CHAPTER 6

- INHIBITION OF NITROSATIONS IN VITRO
  BY SPICES AND LEAFY VEGETABLES
INTRODUCTION

The consumption of nitrite and dietary nitrogen containing compounds might be an etiological factor in the development of some cancers, particularly of the gastrointestinal tract (1,2). Since the first demonstration by Sander and Burckle (3), that ingested secondary amines and nitrite could react in vivo to produce carcinogenic nitrosamines, increasing attention has been focussed on estimating the extent of human exposure to N-nitroso compounds (NOC) formed in in vivo from both exogenous and endogenous precursors (4).

A review of literature shows the ubiquitous occurrence of precursors of carcinogenic N-nitroso compounds (nitrite, nitrate and biogenic amines). It is found that secondary and tertiary amines react readily with nitrite at pH 3 to 6 to produce nitrosamines (5). This pH range includes that prevalent in the mammalian stomach. The nitrosation reaction that takes place in the stomach depends on factors such as pH of the medium, basicity of the amines, microflora, and presence of catalyst (thiocyanate) and inhibitors (ascorbic acid). Many alkaloids, other natural products and numerous drugs are secondary or tertiary amines. Some of these are ingested in substantial doses by many people often for long periods and could interact with nitrite in the stomach. Many compounds that could become incidental food additives such as surfactants, agricultural
chemicals and many drugs are tertiary amines (6,7). Nitrite, nitrate and nitrosating agents can also be synthesized endogenously by reactions mediated by bacteria and activated macrophages (8,9). As a consequence, endogenous nitrosation may occur at many sites in the body, including nitrosation in the oral cavity, urinary bladder, reaction of nitrogen oxides with amino compounds in the lungs, and at organ sites where there is infection or inflammation (10). NOCs, are a versatile class of carcinogens that produce tumours in 40 animal species (5,11). The NOCs produce reactive electrophiles which can bind to cellular nucleophiles, mainly DNA (Fig. 6.1). The carcinogenic potency of NOCs is thought to mainly rest on their ability to form pro-mutagenic DNA adducts (12).

The daily nitrite burden of a human being is estimated to be 10 mg per person (13). Widely occurring dietary nitrosatable precursors are amines, amides, guanidines and ureas. The diet however also contains many components like ascorbic acid which inhibit the nitrosation reaction (5). Therefore, it is essential to identify new compounds which are capable of inhibiting the nitrosation reaction for prevention of alimentary tract cancers.

Nitroso diethanolamine (NDEA) is a potent environmental carcinogen and has been shown to induce tumours in experimental animals (14). It has also been demonstrated that triethanolamine (TELA, a widely used compound) could undergo in vivo nitrosation under simulated gastric
Figure 6.1: Formation of electrophilic products by spontaneous or enzymatic degradation of N-nitroso compounds
conditions to yield NDEA (15). Moreover NDEA is a direct acting mutagen in the Ames Assay, producing dose-dependent effects (16). Methylurea (MU), an amide, is also formed in the diet from creatinine to the extent of 25 mg/kg (17). Moreover nitroso methylurea (NMU) is a direct acting mutagen in *S. typhimurium* strains (18). The effects of plant products on the nitrosation of triethanolamine and methylurea were investigated. The pre incubation method of *S. typhimurium* mutagenicity test was chosen since it permits an accurate estimation of the increase of histidine revertants, per plate (19).

**MATERIALS AND METHODS**

**Chemicals:** Methylurea (MU), N (1 naphthyl)-ethylene-diamine, dimethyl sulfoxide (DMSO) and sulphanilic acid were purchased from Aldrich Chemical Company U.S.A. Triethanolamine (TELA), nitrosomethylurea (NMU), nitroso-diethanolamine (NDEA) were purchased from Sigma Chemical Company, U.S.A. All other chemicals locally purchased were of ANALAR grade.

**Preparation of Plant products:**

The following plant products were used:- Cumin seeds, poppy seeds, asafoetida, kandathipili, turmeric, basil leaves, drumstick leaves, manathakkali leaves and ponnakanni leaves. The spices were powdered and samples weighing 7.5 mg and 5 mg were suspended in 0.5 ml citrate (0.068 M)-phosphate (0.064 M) buffer pH 3.6, for studying their
effects on the nitrosation of MU. To study their effects on the nitrosation of TELA, the spices (7.5 mg and 5 mg) were suspended in 0.5 ml of distilled water. The leafy vegetables (7.5 mg and 5 mg) were made into pastes with 0.5 ml of citrate-phosphate buffer and 0.5 ml of distilled water to study their effects on nitrosation of MU and TELA respectively.

Nitrosation of methylurea:

Reagents:

1. Methylurea - 25 mM
2. Sodium nitrite - 100 mM
3. Citrate (0.068 M) - disodium hydrogen phosphate (0.064 M) - buffer pH 3.6
4. Sodium bicarbonate - 7.5%
5. Phosphate buffered saline - Prepared with the following composition per litre:
   (i) sodium chloride - 80 g
   (ii) potassium chloride - 2 g
   (iii) dibasic sodium phosphate - 11.5 g and
   (iv) monobasic potassium phosphate - 2 g

Method:

The nitrosation of methylurea was performed according to the method of Stich et al (19). The reaction mixture contained 0.5 ml of methylurea (25 mM) and 0.5 ml of sodium nitrite (100 mM) made upto 2 ml with citrate - phosphate buffer pH 3.6. This mixture was incubated at 30 C for 30 min. The mixture was neutralised by adding 0.7 ml of 7.5 %
sodium bicarbonate. The final volume was adjusted to 3 ml by adding 0.3 ml PBS. To test the modulating effects of plant products on the nitrosation reaction, the suspensions of plant products (5 mg and 7.5 mg in 0.5 ml citrate-phosphate buffer) were added to methylurea just prior to the addition of sodium nitrite and made upto 2 ml with citrate-phosphate buffer.

**Nitrosation of TELA** :

**Reagents** :

(i) Triethanolamine - 25 mM

(ii) Sodium nitrite - 100 mM

(iii) Sodium dihydrogen phosphate - 2.5 M, pH 3.6

(iv) Sodium hydroxide - 1 N

(v) PBS

**Method** :

The nitrosation of TELA was done according to the method of Lijinsky et al. (20). The nitrosation mixture contained 0.5 ml of 25 mM TELA, 0.5 ml of 100 mM sodium nitrite, made up to 2 ml with NaH₂PO₄ (2.5 M, pH 3.6). The reaction mixture was incubated at 37 C for 1 h and then neutralised with 0.5 ml of NaOH. The volume was made up to 3 ml with PBS. To test the modulating effects of plant products on the nitrosation reaction, the spices/leafy vegetable suspensions (in 0.5 ml) were added to triethanolamine just prior to the addition of sodium nitrite and the volume made up to 2 ml with 2.5 M NaH₂PO₄.
The nitrosation mixtures (of both MU and TELA) were centrifuged at 500 g for 10 min and the supernatants collected. The supernatants were sterilised by passing through a disposable 0.45 μM filter unit (Millipore Corporation, U.S.A.) for use in mutagenicity assays. The supernatants were used as such for determination of the nitrosamine or nitrosamide content.

Mutagenicity assay:

The mutagenicities of the nitrosation reaction products were assayed by Ames Assay (21) as modified by Stich et al (19). Logarithmically growing cultures of Salmonella typhimurium TA 1535 (2-3 x 10⁹ cells/ml) were pelleted out from the nutrient broth and were resuspended in the nitrosation reaction mixture (NM). It was incubated at 37 C for 30 min. The bacteria were pelleted and washed in PBS and resuspended in PBS at the original cell concentration. Aliquots were diluted with 0.85% NaCl and plated on minimal agar plates for the estimation of histidine revertants. The plates were incubated at 37 C for 48 h and his revertants determined, for each treatment. The per cent inhibition of mutagenicity of the nitrosation reaction mixture (NM) by plant products was determined as follows:

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\% \text{ Inhibition} = 100 - \frac{\text{No. of rev/plate in absence of plant products}}{\text{No. of rev/plate in presence of plant products}} \times 100
\]

Estimation of NDEA in the nitrosation mixture:

The procedure of Lijinsky et al (20) was followed. The
reaction products were evaporated to dryness in a rotary evaporator. The residue was extracted twice with warm acetone. Acetone was removed by evaporation and the oil dissolved in 10 ml methanol. The nitrosodiethanolamine (NDEA) was then estimated by determining the absorbance at 345 nm. A standard of NDEA was used as the reference.

Estimation of NMU in the nitrosation mixture:

Reagents:

1. Acetate buffer - 0.1 M, pH 4

2. Colour Reagent -

   N-(1-naphthyl)-ethylene diamine 0.5 g and sulphanilic acid 2.5 g were dissolved in 1 litre of aqueous acetic acid (30%, v/v).

3. HCl - 6 M

NMU was extracted from the nitrosation mixture with dichloromethane (2 ml), thrice. This extract was transferred to a separating funnel and extracted thrice with 2 ml of 0.1 M acetate buffer, pH 4. The aqueous extract was then taken for the estimation of NMU according to the method of Preussmann and Schaper-Druckery (22). In this method the nitrosamide is hydrolysed under acidic conditions to liberate nitrite which is then detected by a colorimetric procedure (23). One ml of the solution was combined with 5 ml of the colour reagent and 1 ml of 6 M HCl. The mixture was heated for 45 min at 60 °C and cooled. The absorbance of this solution was read at 550 nm with a reagent blank as reference. The absorbance of this solution was compared
with absorbance of a standard solution of NMU treated in the same way.

Assay of scavenging of nitrite by plant products:

Reaction mixtures containing 0.5 ml of 100 mM of sodium nitrite and 0.5 ml of suspensions of plant products (5 mg and 7.5 mg) were incubated at 30 C for 30 min. The reaction mixtures were centrifuged at 500 g for 10 min and the amount of nitrite in the supernatants were estimated colorimetrically (23). The ability of the plant products to deplete nitrite ions from the reaction mixture were determined.

Statistical analysis:

Students "t" test was used to evaluate the significance of the results (24).

RESULTS

The effects of spices and leafy vegetables on the mutagenicity of the nitrosation mixture (NM) of TELA in Salmonella typhimurium TA 1535 are depicted in figures 6.2 and 6.3, respectively. Cumin seeds, poppy seeds and turmeric significantly (p<0.001) inhibited the mutagenicity of the nitrosation reaction mixture (NM) in a dose-dependent manner, percent inhibition at doses of 5 mg and 7.5 mg being 47 and 69, respectively for cumin seeds, 45 and 57 respectively for poppy seeds and 43 and 60 for turmeric respectively. Asafoetida failed to suppress the mutagenicity of the NM at both the doses studied.
Figure 6.2

Inhibition of the nitrosation of TELA by spices (assessed by the mutagenicity of the NM in *S. typhimurium* TA 1535). Mutagenicity was tested without the addition of S9.

NM - Nitrosation mixture alone

CS - Cumin seeds

PS - Poppy seeds

AF - Asafoetida

KT - Kandathipili

TU - Turmeric
Figure 6.3

Inhibition of the nitrosation of TELA by leafy vegetables (assessed by the mutagenicity of the NM in S.typhimurium TA 1535).

Mutagenicity was tested without the addition of S9.

NM - Nitrosation reaction mixture alone
BL - Basil leaves
DL - Drumstick leaves
ML - Manathakkali leaves
PL - Ponnakanni leaves
FIG. 6.3

Leafy vegetables

- 5 mg
- 7.5 mg
Kandathipili, at a dose of 5 mg /NM significantly suppressed the mutagenicity of the NM (p<0.05). At a higher dose of 7.5 mg kandathipili did not have any effect.

Drumstick leaves and ponnakanni leaves significantly (p< 0.05) suppressed the mutagenicity of the NM in a dose-dependent manner, per cent inhibition at 5 and 7.5 mg/NM being 32 and 46 respectively for drumstick leaves, 28 and 51 respectively for ponnakanni leaves. Manathakkali leaves significantly inhibited the mutagenicity of the nitrosation mixture when added at a dosage of 7.5 mg (p< 0.01, % inhibition being 43). Basil leaves inhibited the mutagenicity of the NM at a dose of 5 mg alone, by 30% (p<0.05). At a higher dose of 7.5 mg, basil leaves failed to significantly inhibit the mutagenicity of the NM.

The effects of plant products on the formation of NDEA from its precursor (TELA) are shown in Figures 6.4 and 6.5. Cumin seeds, poppy seeds, kandathipili and turmeric significantly (p<0.05) decreased the formation of NDEA by 54%, 44%, 26%, and 39% at 5 mg/NM and by 60%, 56%, 38% and 49% at 7.5 mg/NM. Asafoetida failed to significantly inhibit the synthesis of NDEA from its precursor.

Among the leafy vegetables, significant inhibition of the formation of NDEA from its precursor were shown by basil leaves,( p< 0.05, % I being 29% and 40% at 5 mg and 7.5 mg/NM respectively), drumstick leaves (% I = 33%, p< 0.05 at 5 mg/NM and % I = 50%, p< 0.001 at 7.5 mg/NM) and ponnakanni
Figure 6.4
Inhibition of the formation of NDEA from its precursor (TELA) by spices.
NM - Nitrosation reaction mixture alone
CS - Cumin seeds
PS - Poppy seeds
AF - Asafoetida
KT - Kandathipili
TU - Turmeric
Inhibition of the formation of NDEA from its precursor (TELA) by leafy vegetables.

NM - Nitrosation reaction mixture alone
BL - Basil leaves
DL - Drumstick leaves
ML - Manathakkali leaves
PL - Ponnakanni leaves
leaves (% I - 37%, p < 0.01 at 5 mg/NM and % I - 56%, p < 0.001 at 7.5 mg/NM).

The effects of spices and leafy vegetables on the nitrosation of MU as measured in Ames test using strain TA 1535 of *Salmonella typhimurium* are shown in Figures 6.6 and 6.7.

Cumin seeds, poppy seeds and turmeric significantly (p < 0.001) suppressed the mutagenicity of the NM at both doses tested, per cent inhibition being 41, 33 and 37 at 5 mg/NM and 79, 43 and 50 at 7.5 mg/NM respectively. Asafoetida failed to significantly decrease the mutagenicity of the NM. Kandathipili significantly decreased the mutagenicity of the NM at 5 mg/NM by 30% (p < 0.01), but at 7.5 mg/NM it failed to significantly decrease the number of his revertants/plate.

Among the leafy vegetables, drumstick leaves and ponnakanni leaves decreased the mutagenicity of the NM by 29% and 50% at 5 mg/NM and by 47% and 85% at 7.5 mg/NM. Basil leaves inhibited the mutagenicity of the NM by 39% at a dose of 5 mg/NM alone (p < 0.001), while manathakkali leaves inhibited the mutagenicity of the NM by 26% at a dose of 7.5 mg alone significantly (p < 0.01).

The effects of spices and leafy vegetables on the formation of NMU from its precursor are depicted in Figures 6.8 and 6.9 respectively.

Cumin seeds, poppy seeds, turmeric and kandathipili significantly (p < 0.001) inhibited the formation of NMU by
Figure 6.6

Inhibition of the nitrosonation of MU by spices (as assessed by the mutagenicity of the nitrosonation mixture in *S. typhimurium* TA 1535).

Mutagenicity was tested without the addition of S9.

NM - Nitrosonation reaction mixture alone
CS - Cumin seeds
PS - Poppy seeds
AF - Asafoetida
KT - Kandathipili
TU - Turmeric
Figure 6.7
Inhibition of the nitrosation of MU by leafy vegetables (assessed by the mutagenicity of the NM in *S. typhimurium* TA 1535).

Mutagenicity was tested without the addition of S9

**NM** - Nitrosation reaction mixture alone

**BL** - Basil leaves

**DL** - Drumstick leaves

**ML** - Manathakkali leaves

**PL** - Ponnakanni leaves
Figure 6.8

Inhibition of the formation of NMU from its precursor (MU) by spices.

NM - Nitrosation reaction mixture alone
CS - Cumin seeds
PS - Poppy seeds
AF - Asafoetida
KT - Kandathipili
TU - Turmeric
Figure 6.9

Inhibition of the formation of NMU from its precursor (MU) by leafy vegetables.

NM - Nitrosation reaction mixture alone
BL - Basil leaves
DL - Drumstick leaves
ML - Manathakkali leaves
PL - Ponnakanni leaves
48%, 37%, 34%, and 23% respectively at 5 mg/NM and by 68%, 46%, 45% and 32% respectively at 7.5 mg/NM. Asafoetida failed to inhibit significantly the formation of NMU.

Among the leafy vegetables, basil leaves, drumstick leaves and ponnakanni leaves decreased the synthesis of NMU from its precursor by 42%, 37% and 46% at 5 mg/NM and by 47%, 56% and 76% at 7.5 mg/NM. Manathakkali leaves inhibited NMU formation significantly ($p<0.001$) only at a concentration of 7.5 mg/NM by 33%.

The ability of various plant products to effect depletion of nitrite ions in the reaction system are depicted in figures 6.10 and 6.11. All the plant products with the exception of manathakkali leaves significantly depleted nitrite ions in the reaction mixture in a dose-dependent manner. Manathakkali leaves however failed to significantly scavenge nitrite ions from the reaction mixture at a dose of 5 mg/NM.

DISCUSSION

Among the 9 plant products tested, cumin seeds, poppy seeds, turmeric, drumstick leaves and ponnakanni leaves significantly inhibited the nitrosation of TELA and MU. This conclusion is further confirmed by the finding that these plant products significantly decreased the formation of NDEA and NMU. The mechanism of action of these plant products might be through scavenging of nitrite ions in the medium, making them unavailable for nitrosation. Turmeric
Figure 6.10

Depletion of nitrite ions by spices.

CS - Cumin seeds
PS - Poppy seeds
AF - Asafoetida
KT - Kandathipili
TU - Turmeric
Figure 6.11
Depletion of nitrite ions by leafy vegetables.
BL - Basil leaves
DL - Drumstick leaves
ML - Manathakkali leaves
PL - Ponnakanni leaves
and its constituents (curcumins) have been shown to be efficient inhibitors of the nitrosation of methylurea (25).

Asafoetida failed to inhibit the nitrosation of TELA and MU. However, it caused significant depletion of nitrite ions in the nitrosation mixture. This can be attributed to the presence of ferulic acid in asafoetida (26), which is known to deplete nitrite ions by reacting with them (27). The failure of asafoetida to decrease the mutagenicity of the NM can be attributed to the fact that certain phenolics (including ferulic acid) can form C-nitroso derivatives which act as powerful nitrosating agents (28). Inhibitory effects of phenolics are generally exerted when the molar ratio (nitrite) : (phenolic)< 1, and inhibition increases when this ratio is lowered. Catalysis of N-nitrosation may occur with phenolics that can form C-nitroso derivatives at molar ratios of (nitrite): (phenolic)> 1 (10). This may account for the failure of asafoetida to inhibit the nitrosation reaction.

Basil leaves and kandathipili inhibited the nitrosation reaction at a dose of 5 mg. At a higher dose of 7.5 mg they were ineffective. However, the amount of NDEA or NMU formed was decreased. These results suggest that both basil leaves and kandathipili might contain nitrosatable precursors which may get nitrosated and contribute to the mutagenicity, or they may contain phenolics which may form C-nitroso derivatives which may in turn catalyse N-
nitrosation reactions. Basil leaves contain eugenol and carvacrol which are phenolic compounds (29).

It has been found using food mixtures that unidentified non-volatile nitrosamines are synthesized in much larger amounts in vivo than known volatile ones (30). Moreover, it has been established that the rate of in vivo nitrosation correlates reasonably well with in vitro kinetic studies (31, 32).

Although a panel of the National Research Council, U.S.A., (1978) (33) suggested that endogenous formation of nitroso compounds from precursors (nitrite, nitrate and amino compounds) is the largest source of exposure for the general population, the extent to which nitrosation reactions occur in humans, ingesting typical levels of nitrite, nitrate and nitrosatable compounds could not be determined until recently. On the basis of animal tests (10, 34), it was concluded that monitoring of urinary levels of N-nitrosaminoacids such as N-nitrosoproline (NPRO) could be a useful procedure for the quantitative estimation of nitrosation in humans in vivo (35). Thus, the amount of NPRO excreted in the 24 h urine from the test dose of proline ingested can be used as an indicator of daily endogenous nitrosation (NPRO test). The NPRO test has demonstrated unequivocally that in vivo nitrosation can occur in humans and can be inhibited by dietary constituents such as vitamins C and E and plant phenols (10). The leafy vegetables especially drumstick leaves contain high levels
of vitamins C and E and β carotene (36). These constituents may be responsible for the efficient inhibition of the nitrosation of TELA and MU. Vitamins C and E are potent inhibitors of the nitrosation reaction (37).

Present knowledge on the etiology of gastric cancer in the Western world and in Japan, suggests that it is initiated early in life, most likely by nitrosamide type carcinogens derived from diets rich in nitrite, nitrate, dried, smoked or cured fish and meat products, or fava beans or when the gastric mucosa is damaged by a high salt diet (10). Mortality rates for oesophageal cancer were found to be associated positively with endogenous nitrosation and negatively with the background ascorbate level present in the plasma (10). Therefore in order to minimise oesophageal and gastric cancer risk, it is essential to identify more dietary inhibitors of the nitrosation reaction and to increase human exposure to them.
SUMMARY

1. The following plant products, cumin seeds, poppy seeds, asafoetida, kandathipili, turmeric, basil leaves, drumstick leaves, manathakkali and ponnakanni leaves were tested for their effects on the nitrosation of TELA and MU in vitro.

2. Cumin seeds, poppy seeds, turmeric, drumstick leaves and ponnakanni leaves significantly inhibited the nitrosation of MU and TELA.

3. Kandathipili and basil leaves inhibited the nitrosation of TELA and MU, when added at a dose of 5mg to the nitrosation mixture. At a higher dose they were ineffective.

4. Manathakkali leaves significantly inhibited the nitrosation of TELA and MU at a dose of 7.5 mg only.

5. Asafoetida failed to inhibit the nitrosation of TELA and MU.

6. All the plant products except manathakkali leaves, significantly depleted nitrite ions from the reaction system in a dose-dependent manner. Manathakkali leaves were efficient only at a dose of 7.5 mg.
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