Protection against 2- acetyl aminofluorene-induced toxicity in mice by garlic (*Allium sativum*), bitter kola (*Garcina kola* seed) and honey

OA Odunola*, A Adetutu*, OS Olorunnisola* and O Ola-Davis#.

Cancer Research and Molecular Biology Laboratories, Department of Biochemistry, College of Medicine, and #Department of Veterinary Physiology and Pharmacology, University of Ibadan, Ibadan, Nigeria.

Summary

The effects of honey (Ho) and aqueous suspensions of garlic (*Allium sativum*) (Ga) and bitter kola (*Garcina kola* seed) (Bi) on the toxicities induced by 2-acetylaminofluorene (2-AAF) a model carcinogen, were investigated in mice. The animals were dosed for seven consecutive days with Ho, Ga and Bi as dietary supplements. They were then challenged with a single intraperitoneal (i.p.) dose of 2-AAF at 50mg/kg bd. wt on the seventh day. The degree of clastogenicity was assessed using the mouse micronucleus assay while liver damage was monitored by measuring the level of gamma glutamyltransferase (γ-GT) in serum and liver homogenates respectively. The results revealed that 2-AAF induced micronuclei formation in the polychromatic erythrocytes (PCEs) of the bone marrow by about five fold in comparison to the PCEs formed in control mice. Ho, Ga, and Bi also induced micronucleus formation on their own. However, feeding of any of Ho, Ga or Bi and the administration (i.p) of 2-AAF reduced significantly, the ability of 2-AAF to induce micronuclei formation in the order Ho>Ga>Bi. Furthermore, 2-AAF induced γ-GT activity in the serum and liver homogenate by about two and a half and three folds respectively. A combination of 2-AAF and any of Ga or Bi or Ho significantly decreased 2-AAF-induced activity of γ-GT in the order Ho>Bi>Ga (serum) and Bi>Ga=Ho (liver). These findings suggest that honey, garlic and bitter kola protect against 2-AAF-induced γ-GTactivity and micronuleated PCEs formation.

Keywords: *2-acetyl aminofluorene, bitter kola, honey, garlic, clastogenicity.*

Résumé

Les effects du miel(Ho) et des suspensions acqueuse de l'aile(*Allium sativum*)(Ga) et le bitter kola (*Garcina Kola seed*)(Bi) sur les toxicités induite par un modéle de carcinogéne: acétylaminofluorene(2-AAF) étaient investigués chez des souris. Les animaux recevaient pendant 7 jours consécutifs du Ho,Ga et Bi comme supplément diététiques. Ensuite, ils recevaient au 7 iéme jour une dose de 2-AAF intapéritonéale(i.p) de 50 mg/kg. Le dégree de clastogénecité était évalué en utilisant la technique micronucleaire au souris alorsque la destruction du foie était surveillée en mésurant le taux du gamma – gultamyltransferase(γ-GT) en sérum et l'homogenéité du foie respectivement.Les résultats revélaient que le 2-AAF induicait des formation micronucleaire dans les erythrocytes polychromatique de la moélle épiniére 5 fois de plus comparé au groupe de controle. Ho, Ga et Bi induicaient également des formations de leur part. Cependant la nutrition d un des supplements et l' administration(i.p) du 2AAF reduicaient significativement l'abilité du 2-AAF d induire cette synthése dans l'ordre du Ho>GA>Bi. En plus, le 2-AAF induicait l' activité du γ-GT dans le serum et l'homogeneite du foie de 2.5 et 3 fois respectivement. La combinaison du 2-AAF et l'un des Ho, GA ou Bi réduicait significativement l'activité du 2-AAF a induire le γ_GT dans l ordre Ho>Bi>Ga (sérum) et Bi>Ga= Ho(foie). Ces données suggérent que le meil, l'aile et le bitter kola protége contre l'activité induite du 2-AAF au γ –GT et la formation des erythrocytes nucleuées et polychromatiques.

Introduction

In addition to exposure to several naturally occurring toxicants resulting from the indiscriminate use of products of herbal medicine, the majority of the people in developing countries of the world are at a high risk of exposure to diverse environmental contaminants [1]. Environmental hazards arising from the ingestion of substances such as mutagens, clastogens and carcinogens occurring as toxicants in drinking water, various foods and food additives are therefore common. For instance, exposure to cigarette smoke, emissions from hazardous waste sites, nonferrous smelters, insecticide and pesticide manufacturing or from consumption of contaminated water [2-4] could lead to the inhalation or ingestion of toxic substances such as polycyclic aromatic hydrocarbons, inorganic arsenic and 2-acetylaminofluorene (2-AAF). Epidemiological evidences also exist associating these exposures to the etiology of human cancer [2-5].

Although the carcinogenicity of 2-AAF in different species of animals is well documented,it is also a suspected human carcinogen due to the fact that human hepatic cells do metabolise it to highly reactive genotoxic metabolites [6,7]. In fact, this aromatic amine is commonly used as a model in experimental liver carcinogenesis studies [8]. In

Correspondence: Dr. Oyeronke A. Odunola, Cancer Research and Molecular Biology Laboratories, Department of Biochemistry, College of Medicine, University of Ibadan, Ibadan, Nigeria. Email: ronodunola@yahoo.com

addition, 2-AAF also induces acute cellular toxicities which include enhancement of production of active oxygen species, [9], immunosuppressive effects [10]and direct inhibition of cell proliferation through interactions with the intracellular signal transduction pathway [11].

Currently, research activities are being directed towards means of minimizing exposure to these agents and searching for substances especially in foods that can block or interfere with their toxicity. In this connection, it has been shown that consumption of certain food(s) may protect against some forms of human cancer [12]. For example, the consumption of garlic (*Allium sativum*) popularly known for its antibiotic, antihelmitic, fungicidal and antithrombic properties [13] has been associated with the reduction of the incidence of breast and rectal cancers in man [14,15]. Other natural food products such as honey is widely used for its antiseptic, decongesting and regenerating properties in wound dressing and ulcer treatment [16-18] while bitter kola (*Garcina kola* seed) is used for the treatment and prevention of dysentery, bronchitis and sore throat [19,20].

In the light of the foregoing, the present study was undertaken to assess the possible preventive effects of honey and the extracts of bitter kola and garlic on the toxicity of 2-AAF.

Materials and methods

Experimental animals

Male albino mice (*Mus musculus*) littermates were bred at the Experimental Animal House of the Department of Biochemistry, University of Ibadan, Nigeria. The mice, 12 weeks old, weighing approximately 22g were kept, five per cage, and were fed pellets (obtained from Ladokun Livestock Feeds Limited, Ibadan, Nigeria) and water *ad libitum* at room temperature 29± 2°C with 12 hours light/dark cycle.

Chemicals

2-aminoacetylfluorene (2-AAF) obtained from Sigma Chemical Co. (St. Louis Mo.) was dissolved in glass-distilled water. Gamma glutamyltransferase reagent kit was also purchased from Sigma Chemical Co. Ltd. St Louis Mo., U.S.A. Giemsa and May-Grunwald stains were obtained from Aldrich Chemical Co.,Inc. Mil. Wisconsin, U.S.A. All other reagents were of the highest purity grade and were purchased from the British Drug Houses Ltd. Poole, England or Hopkin and Williams Essex, U.K.

Preparation of test substances

Garlic bulbs (*Allium sativum*; single clove variety) and bitter kola (*Garcina kola* seed) were purchased from Bodija Market, Ibadan and certified at the herbarium in the Department of Botany and Microbiolgy, University of Ibadan. Pure honey was obtained from the School of Forestry, Eleyele, Ibadan, Oyo-State, Nigeria. Aqueous extract of Garlic (Ga) was prepared from freshly sliced cloves that had been grounded into paste and made up to 2.2 % w/v stock suspension. The suspension was fed to mice by

gavage at a dose of 100 mg/kg body weight corresponding to a daily human intake of 6.0 g garlic by a 60 kg individual. This dose also corresponds to the highest concentration of garlic extract that had been used beneficially against certain ailments [15]. Bitter kola extract (Bi), was also prepared from freshly peeled nuts grounded, air dried for forty-eight hours and powdered. The preparation and administration were as described for garlic extract. Honey (Ho) was fed as purchased at 100 mg/kg body weight of mice.

Experimental protocol

The mice were divided into eight different groups of five mice each. All experimental animals had access to pellets and water *ad libitum* throughout the duration of the experiment, in addition to distilled water and/or test suspensions. The mice in group A were fed with distilled water for seven consecutive days. Those in group B had distilled water for seven days and on the seventh day they were given a single intraperitoneal dose of 2-AAF (50mg/kg body weight). The mice in groups C, D and E were separately fed by gavage with aqueous extract of garlic (Ga) and bitter kola (Bi) and the pure honey (Ho) each at 100 mg/kg respectively, for seven days. The animals in the remaining three (3) groups F, G, H were separately fed with aqueous suspensions of Ga, Bi, and honey respectively, for seven days and on the seventh day they were given a single intraperitoneal dose of 2-AAF (50 mg/kg body weight). Twenty-four hours after the last feeding of the extracts, honey and/or 2-AAF injection, the mice were bled and sacrificed by cervical dislocation.

Micronucleus assay

The assay was performed following the procedure of Heddle and Salamone [21] and as modified by Heddle *et. al.* [22]. Immediately, after sacrifice, the femurs were removed by cutting through the pelvic bones and below the knee. Bones were freed from the muscles and knee. All the surrounding tissues were separated from the shaft in the epiphyseal plate leaving the marrow cavity closed. A needle was inserted into the proximal part of the marrow canal and the marrow was flushed out by gentle aspiration and flushing with fetal calf serum in the syringe. The cell suspension was centrifuged at 1,000 rpm for 5 min. The supernatant was removed and the viscous sediment saved for use. Slides were prepared by smearing the viscous sediment as a thin film on microscope slides. This was followed by fixation in glacial acetic acid-ethanol (1:?,v/v), air drying and pretreatment in undiluted and diluted May- Gruenwald solution for 3 min and 2 min, respectively. The slides were then stained in Giemsa solution. The stained slides were coded and scored under a direct light compound microscope (Leitz Wetzlar model) with the aid of a tally counter for the presence of micronucleated polychromatic erythrocytes.

γ-glutamyltransferase assay

γ-Glutamyltransferase (γ-GT) was assayed by the method of Szasź [23] using the γ-GT diagnostic reagent kit (Sigma

Chemical Co., St Louis, Mo). The reagent was reconsti-tuted by mixing glycylglycine (62 nmol/L) and Tris (95 mmol/L; pH 8.1) together with the substrate L-G-glutamyl-p-nitoanilide (2 mmol/L).

Serum was prepared by allowing the blood samples to clot at room temperature for two hours and at 4 °C over-night for clot contraction. Serum was separated from the clot and saved. The liver was asceptcally removed, rinsed thoroughly in saline at 4°C and then homogenised in a volume of saline three times the weight of the liver. The homogenate was centrifuged at 10,000 x g for 1h while the supernatant so obtained was further centrifuged at 109,000 x g for 10 min. The supernatant was used for γ-GT determi-nation.

γ-GT activity was determined by mixing 1 ml of the reconstituted γ-GT reagent at 30°C with 50 μl of the serum sample or the liver homogenate fraction. The mixture was incubated at 30°C. The initial absorbance was read after 30 seconds at 405nm and subsequently at 1 min interval for 3 mins. γ-GT activity was then calculated and expressed in International Units per liter as described in [23]. The data was statistically analysed using the student t-test.

Table 1: Number of micronucleated polychromatic eryth-rocytes (mPCEs)/1000 PCEs in mouse bone marrow after administration of 2-AAF, extracts and/or honey.* .

Group	Treatment	Number of Micro-nucleated PCEs
A	Distilled Water	7.50 ± 0.89
B	2-AAF only	37.00 ± 5.57
C	Garlic only	20.00 ± 2.96
D	Bitter Kola only	12.50 ± 2.07
E	Honey only	11.25 ± 1.01
F	Garlic + 2-AAF	21.25 ± 3.15
G	Bitter Kola + 2-AAF	26.25 ± 4.01
H	Honey + 2-AAF	18.75 ± 2.08

*Each value is a mean of at least ten different determinations ± standard error. (P<0.05).

Results

The number of micronucleated polychromatic erythrocytes (mPCEs) in mouse bone marrow after administration of 2-acetylaminofluorene (2-AAF), garlic and bitter kola and / or honey are shown in Table 1. From the data, it is evident that 2-AAF significantly (P<0.05) induced micronucleus formation in PCEs by about five folds when compared to the PCEs formed in the negative control mice fed distilled water only. Garlic and bitter kola (Ga;Bi) and honey (Ho) also induced micronucleus formation in the PCEs in the order Ga > Bi> Ho. In this regard, the effect of Ga only was about three- folds while Bi and Ho induced PCEs forma-

tion by about 30% each. Interestingly, the combination of the intraperitoneal (i.p) administration of 2-AAF and the oral administration (p.o.) of any of honey and suspen-sions of either garlic (Ga) or bitter kola (Bi) reduced the potency of 2-AAF to induce micronucleus formation in the order Ho > Ga > Bi. Specifically, Ho and Ga reduced 2-AAF induced formation of PCEs by about 49% and 43%, respectively while Bi diminished the effect of 2-AAF by 29%.

Table 2: γ-Glutamyltransferase activity in the liver homo-genate and serum of experimental mice.*

Group	Treatment	γ-GT activity in the liver homo-genate	γ-GT activity in the serum
A	Distilled Water	4.24 ± 0.20	4.00 ± 0.49
B	2-AAF only	12.72 ± 0.86	10.59 ± 0.99
C	Garlic only	6.00 ± 0.54	4.29 ± 0.65
D	Bitter Kola only	6.08 ± 0.26	6.10 ± 0.91
E	Honey only	4.95 ± 0.15	4.24 ± 0.20
F	Garlic + 2-AAF	9.34 ± 0.76	7.07 ± 0.82
G	Bitter Kola + 2-AAF	8.84 ± 0.67	5.59 ± 0.61
H	Honey + 2-AAF	7.06 ± 0.52	7.05 ± 0.82

*Each value is a mean of at least ten different determinations ± standard error. (P<0.05).

Table 2 shows the activity of gamma glutamyltransferase in the liver homogenate and serum of animals previously exposed to 2-AAF only or in combina-tion with any of Ho, Ga and Bi. The results show clearly that 2-AAF when intraperitonealy administered induced gamma glutamyltransferase activity by about three folds in liver homogenate and by two and a half folds in serum. Although, the adminisration of Ga only induced the activ-ity of gamma glutamyltransferase by about 45% in the liver, the activity of the serum enzyme was not affected by this food condiment. Similarly the administration of Bi only resulted in increases in the activity of gamma glutamyltransferase by about 45% in both liver and serum.In contrast, the administration of Ho only did not yeild any appreciable effect on the activity of gamma glutamyltransferase in liver homogenate and serum. A com-bination of the administration of 2-AAF and Ga or Bi or Ho significantly (P<0.05) decreased the effect of 2- AAF on the induction of the activity of gamma glutamyltransferase in liver homogenate and serum. In this regard, Ga, Bi and Ho reduced the effect of 2-AAF by 27%, 31%, and 45% respectively. Moreover, the effect of 2-AAF on the serum enzyme was reduced by the adminisration of Ga, Bi and Ho by 33%, 47% and 33% respectively.

The histogram shown in Fig.1 is a representation of the comparative effects of Ga, Bi and Ho on the toxicity of 2-AAF. Although Ga, Bi and Ho elicited some effect on

the PCEs, they effectively protected the animals against the toxic effect of 2-AAF; the protection being greatest with Ho (Fig.1). Similar patterns were seen in the effect of the test compounds on 2-AAF induced increases in the activity of gamma glutamyltransferase in liver homogenate, again Ho protected the animals most effectively against hepatotoxic effect of 2-AAF. In contrast the effect of 2-AAF on the serum gamma glutamyltransferase was highly diminished in the presence of Ga while Ho had the least effect.

at this concentration, 2-AAF induced certain specific toxic effects in the liver as shown by the high activity of the liver gamma glutamyltransferase (γ-GT) 24 hours after its administration as compared to control animals. (Table 2, Fig. 2). Several studies have shown that liver damage is usually followed by an increase in the activity of the serum γ-GT arising from an increase in the rate of synthesis and leakage of the enzyme from the liver. [31,32]. The serum level of this enzyme was significantly ($P<0.05$) increased 24 hours after the administration of the insecticide
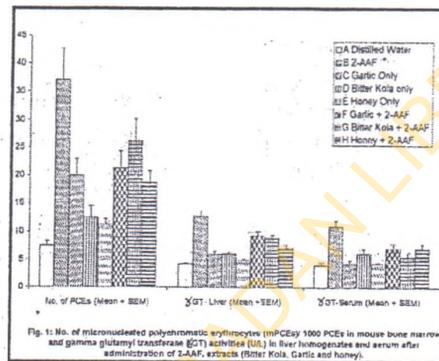

Fig. 1: *No. of micronucleated polychromatic erythrocytes (mPCEs)/1000 PCEs in mouse bone marrow and gamma glutamyl transferase (γGT) activities (U/L) in liver homogenates and serum after administration of 2-AAF, extracts (bitter kola, garlic and honey.*

Discussion
2-Acetylaminofluorene has been used as a model carcinogen in both *in vitro* and *in vivo* assay systems [8,24,25] This compound can act as a direct carcinogen via the generation of electrophilic derivatives which react with DNA to form mutagenic adducts [26] or as a promoter [27] It has been suggested that acute cellular toxicity of 2-AAF which includes inhibition of cell growth and cell death without triggering an apoptotic process, may contribute to its property as a promoter of carcinogenenesis [28]. In the light of the above and given the fact that the molecular basis of 2-AAF induced acute toxicities remains to be fully understood, and more importantly that the insecticide is still widely used in certain developing countries of the world, there is the need to determine whether certain naturally occurring products could protect against the toxic effects of the insecticide.

The results obtained from the present study show that 2-AAF induced micronucleus formation in the polychromatic erythrocytes of the bone marrow cells of mice 24 hours after administration at 50 mg/kg body weight (Table 1, Fig. 1). This is an indication of *in vivo* chromosomal breakage, which is easily detectable using the mouse micronucleus assay [29,30]. Furthermore,

to experimental animals. Taken together, these results may be due to 2-AAF induced formation of cellular reactive oxygen species in cells [9].

The results of preliminary investigation on the clastogenic potentials of the aqueous suspensions (i.e. Ga and Bi) and honey (Ho) show evidence of mild clastogenic activity of the test substances in the order Ga>>Bi>Ho when compared with the negative control group of animals that had only distilled water (Table 1, Fig. 1). In this regard, the clastogenic potentials of bitter kola (Bi) and Honey (Ho) are not markedly different from one another. The results obtained in this study that garlic exhibits the highest clastogenic potential is supported by previous observation that crude extracts of garlic have some degree of clastogenicity in mouse bone marrow cells [33]. An assessment of the ability of the suspensions (Ga and Bi) and Ho to induce liver damage suggested that honey seem not to induce γ-GT synthesis in the liver and serum when compared to the liver and serum γ-GT activity in the negative control group (Table 2, Fig. 2). This suggests that honey does not have any toxic effect at the dosage of 100mg/kg body weight (w/w) on mice. In contrast, garlic and bitter kola have a somewhat very mild ability to induce liver and serum γ-GT activity when compared to the negative control group.

Pre-treatment of the mice by feeding of the crude suspensions (Ga and Bi) and honey (Ho) for seven days before exposure to 2-AAF markedly reduce the population of micronuclei in the PCEs. This is an indication of a decrease in chromosomal damage when compared to the group of animals treated with 2-AAF alone (Table 1, Fig 1). The degree of reduction of 2-AAF induced micronucleus formation by Ga, Bi and Ho is in the order Ho>Ga>Bi. Similarly, 2-AAF induced-synthesis of γ-GT in the liver and serum was reduced by pre-treatment with Ga and Bi and Ho. In this regard, the degree of reduction of 2-AAF induced toxicity is in the order Ho> Bi > Ga for the liver γ-GT activity and Bi > Ho and Ga for the serum γ-GT activity. The reduction of 2-AAF induced clastogenic effects and γ-GT activity in serum by the crude suspensions (Ga and Bi) and honey (Ho) may be attributed to the antioxidant properties of their components. This suggestion is supported by the fact that allicin (thio-2-propene-1-sulfonic acid-S-ally ester) an enzymatic degradative product of allin (S-ally-L-cysteine sulfoxide) present in garlic has been shown to have a great potential in the inactivation of active oxygen species [34]. Indeed, most of the biochemical properties of garlic have also been attributed to allicin [35]. Similarly, the antioxidant properties of a bitter kola extract, kolaviron (a mixture of Garcina biflavonoids 1 and 2 and kolaflavonone) have been documented [36]. Although, there is a dearth of information on the clastogenic and antihepatotoxic effects of honey, its antioxidant properties may also account for its ability to protect against 2-AAF- induced -micronucleus formation and -γ-GT activity. In this regard, further work will be carried out to investigate the antioxidant, anticlastogenic and possible anticarcinogenic potentials of honey.

By simple extrapolation it seems likely that ingestion of garlic, bitter kola and honey may have great potentials in arresting 2-AAF induced toxicities and protect against promotion of carcinogenesis in humans.

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