxin was corrected by addition of propolis to the formula. This means that the stress condition or toxicity of aflatoxin can be tolerated by propolis.

Transaminases:

The activities of the enzymes ALT& AST are indicative to the state of liver. It is a measure of liver function. Addition of propolis in any form to the cake caused a slight decrease in the activity of each of these enzymes. When aflatoxin was added to the diet, the activity of these enzymes became very high. Elevated activities of these enzymes in serum point to cellular leakage and damage of liver cell membranes. Wang *et. al* (2009) reported that repeated butenolide exposure induced a significant liver injury, and oxidative damage. Bhadauria *et al.* (2007), found that alcohol extract of propolis is effective against the injurious action of carbon tetra chloride on hepatocytes in vitro. When propolis was added to the contaminated diet, the activities of these enzymes became relatively lower or started to be normalized. This means that propolis therapy could protect against hazardous effect of mycotoxin on the liver.

Stabilization of AST or ALT activates by propolis ingestion indicate improvement in the functional status of liver cells, which may be attributed to the free radical scavenging action of propolis. The present result insure that propolis either crude or extracts can protect the liver from injurious factors including mycotoxins. The protection may extend to normal state without direct exposure to the

harmful agents. It is noted that rats given the cake with propolis have enzymes activities lower than those fed on the control standard diet. This means that propolis is effective against any inflammatory condition that can affect the liver. The anti-inflammatory effect of propolis was reported before by Teixeira *et al*. (2008).

Gamma Glutamyl transferase:

There was no significant difference between the activity of serum γ-glutamyl transferase of rats given the cake and those given the standard diet table (12). Even when propolis was added no change was observed. When the cake was contaminated with aflatoxins, serum γ-glutamyl transferase was markedly elevated, (13.43 ± 1.047 U/L) relative to a value of (8.63 ± 0.842 μ/L) for the non-contaminated cake.

High level of this enzyme in blood is indicative to liver injury, (Ruppin *et al*. 1982). Gamma glutamyl transferase is elevated in diseases of the bile duct, however, increased level may indicate in general that the liver is being damaged (Gonsales, 2006).This means that the injurious effect of aflatoxins on the liver is marked. However, when propolis was added to the aflatoxin contaminated cake, the activity of the enzyme returned to near normal. This again proves the protective action of propolis against aflatoxins toxicity and support the probability that Propolis have the ability to promote liver metabolic processes that lead to detoxification. This funding agree with similar study (Bhadauria *et al*. 2008), who confirmed the hepato-protective efficacy of propolis.

It is thus proved in this study, that addition of propolis to cake brought about a very good health value, through improvement of beneficial bacteria and diminishing the harmful ones. The toxic hazards of mycotoxin contamination were ameliorated as evidenced by liver and kidney functions.

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Table 1: List of different groups of rats used in animal experiment.

Group	Diet
1	(Control a) the rats fed on basel diet.
2	(Control b) the rats fed on cake alone.
3	The rats fed on cake + crude propolis.
4	The rats fed on cake + water extract of propolis.
5	The rats fed on cake + ethanol extract of propolis.
6	The rats fed on cake + aflatoxins.
7	The rats fed on cake + aflatoxins + crude propolis.
8	The rats fed on cake + aflatoxins + water extract of propolis.
9	The rats fed on cake + aflatoxins + ethanol extract of propolis.

Table2. Coliform and Bifidocount (CFU/g faecal homogenate) in samples obtained from Secum.

Groups	Bifidobacterium	Coliform group
1	50×10^4	39×10^3
2	77×10^4	10×10^3
3	10×10^6	10×10^3
4	10×10^6	39×10^4
5	30×10^6	10×10^2
6	10×10^2	62×10^4
7	20×10^6	40×10^4
8	10×10^6	48×10^4
9	30×10^6	33×10^4

Table 3. Coliform and Bifidocount (CFU/g faecal homogenate) in feces.

Group	Bifidobactereum	Coliform group
1	24×10^5	14×10^5
2	30×10^6	88×10^3
3	30×10^6	10×10^2
4	27×10^6	14×10^3
5	33×10^7	10×10^3
6	60×10^2	62×10^5
7	18×10^6	25×10^4
8	50×10^5	84×10^3
9	32×10^5	33×10^4

Table 4. Gain in body weight (g) and Food Efficiency Ratio of control rats and those fed on diets contaminated with aflatoxins and supplemented with propolis.

Rat No.	Group 1	Group 2	Group 3	Group 4	Group 5	Group 6	Group 7	Group 8	Group 9
Gain in body weight									
Mean	**170.5**	**194.8**	**177.5**	**214.0**	**169.2**	**122.7**	**159.2**	**183.3**	**163.5**
SE	8.45	8.80	6.24	14.87	7.77	6.64	9.93	16.43	7.00
Pa<		0.098	0.234	0.189	0.081	0.000	0.209	0.038	0.695
Pb<							0.015	0.000	0.007
Food Efficiency Ratio									
Mean	0.25	0.33	0.31	0.36	0.30	0.22	0.28	0.31	0.28
SE	1.32	1.51	1.43	2.93	1.42	1.50	1.65	2.87	1.46
Pa<		0.006	0.50	0.241	0.489	0.000	0.244	0.103	0.437
Pb<							0.045	0.001	0.048

Table 5. Liver, Kidney and spleen weights (g) of control rats and those fed on diets contaminated with aflatoxins and supplemented with propolis.

Rat No.	Group 1	Group 2	Group 3	Group 4	Group 5	Group 6	Group 7	Group 8	Group 9
Liver									
Mean	**7.75**	**7.70**	**6.40**	**5.68**	**5.90**	**5.57**	**6.10**	**5.88**	**6.00**
SE	0.321	0.511	0.256	0.865	0.167	0.548	0.192	0.227	0.337
Pa<		0.936	0.040	0.002	0.005	0.001	0.628	0.747	0.872
Pb<							0.391	0.610	0.485
Kidney									
Mean	**1.617**	**1.617**	**1.467**	**1.883**	**1.483**	**1.283**	**1.350**	**1.317**	**1.483**
SE	0.075	0.098	0.067	0.471	0.024	0.060	0.034	0.031	0.154
Pa<		1.000	0.545	0.284	0.590	0182	0.638	0.026	1.000
Pb<							0.788	0.893	0.420
Spleen									
Mean	**0.917**	**0.633**	**0.633**	**0.450**	**0.533**	**0.500**	**0.400**	**0.517**	**0.483**
SE	0.120	0.076	0.076	0.096	0.033	0.089	0.037	0.060	0.098
Pa<		0.017	1.000	0.115	0.386	0.249	0.047	0.562	0.664
Pb<							0.386	0.885	0.885
Liver /body weight Ratio									
Mean	**3.34**	**3.99**	**3.19**	**3.23**	**3.20**	**3.44**	**3.34**	**3.20**	**3.18**
SE	0.194	0.136	0.264	0.609	0.070	0.211	0.143	0.131	0.103
Pa<		0.079	0.032	0.042	0.035	0.135	0.678	0.938	0.949
Pb<							0.788	0.518	0.478

Table 6. Serum AST,ALT and GT Activities (U/ml) of control rats and those fed on diets contaminated with aflatoxins and supplemented with propolis.

Rat No.	Group 1	Group 2	Group 3	Group 4	Group 5	Group 6	Group 7	Group 8	Group 9
AST									
Mean	92.95	87.34	80.38	77.73	76.49	141.46	97.13	91.25	85.51
SE	5.26	6.61	12.18	12.47	4.97	4.16	5.19	6.86	9.28
Pa<		0.622	0.543	0.401	0.373	0.000	0.146	0.293	0.430
Pb<							0.000	0.000	0.000
ALT									
Mean	**21.17**	**15.72**	**13.83**	**15.68**	**13.35**	**33.86**	**18.66**	**18.30**	**14.89**
SE	2.68	2.04	0.46	2.14	0.55	4.02	2.37	2.36	0.42
Pa<		0.088	0.549	0.990	0.452	0.000	0.130	0.406	0.624
Pb<							0.000	0.000	0.000
GT									
Mean	**8.89**	**8.63**	**8.484**	**8.03**	**8.21**	**13.43**	**8.85**	**8.228**	**8.48**
SE	1.069	0.842	1.171	0.695	0.685	1.047	0.769	0.867	0.700
Pa<		0.837	0.909	0.636	0.739	0.001	0.769	0.876	0.827
Pb<							0.001	0.000	0.000

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