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Nitrosamine formation from different *Catha edulis* leaves extracts under simulated gastric condition

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Abstract

Nitrosamines are well-known carcinogenic and toxic compounds for humans and animals. The aim of this study was to investigate the possible formation of nitrosamine compounds from aqueous extracts of five different types of *Catha edulis* leaves using nitrite as a nitrosation agent, either in aqueous solution or under simulated normal fasting stomach conditions (at 37 °C and pH 2) for 1 h. Nitrosoephedrine was used as a reference compound in this study. Nitrosation of aqueous extracts of the different types of *Catha edulis* leaves with constant concentration of nitrite (14.4 mM) in aqueous solution showed total apparent nitrosamine compounds to be in the range 94–319 mg/100 g DM (dry matter) of CE (*Catha edulis*) leaves. In contrast, nitrosamines formed in simulated gastric fluid were much lower, in the range 23–79 mg/100 g DM of CE leaves. Based on the moderate formation of nitrosamines with the Sabri *Catha edulis* leaves, the latter was selected for investigating the effect of varying levels of nitrite in aqueous solution and simulated gastric juice. The nitrosation of aqueous extracts of Sabri *Catha edulis* with different levels of nitrite in aqueous solution yielded a dose-dependent amount of total apparent nitrosamine compounds, these being undetected at $\leqslant$0.5 mM sodium nitrite in aqueous solution and $\leqslant$1 mM in simulated gastric juice. This raises the question of whether the observed high incidence of esophageal and forestomach carcinomas in Yemen could be attributed to the formation of nitrosamines in vivo from the secondary amines present in *Catha edulis* leaves.

Keywords: *Catha edulis*; Khat; Nitrosation; Total apparent nitrosamines; Carcinogens

1. Introduction

The habit of khat chewing has prevailed for centuries among population in the horn of Africa and the Arabian Peninsula, including the Yemen. Fresh leaves of khat (*Catha edulis* Forsk) are customarily chewed to attain a state of stimulation (Al-Motarreb, Baker, & Broadley, 2002). The facts that cathinone has a close structural similarity to amphetamine, and that both share common pharmacodynamic features, led to the conclusion that cathinone is the most important active ingredient of khat, which causes major pharmacological effects (Hollister, 1995). The common adverse effects of khat are wide and variable (AL-Habori, Al-Aghbari, Al-Mamary, & Baker, 2002; AL-Habori & AL-Mamary, 2004; Al-Mamary, Al-Habori, Al-Aghbari, & Baker, 2002; Al-Meshal, Ageel, Tariq, & Parmer, 1983, 1985; Brenneisen, Fish, Koebling, Geisshusler, & Kalix, 1990; Connor, Makonnen, & Roston, 2000; Heymann et al., 1995; Wilder, Mathys, Brenneisen, Kalix, & Fisch, 1994). Among these is the suggestion that the high tannin content of the leaves is responsible for the observed gastritis (Halbach, 1972) and the apparently high prevalence of oesophageal carcinoma in Yemen (Drake, 1988; Gunaid

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et al., 1995). Gunaid et al. (1995) found indications of an increase in cancer in the cardia and gastroesophageal junction in individuals from Yemen who chewed khat and smoked water pipes, but the number of cases (in total 20) was insufficient to identify independent effects of the two factors. Later, Kassie, Darroudi, Kundi, Schulte-Hermann, and Knasmuller (2001) suggested that khat consumption, especially when accompanied by alcohol, and tobacco consumption might be a potential cause of oral malignancy.

Catha edulis leaves contain some primary amines, such as cathinone, cathine, and norephedrine (Geisshusler & Brenneisen, 1987; Schorno, Brenneisen, & Steinegger, 1982) and secondary amines, such as ephedrine and pseudoephedrine (Caveney, David, Helmut, Maria, & Alvin, 2001), which may be considered to be precursors of nitrosamines (potent carcinogens) in the presence of nitrite. Significant concentrations of nitrite are present in the dietary intake (Walters, Dyke, Saxby, & Walters, 1976), added either as a preservative or formed by the action of nitrifying bacteria as an intermediate stage in the formation of nitrates. The aim of the present study is to evaluate the formation of nitrosamine compounds from the nitrosation of the aqueous extracts of different types of *Catha edulis* leaves in vitro and in simulated gastric fluid.

2. Materials and methods

2.1. Materials

Five different types of *Catha edulis* leaves were collected (Sabri, Sauti, Ansi, Baladi and Hamdani) from different areas in Yemen namely Taiz, Hajah, Dhamar and Sana'a, respectively. All samples were air-dried and ground using a coffee grinder.

2.2. Synthesis of N-nitrosoephedrine

N-Nitrosoephedrine was synthesised by the reaction of nitrous acid, either with 2 M acetic acid or with 2 M HCl at 5 °C. The formed pale yellow precipitate was washed with a saturated solution of sodium bicarbonate to remove the excess of acid, followed by distilled water. Purification was carried out by recrystallisation from ethanol (95%) and the yield was 82% from the reaction with acetic acid and 42% from that with HCl (Fig. 1).

The identity of the nitrosoephedrine was checked by IR spectroscopy and m.p. and the results were similar to those obtained previously (Wogan, Paglialunga, Archer, & Tannenbaum, 1975).

2.3. Nitrosation in aqueous solution with constant amount of sodium nitrite

About 2 g of powder of each type of air-dried *Catha edulis* leaves were boiled with 200 ml of distilled water for 5 min and allowed to cool to room temperature. The residue was removed by filtration and the aqueous extract was acidified to pH 2 with 2 M HCl. The flasks containing the extracts were incubated in a water bath at 37 °C. 50 ml of aqueous solution containing 1 g sodium nitrite were added to each flask, in small portions, to avoid, as much as possible, any loss of nitrous acid and to secure the nitrosation of secondary amines present. The reaction was carried out for 1 h with continuous shaking. The reaction mixture was extracted with diethyl ether (3×50 ml) and the ethereal fractions were pooled and evaporated to dryness under reduced pressure. Total apparent nitrosamine compounds were determined colorimetrically as nitrosoephedrine, which was used as the standard (see below). An aliquot of 200 ml of the decoction was treated similarly, but without the addition of sodium nitrite and served as a blank. Each sample was determined in triplicate.

2.4. Nitrosation under simulated gastric conditions

Simulated gastric juice was prepared according to the method described by Gillat, Palmer, Smith, and Walters (1985). The composition of the simulated gastric juice was based on a 0.05 M KCl/HCl system buffered to pH 2, with a total chloride concentration of 125 mM as well as sodium (70 m M), potassium (60 mM), D-glucose (2.2 mM), pepsin (0.14 mM), D-(−)-lactic acid (1.1 mM), and nitrosation catalyst (1.5 mM thiocyanate and 0.14 mM catechin). The mixture was adjusted to pH 2 with HCl.

About 2 g of powder of each type of air dried *Catha edulis* leaves were added to 200 ml of simulated gastric fluid and the acidity was corrected to pH 2 with 2 M HCl. 50 ml of aqueous solution containing 1 g sodium

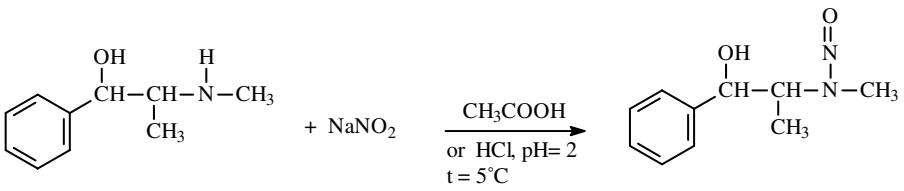

Fig. 1. Synthesis of *N*-nitrosophedrine.

nitrite were added to each flask, in small portions, to avoid, as much as possible, any loss of nitrous acid and to secure the nitrosation of secondary amines present. The reaction was then carried out as described for aqueous nitrosation and samples without addition of sodium nitrite served as a blank.

2.5. Nitrosation of Catha edulis extracts in aqueous solution or under simulated gastric conditions with different amounts of sodium nitrite

One gramme of Sabri Catha edulis was either boiled with 100 ml of distilled water for 5 min and allowed to cool to room temperature or incubated with 100 ml of simulated gastric fluid. The residue was then removed by filtration and the aqueous extract acidified with 2 M HCl to pH 2. To each extract, 0.05, 0.1, 0.5, 1, 2.5, 5 or 10 mM of sodium nitrite were added. Reactions were then carried out as described for aqueous nitrosation and samples without addition of sodium nitrite served as a blank.

2.6. Colorimetric determination of N-nitrosamines

Total apparent nitrosamine compounds were determined according to the method described by Eisenbrand and Preussmann (1970) and modified by Alwan, Hindawi, Abdul-Rahman, and AL-Sarraj (1986). To the dry extracts of nitrosamines were added, 1 ml of glacial acetic acid, 3 ml of HBr (4%) in glacial acetic acid and 6 ml of Griess reagent. The mixture was then heated at 80 °C for 10 min and the absorbance of the developed purple colour was measured at 520 nm. A standard curve was prepared and total apparent nitrosamine compounds were calculated as nitrosoephedrine (mg/100 g dry matter of *Catha edulis* leaves).

2.7. Statistical analysis

Differences in the amounts of nitrosamine formed in the extracts of the different types of *Catha edulis* leaves were tested by one-way analysis of variance using the SPSS statistical package. Significance level was at $p < 0.05$ unless otherwise indicated.

3. Results and discussion

Due to the lack of a suitable technique for the exact determination of the formed individual nitrosamines and, consequently, the amines existing in *Catha edulis* leaves, the results in this study report total apparent nitrosamine compounds, expressed as nitrosoephedrine equivalents, since the latter was used as a reference in this study. The results of nitrosation products of aqueous extracts from different types of *Catha edulis* leaves, with a constant concentration of $NaNO_2$, either in aqueous solution or under simulated gastric juice conditions, are shown in Table 1.

Table 1
Total apparent nitrosamine compounds (mg/100 g of *Catha edulis* as DM) formed by the addition of 1 g of sodium nitrite to different types of *Catha edulis* extracts, either in aqueous solution or in simulated gastric juice

Type of *Catha edulis*	Total apparent nitrosamines as nitrosoephedrine[A]	
	In aqueous solution ($n = 3$)	In simulated gastric juice ($n = 3$)
Saotti	319 ± 44.6^{a}	79.4 ± 11.5^{a}
Baladi	300 ± 35.0^{a}	55.0 ± 10.6^{b}
Ansi	200 ± 0.01^{b}	22.5 ± 5.3^{d}
Sabri	200 ± 35.4^{b}	23.1 ± 2.7^{d}
Hamdani	93.8 ± 8.8^{c}	35.6 ± 4.4^{c}

Values (means ± SD) in the same column followed by the same letter are not significantly different at $P < 0.05$.
[A] Hot water extraction was used in the aqueous solution but not under the simulated gastric conditions.

The amounts of total apparent nitrosamines resulting from nitrosation of aqueous extracts from five different types of *Catha edulis* leaves, namely Saotti, Baladi, Ansi, Sabri, and Hamdani, were in the range 93.8–319 mg/100 g DM (dry mater) of CE (*Catha edulis*) leaves. The values obtained from the Saotti and the Baladi types of CE leaves were the highest and not significantly different from each other, but both values were significantly different from the other three types of *Catha edulis* leaf extracts.

The total apparent of nitrosamines obtained by nitrosation of *Catha edulis* leave extracts with constant concentration of $NaNO_2$ in simulated gastric juice were much lower than those obtained in aqueous solution and this could be attributed to the hot extraction with distilled water before the incubation, when the hot extraction may give higher amounts of extracted amines which, in turn, may convert to nitrosamines by nitrosation with $NaNO_2$. The results obtained by this treatment followed the same trend as those obtained in aqueous solution nitrosation with the exception of the Hamdani (Table 1). The highest value was obtained from Saotti CE leaves extract and was significantly different from all others. The second highest value was obtained from Baladi CE leaves extract and was significantly different from those of the Ansi, Sabri and Hamdani, but the amounts of total nitrosamines resulting from the Ansi and Sabri were not significantly different from each other, but they were lower and significantly different from the result obtained for the Hamdani CE leaves extract. The reason for this discrepancy, in comparison with the result obtained in the aqueous solution condition, is difficult to explain.

Table 2 shows, the amounts of total apparent nitrosamines obtained by nitrosation of aqueous extracts of the Sabri type of *Catha edulis* leaves using different con-

Table 2
Total apparent nitrosamine compounds (mg/100 g of *Catha edulis* as DM) formed by the addition of different amounts of sodium nitrite to Sabri *Catha edulis* extract, either in aqueous solution or in simulated gastric juice

Amount of sodium nitrite added (mM)	Total apparent nitrosamines as nitrosoephedrine[A]	
	In aqueous solution ($n = 3$)	In simulated gastric juice ($n = 3$)
10	70.8 ± 5.8^{a}	10.9 ± 2.7^{a}
5	27.5 ± 5.8^{b}	6.5 ± 1.2^{b}
2.5	17.5 ± 3.7^{c}	4.3 ± 0.7^{c}
1	5.0 ± 1.1^{d}	ND
0.5	ND	ND
0.1	ND	ND
0.05	ND	ND

Values (means ± SD) in the same column followed by the same letter are not significantly different at $P < 0.05$.
[A] Hot water extraction was used in the aqueous solution but not under the simulated gastric conditions.

centrations of $NaNO_2$, either in aqueous solution or under simulated gastric juice conditions. The total apparent nitrosamines obtained from both treatments were directly proportional to the concentrations of $NaNO_2$. The results obtained in the aqueous solution nitrosation were much higher (4- to 6.5-fold) than those obtained in simulated gastric juice (Table 2), which can be attributed to the hot extraction, as explained earlier. The amounts of total apparent nitrosamines in each treatment were significantly different from each other. The minimum amounts of $NaNO_2$ giving a detectable level of nitrosamines, either in aqueous solution or in simulated gastric juice, were 1 and 2.5 mM, respectively. The total apparent nitrosamines obtained from all types of *Catha edulis* used in our study, either in aqueous solution or simulated gastric juice, were much higher than that obtained from the nitrosation of tea made from the plant of *Ephedra Altissima*, either in vitro or in simulated gastric juice (Tricker, Wacker, & Preussman, 1987), as well as that obtained from *Ephedra Foliata* (Alwan et al., 1986). The former study showed that nitrosamines, such as *N*-nitrosoproline, *N*-nitrosoephedrine, *N*-nitrosopseudoephedrine and *N*-nitrosomethylbenzylamine can be formed under the simulated gastric conditions using a constant nitrite concentration of 25 μM (Tricker et al., 1987), which is representative of the upper range in the normal acidic fasting stomach with continuous replenishment of nitrite from saliva (Gillat et al., 1985). The nitrosamines formed due to nitrosation in the earlier studies have been implicated in the incidence of liver, lung, pharyngeal, esophageal and forestomach carcinomas (Druckrey, Preussmann, Schmahl, & Ivankovic, 1967; Eisenbrand, Preussmann, & Schmhl, 1978; Pinto et al., 2003; Wogan et al., 1975). Moreover, among all of the etiological factors associated with the disease, nitrosamines are capable of inducing tumours in the esophagus of experimental animals (Craddock, 1993), independent of the route of administration (Lijinsky, 1992).

In the light of the findings it seems that the pH in the gastro-esophageal junction and the cardia of the stomach favours nitrosation, as it escapes the buffering effect of food (Fletcher, Wirtz, Young, Vallence, & McColl, 2001); also this anatomical site corresponds with increasing incidence of mutagenesis and carcinogenesis (Hansson, Sparen, & Nyren, 1993; Hansen, Wijg, Giercksky, & Tretli, 1997; Mayne et al., 2001). This then raises the question of whether the observed high incidence of esophageal and forestomach carcinomas in Yemen (Gunaid et al., 1995) could be attributed to the formation of nitrosamines in vivo from the secondary amines present in Catha edulis leaves.

The exposure to nitrite could be accounted for from nitrite-containing foods, such as preserved meat and certain vegetables (Gangolli et al., 1994; MAFF, 1992) as well as the reduction of nitrate (NO_3^-) to nitrite (NO_2^-) by oral bacteria (Stephany & Schuller, 1980; Walters & Smith, 1981). Nitrate in food or drinking water is absorbed in the small intestine and about 25% of the intake is actively secreted by the salivary glands into the oral cavity, where approximately 20% of this fraction is converted into nitrite by oral bacteria (Spiegelhalder, Eisenbrand, & Preussmann, 1976). This nitrite gradually enters the stomach after swallowing of oral fluid where, at low pH, nitrite will be converted to nitrous acid which reacts with secondary amines to give nitrosamines (Mirvish, 1983). It is known that khat chewers consume large amounts of water during and after khat chewing (amount of nitrate in water = 80–125 mg/l), which means increased nitrate intake and, consequently, increased production of nitrite and nitrosamines. Excessive use of nitrogen fertilisers may also be one of the reasons for nitrate increase in water. Nitrogenous compounds are converted, in many soils, into inorganic forms, mainly ammonium ion, by a host of living organisms, but the ammonium does not accumulate because of the activity of the nitrifying bacteria which rapidly convert it into nitrite and then into nitrate (Lewis, 1986). In conclusion, the new findings of nitrosamines formation from *Catha edulis* leaves may be a likely explanation of the observed high incidence of esophageal and stomach carcinomas in Yemen, along with the high tannin content of *Catha edulis* leaves.

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