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Effect of Certain Plant Phenolics on Nitrosamine Formation[1]

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Phenols are known to modify the nitrosation of amines, as catalysts or inhibitors, depending on their structure, reaction conditions, pH, and nitrite concentration. In the present work, the roles of catechol (CA), 4-hydroxychavicol (HC), eugenol (EU), and methyleugenol (MEU) on the nitrosation of model amines, viz. pyrrolidine (PYR), piperidine (PIP), and morpholine (MOR), were studied. The modifying effects of these phenolics were compared to that of ascorbic acid (AA). It was observed that HC and CA were excellent inhibitors of the nitrosation reaction while EU was less effective. MEU was a poor inhibitor.

N-Nitroso compounds (NOC) are known to be strong carcinogens in various animals including primates (Preussmann and Stewart, 1984). Human exposure to these compounds can be by ingestion or inhalation of preformed NOC or by endogenous nitrosation. It has been established unequivocally that NOC are formed in the body from precursors present in normal diet (Bartsch et al., 1984). Essentially they are formed by the reaction of amines, especially secondary and tertiary amines, or amino group containing compounds, such as dialkylamines, alkylarylamines, piperazine, pyrrolidine, etc., with nitrite. While amines are present in foods, wine, tobacco products, drugs, and other environmental chemicals such as pesticides, sources of nitrite vary (Fine, 1979; Shephard et al., 1987; Tannenbaum, 1979).

N-Nitrosation is influenced by many factors such as pH, amounts of precursors, basicity of the nitrosatable amines, and presence of catalysts and inhibitors (Challis, 1981). Simple phenols and polyphenolic compounds can decrease or increase the rate of N-nitrosation reactions depending on their structure and reaction conditions, especially pH (Douglass et al., 1978; Pignatelli et al., 1980; Mirvish, 1981; Challis, 1981). Certain polyphenolic compounds react with nitrite to give *C*-nitroso derivatives, which can act as catalysts of nitrosation (Pignatelli et al., 1982, 1984). However, phenols and polyphenolic compounds that are readily oxidized inhibit nitrosation reactions (Stich et al.,

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1983). A number of compounds, viz. sulfur dioxide, bisulfite, α-tocopherol, ascorbic acid, and glutathione, are known to inhibit N-nitrosation (Mirvish, 1981; Cooney et al., 1986).

The present study was undertaken to assess the role of certain phenols in modifying the rates of nitrosamine formation. The phenols studied were 4-hydroxychavicol (HC), eugenol (EU), methyleugenol (MEU), and catechol (CA). While HC, EU, and MEU are present in betel leaves and bay leaves, eugenol is a major constituent of clove oil (Atal et al., 1975; Evans et al., 1984). Ascorbic acid and CA were included in the study as reference inhibitors. The total phenolic fraction from betel leaf, which contains some of the phenolic compounds studied, was also assessed for its modifying effect on nitrosamine formation. Pyrrolidine (PYR), piperidine (PIP), and morpholine (MOR) were chosen as model amines. This study would be extended to assess the modifying role of the above phenolics on the formation of nitroso derivatives of other food- and tobacco-based amines.

MATERIALS AND METHODS

Reagents. Pyrrolidine was purchased from Riedel-De-Haenag, Seelze, Hannover; PIP from Fluka A 6; MOR from Pfizer Ltd. All three amines were distilled prior to use (Vogel, 1986a). *N*-Nitrosopyrrolidine (NPYR), *N*-nitrosopiperidine (NPIP), and *N*-nitrosomorpholine (NMOR) were procured from Sigma Chemical Co. Eugenol and CA were obtained from E. Merck, ascorbic acid from Sarabhai Chemicals, and sodium nitrite from British Drug House.

Caution! All the nitrosamine standards mentioned above are potent carcinogens. Utmost caution should be taken while working with these chemicals. Contact with skin and inhalation of vapors must be avoided.

Methyleugenol was synthesized by methylating EU with dimethyl sulfate (Vogel, 1986b), and its identity and purity were confirmed by chromatographic and spectral comparison with a standard MEU standard sample obtained from Sisco Research Laboratories. Catechol was purified by sublimation (Vogel, 1986c), and HC was synthesized by demethylating EU with aluminum iodide as the demethylating agent (Bhatt and Babu, 1984).

Synthesis of 4-Hydroxychavicol. Dry aluminum foil (500 mg, 18.6 mmol) and iodine (3.8 g, 30 mmol) were refluxed with stirring in dry acetonitrile (25 mL) until the iodine color disappeared (3.5 h). To this freshly prepared solution of aluminum iodide was added EU (1.64 g), and the resultant mixture was refluxed until all the EU was used (45 min). The reaction mixture was treated with ice and extracted with ethyl acetate (3 × 200 mL). The ethyl acetate extract was washed with 15% sodium thiosulfate solution (3 × 15 mL) and dried over anhydrous sodium sulfate. The solvent was removed completely, and the product was purified by column chromatography (silica gel G, 4 × 60 cm column). The compound was eluted with benzene initially, gradually increasing the polarity with chloroform (benzene to chloroform ratios used were 99:1, 98:2, ..., 90:10). The compound eluted completely when 10% chloroform was used. The compound was crystallized with benzene–petroleum ether. It gave a single spot on a TLC plate (silica gel, benzene:ethyl acetate:acetic acid = 8.4:1.4:0.1), and its melting point was 48–48.5 °C (lit. mp 48 °C (Sethi and Rao, 1964)).

Spectral Analysis. The NMR spectrum was recorded on a Varian Associates Model 360 NMR spectrophotometer (60 MHz with a permanent magnet). The internal standard was tetramethylsilane, and the solvent used was deuterated chloroform. The NMR spectrum of HC showed a methylene two proton doublet at 3.4 ppm, a two-proton doublet at 5.15 ppm, and a one-proton multiplet around 5.95 ppm, indicating an allyl group. The presence of a trisubstituted ring was inferred from the three aromatic protons around 6.8 ppm. Absence of the methoxy group proton signal at 3.8 ppm indicated that the methoxy group was converted to the corresponding hydroxy group. The presence of the hydroxy proton was noted by their exchange with deuterated water.

The IR spectrum was recorded on a Perkin-Elmer Model 783 spectrophotometer. The spectrum showed a strong hydroxy group absorbtion around 3300 cm^{-1}. The presence of an allylic group was indicated by the olefinic C-H stretch absorption at 3050 cm^{-1} and out-of-plane C–H bend peaks at 910 and 990 cm^{-1}.

The mass spectrum of this compound was recorded on a VG Micromass Model 7070 with electron impact (EI) ionization. The mass fragments obtained were M^+ (150), base peak; $M^+ - OH$ (133); and $M^+ - -CH_2CH{=}CH_2$ (109).

On methylation of this compound with dimethyl sulfate, HC obtained as above yielded a product whose retention time on GLC was the same as that of MEU. The IR spectrum of the methylated compound was identical with that of authentic MEU.

Gas chromatography was carried out on GC Shimadzu Model 7C. Nitrosamines were quantitated by gas chromatography using 3% OV-17 on Gas Chrom Q (80/100) glass column (3.8 m × 5 mm (o.d.)) at 120 °C. The nitrogen carrier gas rate was 40 mL/min. The chromatogram was recorded on Chromatopac C-RIB (Shimadzu). The detector used was a flame ionization detector.

Extraction of Betel Leaf Phenolic Fraction. Fresh betel leaves (23 g), Madras variety, were blended in a blender with acetone (3 × 100 mL). The solvent was removed completely on a flash evaporator. The residue was made alkaline (0.5 N NaOH) and extracted with chloroform (3 × 50 mL). The organic phase was discarded, the aqueous portion was made acidic with 0.5 N acetic acid, and the phenols were extracted with chloroform (3 × 25 mL). The same results were obtained when carbon dioxide gas was passed through solution instead of adding acetic acid. The phenolic fraction was first washed with 10% sodium bicarbonate solution (3 × 5 mL) and then with saturated sodium chloride solution. The extract was dried over anhydrous sodium sulfate and the solvent completely removed by a flash evaporator. The weight of the brownish viscous liquid so obtained was 289 mg, and this fraction was used to study its modifying action on nitrosamine formation. 4-Hydroxychavicol was the major component in this fraction, while EU and other uncharacterized compounds were present as minor constituents.

Nitrosation Reaction. Nitrosation was initiated by adding a solution of sodium nitrite to a solution of the amine or to a solution of amine and the modifier. The reaction was carried out in a buffer system of citric acid–sodium citrate, at pH 3.5, in a stoppered conical flask and shaken on a shaker for 1 h in the dark, at room temperature (25 °C). The volume of the reaction mixture was 5 mL. The concentrations of amine and nitrite were 50 and 100 mM, respectively, when nitrosation of PYR and PIP was studied and 25 and 50 mM when nitrosation of MOR was studied. The reaction was stopped at the end of 1 h by adding an aqueous solution of ammonium sulfamate (100 mM, to stop NPYR and NPIP formation and 50 mM to stop NMOR formation). The reaction mixtures were then extracted with chloroform (2 × 3 mL) and concentrated to suitable volumes required for GLC analysis.

Amounts of nitrosamine formed were estimated by means of calibration curves prepared by analyzing standard nitrosamines under identical conditions (external standard method).

Inhibition was calculated on the basis of the difference between the amount of nitrosamine formed in the presence and absence of added modifier, by taking the yield of the reaction in the absence of the modifier as 0% inhibition.

The percent yield as shown in Tables II–IV is given by (mmol of nitrosamine formed/mmol of amine used) × 100.

RESULTS AND DISCUSSION

The structures of the phenols used are shown in Figure 1.

Effect of Phenolic Fraction from Betel Leaves on Nitrosamine Formation. The effect of the phenolic fraction, obtained from betel leaves, on the nitrosation of PYR, PIP, and MOR is shown in Table I. When 100 mg of the fraction was used, it blocked PYR and PIP nitrosation completely and that of MOR by 79%. The two compounds characterized in betel leaf extract were HC (major) and EU (minor). The other unidentified compounds in the fraction could be chavicol, chavibetol, carvacrol, estragole, etc. (Atal et al., 1975). The strong inhibitory effect of this fraction on the nitrosation reactions

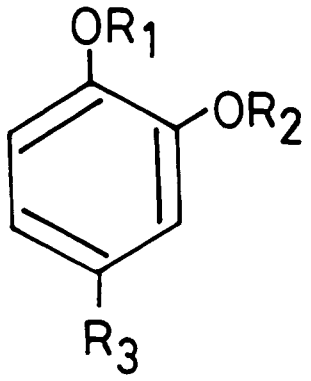

Figure 1. Chemical structures: catechol, R_1, R_2, R_3 = H; 4-hydroxychavicol, R_1, R_2 = H, R_3 = $-CH_2CH{=}CH_2$; eugenol, R_1 = H, R_2 = CH_3, R_3 = $-CH_2CH{=}CH_2$; methyleugenol, R_1, R_2 = CH_3, R_3 = $-CH_2CH{=}CH_2$.

Table I. Effect of Betel Leaf Phenolic Fraction on Formation of NPYR, NPIP, and NMOR

concn of betel leaf phenolics, mg/mL	% inhibition ± SD[a]		
	NPYR	NPIP	NMOR
10	59 ± 1.5	58 ± 2.5	43 ± 1.9
20	100 ± 0	100 ± 0	79 ± 2.0

[a] Standard deviation of three experiments.

could be largely attributed to HC and to a lesser extent to EU and the other phenolics. Synthetically prepared and purified samples of HC and EU were also used for studying their inhibitory effect on the nitrosation reaction. This has been discussed below along with other modifiers.

Effect of Modifiers on NPYR Formation from PYR and Nitrite. Figure 2 indicates the action of CA, HC, EU, MEU, and AA on the nitrosation of PYR. 4-Hydroxychavicol and CA, both of which have two hydroxy groups ortho to each other, were very good inhibitors of the nitrosation reaction. Their modifying effect was comparable to that of ascorbic acid. At 100 mM concentrations, there was 100% blocking by these two phenols and 60% inhibition by EU. Methyleugenol was a poor inhibitor of the reaction. The percentage yield of NPYR in the presence and absence of modifiers at different concentrations is tabulated in Table II.

Effect of Modifiers on NPIP Formation from PIP and Nitrite. In Figure 3 it can be seen that CA and HC are very good blocking agents of the nitrosation reaction.

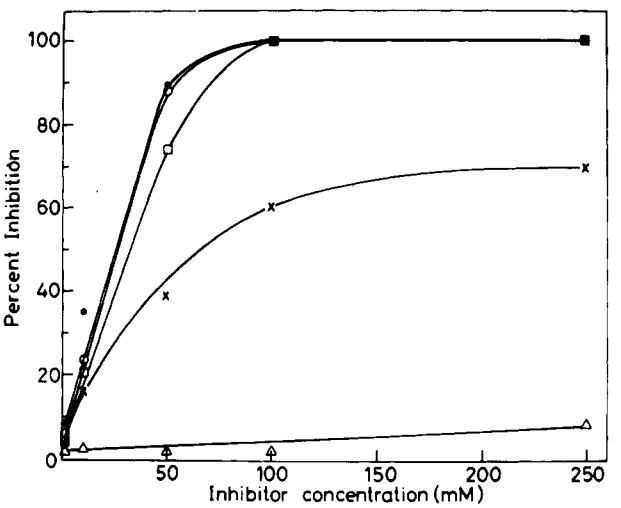

Figure 2. Effect of various concentrations of catechol (○), 4-hydroxychavicol (□), eugenol (×), methyleugenol (△), and ascorbic acid (●) on NPYR formation.

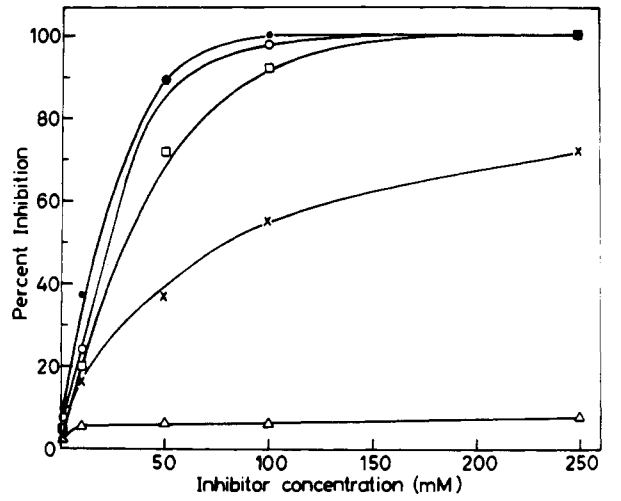

Figure 3. Effect of various concentrations of catechol (○), 4-hydroxychavicol (□), eugenol (×), methyleugenol (△), and ascorbic acid (●) on NPIP formation.

These two inhibited nitrosation of the amine by 98% and 93%, respectively, at 100 mM concentrations. Eugenol at the same concentration inhibited NPIP formation by 55%.

Table II. Effect of Modifiers on NPYR Formation[a]

concn of modifier, mM	% yield ± SD of NPYR in presence of modifiers				
	CA	HC	EU	MEU	AA
0	0.51 ± 0.05	0.51 ± 0.05	0.51 ± 0.05	0.51 ± 0.05	0.51 ± 0.05
1	0.48 ± 0.02	0.49 ± 0.04	0.49 ± 0.01	0.50 ± 0.01	0.46 ± 0.01
10	0.39 ± 0.02	0.40 ± 0.02	0.43 ± 0.02	0.47 ± 0.02	0.33 ± 0.02
50	0.06 ± 0.01	0.13 ± 0.01	0.31 ± 0.02	0.50 ± 0.01	0.05 ± 0.02
100	nd	nd	0.21 ± 0.03	0.50 ± 0.02	nd
250	nd	nd	0.15 ± 0.02	0.46 ± 0.02	nd

[a] Key: nd, not detectable; SD, standard deviation of three experiments.

Table III. Effect of Modifiers on NPIP Formation[a]

concn of modifier, mM	% yield ± SD of NPIP in presence of modifiers				
	CA	HC	EU	MEU	AA
0	0.49 ± 0.02	0.49 ± 0.02	0.49 ± 0.02	0.49 ± 0.02	0.49 ± 0.02
1	0.45 ± 0.02	0.47 ± 0.01	0.47 ± 0.03	0.47 ± 0.01	0.44 ± 0.01
10	0.37 ± 0.01	0.40 ± 0.02	0.41 ± 0.02	0.46 ± 0.04	0.30 ± 0.02
50	0.05 ± 0.01	0.13 ± 0.01	0.30 ± 0.02	0.45 ± 0.03	0.05 ± 0.01
100	0.001 ± 0	0.04 ± 0.01	0.22 ± 0.03	0.45 ± 0.02	nd
250	nd	nd	0.14 ± 0.01	0.44 ± 0.03	nd

[a] Key: nd, not detectable; SD, standard deviation of three experiments.

Table IV. Effect of Modifiers on NMOR Formation[a]

concn of modifier, mM	% yield ± SD of NMOR in presence of modifiers				
	CA	HC	EU	MEU	AA
0	21.0 ± 0.5	21.0 ± 0.5	21.0 ± 0.5	21.0 ± 0.5	21.0 ± 0.5
1	19.8 ± 1.3	20.1 ± 2.1	20.5 ± 1.5	20.8 ± 2.0	20.2 ± 2.2
10	16.1 ± 0.5	18.5 ± 1.1	20.3 ± 1.4	20.8 ± 2.0	17.6 ± 2.3
50	0.5 ± 0.0	4.78 ± 0.3	19.7 ± 0.05	20.8 ± 2.1	13.1 ± 1.1
100	nd	nd	17.8 ± 1.1	19.2 ± 0.5	6.9 ± 1.1
250	nd	nd	15.5 ± 1.9	19.5 ± 2.7	nd

[a] Key: nd, not detectable; SD, standard deviation of three experiments.

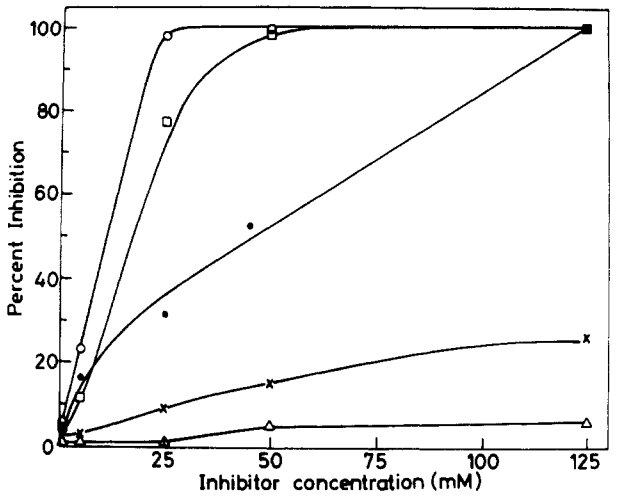

Figure 4. Effect of various concentrations of catechol (○), 4-hydroxychavicol (□), eugenol (×), methyleugenol (△), and ascorbic acid (●) on NMOR formation.

Formation of NPIP in terms of percentage yield in the presence and absence of modifiers is tabulated in Table III.

Effect of Modifiers on NMOR Formation from MOR and Nitrite. From Figure 4 it becomes clear that CA and HC inhibited nitrosation of MOR more efficiently than ascorbic acid. At 50 mM concentration these two phenols almost completely blocked NMOR formation whereas AA and EU did so to the extent of 66% and 15%, respectively. Table IV gives the percentage yield of NMOR, in the presence and absence of modifiers at different concentrations.

One of the reasons why eugenol inhibits the formation of NPIP and NPYR efficiently but not that of NMOR may be due to the difference in the basicities of the amines. Morpholine, being a less basic amine compared to the other two, gets nitrosated at a faster rate (Mirvish, 1981). Our data imply the same. Morpholine may be reacting with the nitrite at a faster rate than eugenol.

Certain phenolic compounds under acidic conditions are known to compete more efficiently for the available nitrite than the amines (Challis, 1973; Massey et al., 1978; Walker et al., 1982; Virk and Issenberg, 1985). The inhibition of nitrosation occurs in most cases because the nitrosating agents are converted to innocuous products such as nitric oxide, nitrous oxide, or nitrogen (Archer et al., 1984). Ascorbic acid, included in this study as a reference inhibitor, also reduces the nitrosating agent to nitric oxide and inhibits nitrosation reaction (Mirvish, 1981).

It would appear that 1,2- and 1,4-dihydroxyphenols are oxidized to the corresponding quinones by nitrous acid, which in turn gets converted to the ineffective nitric oxide (Pignatelli et al., 1980, 1982).

Catechol, HC, and EU may be getting oxidized by the nitrosating agent to quinones, thus depriving the amine of the available nitrite. Catechol and HC are antioxidants by virtue of their two hydroxy groups ortho to each other and may be reducing the nitrosating agent to the ineffective nitric oxide. Various aspects of these possibilities are under investigation and will be reported later. Another reason why EU is a less effective inhibitor than HC may be because it has only one free hydroxy group. Methyleugenol, which has no free hydroxy group, does not have any affect on the nitrosation reaction.

Many naturally occuring phenols, present in vegetables, fruits, etc., contain the catechol moiety. 4-Hydroxychavicol and EU occur in betel leaves and bay leaves. EU is a major constituent of clove oil (*Eugenia caryophylata*). They have been reported to be nonmutagens as well as antimutagens (Amonkar et al., 1986). Since these phenolics are ingested by man as food components, they may modify the endogenous formation of nitrosamines to a considerable extent.

The chewing of betel quid containing tobacco is associated with oral and esophageal cancer (Bhide et al., 1984; Brunneman et al., 1985). One of the causative agents of these cancers could be the nitrosamines present in tobacco or those formed endogenously in the oral cavity and/or stomach by the interaction of tobacco and areca nut amines with salivary nitrite (Hoffmann et al., 1985). However, the other quid components, betel leaf, clove buds, and catechu, could have a protective effect against nitrosation. The protective effect of betel leaf, in oral cancer, has been reported (Khanolkar, 1944). It would appear that the overall mutacarcinogenic potential of the quid may determine the ultimate health hazard.

The present study indicates that plant phenolics can render protection against endogenous nitrosation. Further in vivo studies would determine the level of protection offered by these phenolics against nitrosamine formation.

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Volatile Compounds from Garlic

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Volatile components of crushed garlic were obtained by water distillation, steam distillation, and Likens–Nickerson (L–N) distillation/solvent extraction with or without steam, respectively. The volatile components were analyzed and identified by capillary gas chromatography (GC) and combined gas chromatography–mass spectrometry (GC–MS). The yield of garlic essential oils was 0.22 ± 0.01% (w/w). Monosulfides, disulfides, and trisulfides were the major volatile components in garlic essential oils. Of 28 components, 13 were being reported for the first time as components of garlic. The essential oils obtained by L–N distillation/solvent extraction contained more 2,4-dimethylfuran, 2-propen-1-ol, aniline, and 3,5-diethyl-1,2,4-trithiolane than those from water distillation and steam distillation. Water layer of garlic distillate contained more 3,5-diethyl-1,2,4-trithiolane and 2-propen-1-ol than its oil layer. The essential oils obtained by steam distillations contained more high-volatility compounds and less low-volatility compounds than those by water distillations.

Garlic (*Allium sativum* Linn.) is one of the most important spices used in Chinese food. In some Chinese foods cooking, people put crushed garlic cloves into the wok containing hot vegetable oil first and then put the vegetables and other foods or ingredients in and fry them.

The major volatile compounds of garlic were sulfur-containing compounds. Semmler (1892) established the importance of diallyl disulfide and diallyl trisulfide in the flavor of garlic distillate. Stoll and Seebeck (1948) showed that garlic contains *S*-allylcysteine sulfoxide (alliin) and an enzyme, allinase. By the action of allinase on alliin, diallyl thiosulfinate (allicin) is formed, a volatile compound.

Diallyl thiosulfinate was found as a major constituent

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