Caffeine, quercetin and alizarin stimulate the exhalation of metabolic products of [14C]-N-nitrosodiethylamine in mice

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Naturally occurring plant products belonging to different chemical classes namely alizarin, an anthraquinone, caffeine, a methylxanthine derivative and quercetin, a flavonol were studied for their effect on elimination of metabolites of [14C]-N-nitrosodiethylamine (14C-NDEA) through respiration in mice. Treatment with caffeine, quercetin and alizarin at doses of 200, 9 and 9 Jg/ml respectively, in drinking water enhanced the exhalation of 14CO2, one of the major end products of NDEA metabolism. Radioactive CO2 exhaled in 60 min increased by 2, 1.61 and 1.4-folds in animals treated with caffeine, quercetin and alizarin for 8 weeks respectively. This increase in exhalation in caffeine-treated animals was achieved even in 2 weeks. These compounds had no adverse effects on the absorption of radioactive NDEA from the gut of the animals as shape and time of 14CO2 peak was similar in ip and orally administered [14C-NDEA]. Increased detoxification/elimination of the carcinogen could be one of the mechanisms for the anticarcinogenic properties of these phytochemicals in lung tumorigenesis induced by orally administered NDEA.

Marked international variations in the number of deaths from cancer may be attributed to different environmental factors, life style and nutritional habits. Therefore, nutritional oncologists these days are trying to identify the naturally occurring dietary factors, which are/or may be anticarcinogens. The identification and characterization of these anticarcinogens in the diet may lead to new strategies for reducing the risk of human cancer. Any compound that can block the metabolic activation step, scavenge the reactive intermediates or enhance detoxification would be a potential chemopreventive agent1-4. Human diet contains a complex mixture of phenols and estimated individual dietary consumption is as much as 1g of plant phenols per day5. Many of these plant phenolics have been reported to exhibit chemopreventive properties against neoplasm. The two main classes of plant phenols are the plant pigments, flavonol and anthraquinone. These phenolics, which are widely distributed in plant kingdom, seem to be cancer-preventive substances because of their strong antioxidative activity and free radical scavenging potency6-8. All flavonoids may not be necessarily equally protective against all cancers or other chronic diseases and may also not be equally effective in modifying the mechanistic factors. Three dietary compounds, i.e. caffeine, quercetin and alizarin, which are widely distributed in plant kingdom, were selected for mechanistic studies related to anticarcinogenic potential of any agent.

Caffeine belongs to a group of compounds known as methylxanthine and has been proved non-carcinogenic in animal models9-11. It has also been reported to antagonize the carcinogenic effects of chemicals in vitro12. Under in vivo conditions, caffeine has been reported to enhance or inhibit tumorigenesis induced by various carcinogenic agents13,14. Quercetin, another test compound in the present study is one of the most common flavonol and widely distributed in human diet. It has been found to be non-mutagenic and lack DNA-damaging activity15. Similarly, alizarin is also a naturally occurring plant phenol with 1.2-OH phenolic anthraquinone and is known to possess radical scavenging capacity16 and antiviral activity17. Although, carcinogenicity has been reported for anthraquinones substituted with amino or nitro group, there is no carcinogenicity data on phenolic anthraquinones.

The metabolic activation of procarcinogens or generation of reactive intermediates of complete carcinogens after metabolism is a key step in the process of carcinogenesis. Thus, the present study has been designed to evaluate the effect of feeding these dietary compounds, capable of inhibiting NDEA-induced lung
tumors in mice\textsuperscript{18,19}, on the metabolism of N-nitrosodiethylamine. For this, exhalation of $^{14}$CO$_2$, one of the major end products of NDEA metabolism was taken as an index.

**Materials and Methods**

2,5-Diphenyloxazole (PPO), 1,4-bis-2(5-phenyloxazole)-2,5-diphenyloxazole (POPOP), quercetin, alizarin, caffeine and N-nitrosodiethylamine (NDEA) were procured from Sigma Chemical Company USA. [$^{14}$C]-N-Nitrosodiethylamine (specific activity 57 mCi/mmol) was purchased from Amersham, UK. All other reagents of analytical grade were purchased locally.

a. Animal model

All experiments were conducted on Swiss NMRI mice weighing 20-25 g. Mice were housed in plastic cages bedded with rice husk and were supplied pellet diet and water ad libitum. Changes in body weights during experimental period were recorded weekly. The record for the average daily water consumption of animals was maintained throughout the study.

b. Radiorespirometric studies

Animals were divided into four groups, of six animals each. Animals were fed either with plain drinking water, caffeine (200 \( \mu \)g/ml), quercetin (9 \( \mu \)g/ml) or alizarin (9 \( \mu \)g/ml) through drinking water in respective groups. The group on plain drinking water acted as a control to check if there were any changes in the metabolism of NDEA due to ageing (during the experimental period). Drinking water containing the test compound was prepared fresh daily. In this study, each animal acted as its own control and test. The study at 0 week represents the experiment conducted a day before the start of treatment of the mice with the test compound. Each animal was intraperitoneally (ip) administered [$^{14}$C]-N-nitrosodiethylamine at a dose of 0.2 \( \mu \)Ci/2 ml saline/100g body weight (2 \( \mu \)Ci/\( \mu \)mol) and was immediately put (one animal at a time) in an airtight perspex chamber having an inlet and outlet for air. A steady flow of 80 ml/min was maintained in the chamber with the help of a pump throughout the experiment. The expired air was passed through a CaCl$_2$ column in order to remove water vapours and thereafter, trapped in test tubes arranged in series, and containing 1 ml of mixture of the trapping reagent consisting of ethanolamine:ethyl glycol monomethylether: Triton X-100: (3:1:2 v/v/v). To each test tube 0.125 ml of saturated solution of NaOH was also added and the contents were thoroughly mixed. Trapping reagent was changed after every 10 min for 90 min. The spent agent was decanted into scintillation counting vials, containing 10 ml scintillation fluid. Radioactivity of $^{14}$CO$_2$ was measured in a liquid scintillation counter (LKB) with in-built CPM to DPM converter.

The experiments were repeated 2, 4, 6 and 8 weeks after the start of treatment of animals with the test compounds. The results were analysed in terms of appearance of peak exhalation of $^{14}$CO$_2$ and total $^{14}$CO$_2$ exhaled during the period of radiorespirometric assay.

For evaluation of effects, if any, of these test compounds on absorption of N-nitrosodiethylamine from gastrointestinal tract, an oral dose of 0.05 \( \mu \)Ci [$^{14}$C]-NDEA/0.5 ml was administered by stomach tube. Rest of the procedure was the same as described for ip administration of the carcinogen. For statistical analysis, Student's paired $t$ test was used for the values (mean \( \pm \) SD).

**Results**

The consumption of water by control and animals treated with quercetin and alizarin varied between 5 and 7 ml/animal/day, whereas, in animals kept on caffeine treatment, the intake of caffeine treated water was found to be 10 ml (average). The average amount of caffeine, quercetin and alizarin consumed by each animal per day was found to be 2.03 mg, 55.7 \( \mu \)g and 56.5 \( \mu \)g respectively.

**Influence of caffeine, quercetin and alizarin on [$^{14}$C]-N-nitrosodiethylamine metabolism after its ip administration**—Radiorespirometric studies performed after ip administration of [$^{14}$C]-labelled NDEA at a dose of 0.2 \( \mu \)Ci/100 g body weight revealed that the peak exhalation of $^{14}$CO$_2$ was achieved between 11-20 min., and thereafter, a slow decline in $^{14}$CO$_2$ formation was observed up to 60 min. In the control group there was overall no change in the $^{14}$CO$_2$ exhaled during 8 weeks of experimental period (unpublished data). The treatment of animals with caffeine, quercetin and alizarin at doses of 200, 9 and 9 \( \mu \)g/ml drinking water for 8 weeks enhanced the metabolism of [$^{14}$C]-NDEA (Figs 1-3). It was observed that caffeine, quercetin and alizarin significantly ($P<0.05$) increased the levels (% of the administered activity) of exhaled $^{14}$CO$_2$ after 2, 4, 6 and 8 weeks respectively (Table 1). It is evident that caffeine treatment for 2 weeks increased $^{14}$CO$_2$ exhalation to almost 2-fold. Further feeding of caffeine up to 8 weeks led to a non-significant increase in $^{14}$CO$_2$
exhalation. On the other hand, feeding of quercetin and alizarin led to a continuous enhancement in $^{14}$CO$_2$ formation up to 8 weeks of feeding. The increase in $^{14}$CO$_2$ at the end point (8 weeks) of the experiment was found to be 161% and 140% in quercetin and alizarin treated animals respectively.

The enhancement in $^{14}$CO$_2$ formation in the individual animals at different time intervals of treatment with the test compound was calculated. The average increase at 2, 4, 6 and 8 weeks was 2.06, 2.0, 2.05 and 2.29-fold in caffeine treated group; 1.45, 1.76, 2.16 and 2.66-fold in quercetin treated group and 1.20, 1.91, 2.16 and 2.41 fold in alizarin treated animals respectively.

Influence of caffeine, quercetin and alizarin on $[^{14}C]$-N-nitrosodiethylamine metabolism and absorption from the gastrointestinal tract—Intragastric administration of $[^{14}C]$-NDEA produced the similar shape of $^{14}$CO$_2$ exhalation curve as was observed in ip administered $[^{14}C]$-NDEA. The peak exhalation of $^{14}$CO$_2$ occurred between 11-20 min. Treatment of animals for 8 weeks with caffeine, quercetin and alizarin at doses of 200, 9 and 9 μg/ml drinking water respectively enhanced the metabolism of $[^{14}C]$-NDEA in a similar fashion as was observed in ip administered NDEA. Total (mean±SD) amount (% of $^{14}$CNDEA administered) of $^{14}$CO$_2$ exhaled in 90 min increased from 15.4±1.94 to 34.4±5.36 from 15.0±1.77 to 35.7±4.56 and from 13.8±0.88 to 31.3±5.31 after 8 weeks of caffeine, quercetin and alizarin feeding respectively (Fig. 4).

**Discussion**

Caffeine and quercetin have been found to have anticarcinogenic effects against N-nitrosamines, polycyclic aromatic hydrocarbons and other carcinogens

![Graph](image-url)

Fig. 1—Effect of caffeine treatment at a dose of 200 μg/ml for various time periods on exhalation of $^{14}$CO$_2$ after ip administration of $[^{14}C]$-NDEA. Values are the average of six animals.

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<tr>
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Table 1—Effect of caffeine, quercetin and alizarin feeding for different time periods on exhalation of $^{14}$CO$_2$ (% of administered radioactive NDEA)

[Values are mean ± SD of 6 animals]
responsible for induction of tumors in animals. For example, lung tumorigenesis induced by NDEA could be inhibited by prefeeding of quercetin and alizarin. Similarly, caffeine when given in drinking water at a concentration identical to that found in 2% tea was able to inhibit lung tumours induced by 4-(methylnitrosoamino)-1-(3-pyridyl)-1-butanone (NNK).

Moreover, caffeine inhibited hepatocarcinogenesis induced by 2-acetylaminofluorene. The mechanisms for protection against carcinogens including NDEA-induced lung carcinogenesis could be multiple. It is well known that NDEA requires metabolic activation to form its reactive intermediates, cation radicals. The other mode of activation involves peroxidative

![Figure 2](image1.png)

Fig. 2—Effect of quercetin treatment at a dose of 9 μg/ml for various time periods on exhalation of $^{14}$CO$_2$ after ip administration of $[^{14}C]$:NDEA. Values are the average of six animals.

![Figure 3](image2.png)

Fig. 3—Effect of alizarin treatment at a dose of 9 μg/ml for various time periods on exhalation of $^{14}$CO$_2$ after ip administration of $[^{14}C]$:NDEA. Values are the average of six animals.
cooxygenation by either prostaglandin synthetase or lipid peroxidation, i.e., via a free radical mediated nonenzymatic process. Besides these pharmacological and toxicological effects, xenobiotics are affected markedly by their absorption, distribution, excretion and binding with macromolecules. The potency and organ specificity of procarcinogens to a great extent are determined by the chemical reactivity and the availability of sufficient amounts of ultimate carcinogenic forms in the cells of target tissues. The amount of ultimate carcinogen, in turn, is a function of activities of metabolic pathways and also of the biological half lives of all of the metabolic species involved. Most of the chemopreventive strategies have been based on modification of metabolism at one or many steps such that these agents can block the metabolic activation steps, scavenge the reactive intermediates or enhance the detoxification.

The present study clearly demonstrates that the anticarcinogenic compounds tested in this study did not affect the absorption of radioactive NDEA from gastrointestinal tract, as the peak exhalation time of 14CO2 was similar to that of control animals. On the other hand, exhalation of end product (CO2) of NDEA was significantly increased due to treatment with the test compounds, which clearly indicates the increased metabolic detoxification of NDEA. Earlier studies have shown that caffeine is an inducer of hepatic and pulmonary Cytochrome(Cyt) P450 and hepatic aryl hydrocarbon hydroxylase activity (AHH) in mice. The increased activity of AHH is important in relation to chemical carcinogenesis since this enzyme is responsible for the formation of reactive as well as inactive metabolites of the carcinogen. Studies have shown that it is possible to protect against the process of chemical carcinogenesis by elevation of AHH (hydroxylating enzyme activity) but the elevation should be in such a way that isozymes of Cyt P450 responsible for formation of inactive products of the carcinogen are largely produced. Since, the metabolism of NDEA is through the hydroxylating enzymes, it is likely that the increased exhalation of 14CO2 due to the administration of 14C-NDEA was largely due to the increased levels of Cyt P450 by caffeine feeding. On the other hand, earlier studies have shown that quercetin was unable to change the levels of hepatic and pulmonary AHH and Cyt P450 levels. This was surprising that in spite of no alterations in the drug activating enzymes 14CO2 exhalation was largely increased by quercetin feeding. The possibility could be that under certain conditions phenolic antioxidants can initiate an autooxidative process and behave like prooxidants, which may be involved in the nonenzymatic oxidation of NDEA.

Similarly, we can't rule out the possibility of the increased formation of end products of NDEA by alizarin treatment with the alteration in peroxidative pathway. Induction of xenobiotic detoxifying enzyme is an additional mechanism by which plant products may act as anticarcinogens, since this induction of detoxifying enzymes is capable of competing with steps in xenobiotic activation. Caffeine, quercetin and alizarin (unpublished results) have been found to increase GST and reduced glutathione in liver and lungs of mouse. Another possible mechanism of action of the polyphenols (quercetin and alizarin) used in this study is that these polyphenols may bind to DNA at the carcinogen binding site effecting carcinogen-DNA binding, a crucial step for initiation of carcinogenesis. Alternatively, when the phenolics bind to DNA, its molecules could be positioned in such a way so as to effectively scavenge reactive intermediates (cation radical) that approach the critical sites in DNA or phenolics may directly interact with the ultimate reactive metabolite of the carcinogen by donating their electrons and rendering it inactive.
In conclusion, increased detoxification/elimination of NDEA in caffeine-, quercetin- and alizarin-treated mice may lead to inhibition in NDEA-induced carcinogenesis.

References