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NUTMEG OIL: IDENTIFICATION AND QUANTITATION OF ITS MOST ACTIVE CONSTITUENTS AS INHIBITORS OF PLATELET AGGREGATION

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Summary

Three distilled or commercially available nutmeg oils were analysed and their chemical composition compared with their capacity to inhibit platelet aggregation in vitro. It could be clearly shown that eugenol and isoeugenol play the major role in the detected activity of nutmeg. Medicinally, it appears that nutmeg oil and nutmeg powder can be replaced by eugenol and/or isoeugenol.

Introduction

In traditional medicine, nutmeg, i.e. the dried kernels of the ripe seeds of *Myristica fragrans* Houtt. (family Myristaceae), is used for several purposes. It is well known as a carminative and stomachic. In China, the powdered nut is used as a warming and astringent remedy against dysentery, especially in children and the elderly. It is a remedy for stomach cramps due to cold and also as a stimulant for the treatment of chronic rheumatism. In Indochina, powdered seeds in boiled rice are used as a remedy against dysentery, anorexia and colics. It is further used to treat malarial debility, especially the oil (Perry, 1980).

Recently, several scientific reports have been published on the therapeutic value of nutmeg in veterinary and medical practices (Fawell and Thompson, 1973; Barrowman et al., 1975; Shafran et al., 1977; Stamford et al., 1978, 1980). Nutmeg has shown beneficial effects in severe diarrhoeal conditions.

Crude extracts of nutmeg inhibit formation of prostaglandin-like

compounds in vitro (Bennett et al., 1974). Misra et al. (1978) have localised this pharmacological activity of nutmeg in the petroleum ether fraction. Rasheed et al. (1984a) reported strong evidence that the essential oil is the most important part of nutmeg as to its pharmacological activity. They investigated the pharmacological activities of the many constituents of nutmeg oil and found eugenol and isoeugenol to be the most potent compounds in vitro. Bennett et al. (1988) have enlarged the knowledge on the biological activity of eugenol.

Up to now, no detailed quantitation of the aromatic constituents of nutmeg oil has been done in relation to their pharmacological activity. The present study compares the chemical composition of several nutmeg oil samples with their in vitro antiaggregating activity.

Materials and methods

Chemicals

3-*t*-Butyl-4-hydroxyanisole (Fluka), camphene (Fluka), elemicin (Drug Synthesis and Chemical Branch, National Cancer Institute, Bethesda, U.S.A.), eugenol (Merck), isoeugenol (Fluka), limonene (Janssen Chimica), linalool (Aldrich), myristicin (Saber Laboratories, U.S.A.), α-pinene (Fluka), β-pinene (Janssen Chimica), safrole (Fluka), α-terpineol (Merck) and terpinen-4-ol (Janssen Chimica) were purchased as pure analytical standards. Indomethacin was kindly supplied by Merck Sharp and Dohme. Concentrations of these standards were 0.1 or 1% (weight/volume) in absolute ethanol (pro analysi, Merck). All solvents used (ethanol, ethyl acetate, chloroform, dichloromethane, acetic acid, sulphuric acid) were of analytical grade.

Plant materials

The authentic nutmeg screened for its pharmacological activity was purchased from Donck Food Antwerp (imported from Singapore) and from Liebig Antwerp. The latter also supplied us with pure nutmeg oil (Universal Flavour).

Preparation of the essential oil

About 100 g of dried seeds were powdered and mixed with 500–600 ml of deionised and distilled water. The mixture was gradually heated up to 150 °C on a paraffin oil bath and was distilled for 6–8 h. The commercially available nutmeg oil (Universal flavour) was used without further purification.

Column and thin layer chromatography

Nutmeg oil (10 ml) was dissolved in 10 ml of benzene and chromatographed on a silica gel column (50 cm × 2 cm) packed with 50 g silica gel (60 reinst, Merck, 70–230 mesh). The column was eluted with benzene, chloroform and ethyl acetate, 750 ml of each. The average flow rate amounted to 2.5 ml/min and fractions of 10 ml were collected.

Preliminary sample monitoring was performed by TLC analysis of every third fraction on a silica gel plate (Merck, 60 F 254, 0.25 mm layer thickness) in dichloromethane. Between 2 and 5 μl of oils and standard solutions were applied. The spots were visualized under UV (254 and 366 nm) and by spraying with anisaldehyde reagent (anisaldehyde/acetic acid/sulphuric acid; 0.5:50:1), followed by heating at 100–110 °C for 10 min. Fractions having the same composition were combined and pharmacologically investigated (see below).

Qualitative analysis of the fractionated nutmeg oil

All active fractions were characterized by GC/MS. A Hewlett-Packard quadrupole 5990A instrument was used, equipped with a 0.9 m × 2 mm glass column (3% Dexsil 300 GC on Chromosorb WHP, 100–200 mesh) and an electron impact ionisation source. Mass spectra were automatically recorded at the top of each GC peak, scanning from 50 to 400 atomic mass units. The oven temperature was programmed from 100° up to 200 °C at a rate of 10 °C/min; helium gas was used as a carrier gas at a flow rate of about 20 ml/min.

Quantitative analysis of nutmeg oils

For the quantitative analysis of the most active constituents of nutmeg oils, a Hewlett-Packard gas chromatograph Model 5700A equipped with flame ionisation detector was used. The apparatus was equipped with a siliconised glass capillary column (25 m × 0.3 mm internal diameter), 0V-17 as stationary phase and an injection splitter (Chrompack model C). The split ratio was 40:1. The initial oven temperature of 100 °C was held for 4 min; then it was programmed up to 200 °C at a rate of 4 °C/min. The injection block temperature was 300 °C. The detector was held at 250 °C. Helium was used as carrier gas at a flow of 1.5 ml/min. A calibration curve was constructed daily, containing 2, 20 and 40 μg/μl eugenol using reference mixtures and 20, 40 and 200 μg/μl terpinen-4-ol and safrole. To all standard solutions and samples (1 ml of oil, ± 0.9 g), 1 ml of a 3-*t*-butyl-4-hydroxyanisole solution (100 μg/μl) was added as the internal standard. An identical (FID detector) response was assumed for: eugenol and isoeugenol; terpinen-4-ol and terpineol; safrole, myristicin and elemicin. Peak areas were integrated automatically (Hewlett-Packard model 3380A). Special care was taken to avoid losses by volatilization or photo-oxidation: samples and standards were stoppered and stored in the dark in a refrigerator.

Pharmacologic screening

Rabbit platelet aggregation in vitro was used to quantify the pharmacological activity of the nutmeg oil samples. Rabbits (2–5 kg) were anaesthetised with pentobarbital sodium (30 mg/kg, intravenously). A commercially available solution (60 mg/ml, Nembutal, Abbott) was diluted 1:1 with 0.9% NaCl in order to avoid haemolysis. Heart puncture was done and blood (9-ml portions) was collected in plastic tubes with 1 ml 3.8% trisodium

citrate (Merck). As much as 120 ml blood can be obtained in this way from one animal. The tubes were gently mixed and centrifuged at 1400 rev./min for 10 min (MSE centrifuge) to obtain Platelet Rich Plasma (PRP). This PRP was kept under a carbogen atmosphere throughout the experiment. Platelet Poor Plasma (PPP) was prepared by centrifugation at 3000 rev./min for 10 min. Platelet aggregation was performed using 0.2 ml of PRP and a Payton dual channel aggregometer according to the turbidimetric method of Born (1962). Stirring speed of the magnetic mixing was fixed at 900 rev./min. Aggregation curves were recorded on a Kipp BD9 dual-channel recorder.

Arachidonic acid (AA, Sigma) was used as aggregating agent. A stock solution (10 mg/ml) in hexane (pro analysi, Merck) was kept in the refrigerator at −20 °C. Working dilutions were prepared each day. Portions of 100–200 µl were evaporated to dryness under nitrogen and 100 µl of a 1-M Tris buffer (pH = 8.3 at 25 °C) was added to the residue. Once the salt formed, it was mixed with 0.9 ml of NaCl 0.9%. The emulsion was kept in ice during the experiments and 25 µl was injected in the cuvet to produce an aggregation (100 µg/ml or 3.3×10^{-4} M, AA final concentration).

Essential oils or standard compounds were dissolved in absolute ethanol and diluted with 0.9% NaCl solution. For concentrated solutions, polysorbate 80 (Tween 80) was used as an emulsifying agent. PRP (200 µl) was incubated for 5 min with 25 µl oil or standard solution before AA was added to the cuvet.

Aggregations after preincubation with the same volume and concentration of ethanol or polysorbate 80 were regularly performed in order to control the solvent effect and the normal reactivity of the platelets.

Results

Qualitative and quantitative analysis of nutmeg oil

The average volume of oil recovered from purchased seeds amounted to 0.061 ± 0.004 ml/g (N = 5; mean ± S.E.M.). The pure nutmeg oils showed an inhibitory activity against AA-induced platelet aggregation. An inhibition

TABLE 1

PLATELET AGGREGATION IC_{50} VALUES (g/ml) OF NUTMEG ESSENTIAL OIL FROM THREE SOURCES

Source	ED_{50} ± S.E.M. ($\times 10^{-5}$)[a]
Donck	1.71 ± 0.63
Liebig	1.35 ± 0.89
Universal Flavour	1.59 ± 1.16

[a]Each value was calculated from six experiments per oil. The oil from Universal Flavour was commercially prepared.

TABLE 2

TLC R_f VALUES OF NUTMEG STANDARD COMPONENTS

Reference standard	Concentration in ethanol (%)	R_f
Safrole	1.0	0.70
Myristicin	0.1	0.60
Isoeugenol	1.0	0.45
Eugenol	1.0	0.45
Terpinen-4-ol	1.0	0.27
Elemicin	0.1	0.25
α-Terpineol	1.0	0.18

of 50% (IC_{50}) was obtained with concentrations of 1.35 to 1.71 $\times$ 10^{-5} g/ml. Singapore oil (Donck) had the lowest activity, but this difference was insignificant (Table 1). In this instance, differences between IC_{50} values should not be taken into account as the spread of the methodology exceeds them.

Qualitative composition of the oils was roughly checked by means of thin layer chromatography (TLC). In Table 2, the R_f values obtained are given. The following components could be compared with standard spots: safrole, myristicin, eugenol and/or isoeugenol, terpinen-4-ol, elemicin, α-terpineol.

A more detailed investigation of the composition of the active fractions was carried out by GC/MS. An aliquot of every third batch collected after preparative column chromatography was examined for its pharmacological activity. Only five batches were found to be active: (i) Fraction A: contained large amounts of myristicin and a little safrole; (ii) Fraction B: contained equal amounts of isoeugenol and myristicin; (iii) Fraction C: contained methyleugenol and a trace of eugenol and isoeugenol; (iv) Fraction D: contained myristicin, a small amount of eugenol but no isoeugenol; (v) Fraction E: contained elemicin. Other volatile monoterpenes, such as α- and β-pinene were also found, but these showed no pharmacological activity. No other oxygen-containing aromatics were found in the same concentration range. Methyleugenol showed a relatively low activity as compared to eugenol and isoeugenol. Since its concentration was roughly the same as that of elemicin (Forrest and Heacock, 1972), its contribution to total platelet aggregating activity was considered unimportant in a first approach.

These compounds were quantified by capillary GC/FID, using 3-*t*-butylhydroxyanisole as the internal standard. Unfortunately, pure methyleugenol was not available for peak identification or quantitation. There is a good linearity for detection of the aromatic compounds in the working concentration range (correlation coefficient of linear regression averaged 0.992 $\pm$ 0.011, N = 6). Identification of the pharmacologically tested products was carried out by calculation of the relative retention times with 3-*t*-butyl-hydroxyanisole as

TABLE 3

MEAN (±S.E.M.) CONCENTRATIONS (g/100 ml) OF PHARMACOLOGICALLY IMPORTANT CONSTITUENTS OF NUTMEG OIL

Constituents	Donck (N = 4)	Liebig (N = 3)	Universal Flavour (N = 9)
Terpinen-4-ol	6.70 ± 0.30	7.20 ± 0.20	10.50 ± 0.90
α-Terpinenol	0.74 ± 0.03	0.79 ± 0.04	1.12 ± 0.09
Safrole	3.40 ± 0.02	2.90 ± 0.10	2.40 ± 0.20
Eugenol	0.42 ± 0.09	0.33 ± 0.05	0.44 ± 0.06
Isoeugenol	0.38 ± 0.17	0.17 ± 0.00	0.24 ± 0.05
Myristicin	11.20 ± 0.10	8.10 ± 0.10	8.40 ± 0.50
Elemicin	0.38 ± 0.01	0.35 ± 0.01	1.45 ± 0.07

internal standard. The relative retention times were 0.765 ± 0.002 (N = 6) for eugenol and 0.910 ± 0.002 (N = 6) for isoeugenol. At the retention time of the internal standard no important peaks were present on the chromatogram. In Table 3 the calculated concentrations for the three sources of nutmeg oil are given.

Inhibition of platelet aggregation

It has already been reported that eugenol and isoeugenol are the most potent constituents of nutmeg oil for the inhibition of platelet aggregation induced by arachidonic acid (Rasheed et al. 1984a), and that all other constituents are 100 to 1000 times less potent. In the present study, no IC_{50} could be determined for linalool, α- and β-pinene, α-terpineol, terpinene-4-ol and camphene, because they lacked activity. There was no potentiating action of one compound on another, when equal amounts of eugenol, safrole,

TABLE 4

PLATELET AGGREGATION IC_{50} VALUES OF NUTMEG OIL CONSTITUENTS AND INDOMETHACIN

Product	IC_{50} (M)
Eugenol	3.0×10^{-7}
Isoeugenol	7.2×10^{-7}
Safrole	1.1×10^{-4}
Myristicin	2.5×10^{-4}
Elemicin	3.6×10^{-4}
Indomethacin	2.2×10^{-7}

myristicin and α-terpineol ($N = 8$) were mixed. In Table 4 the molar IC_{50} values are given in molar terms in order to compare the activity of the different compounds on a molecular basis.

Discussion

Power and Salway (1907) were among the first to study the chemical composition of the essential oil of nutmeg. They found 6.94% of oil on a weight basis relative to nutmeg powder. They identified myristic acid, eugenol, isoeugenol, pinene, camphene, linalool, geraniol and safrole. These identifications were mainly made by chemical reactions, elemental analysis, melting and boiling points. Basically, the oil was shown to consist of a monoterpene hydrocarbon fraction (± 80%), a monoterpene alcohol fraction (± 4%) and an aromatic ether fraction (± 11%), together with small quantities of miscellaneous compounds. More recently Forrest and Heacock (1972) could identify 20 compounds by mass spectrometry peak enrichment. Among them, α-pinene (26.7%), β-pinene (20.7%), sabinene (14.5%) and limonene (9.4%) were most prominent. Their results were further supported by Davis and Cooks (1982) who applied MS-MS to analyze nutmeg oil components. Harvey (1975) identified 11 diphenylpropanoids in ethyl acetate extracts of nutmeg samples. He examined 13 samples and found that there was considerable variation in the total percentage of diphenylpropanoids present in the different samples. The presence of diphenylpropanoids did not exceed 2.1% in the extracts. Schenk and Lamparski (1981) identified 7 new sesquiterpene hydrocarbons in nutmeg oil. The total sesquiterpene fraction represents 1% of the oil.

Very recently the Essential Oils Subcommittee of the Royal Society of Chemistry of the U.K. published a monograph on the application of GC to the analysis of essential oils such as nutmeg oil. The GC fingerprint chromatograms of nutmeg oil using two different stationary phases showed the presence of 25 and 31 compounds, respectively, which were identified by GC/MS (Analytical Methods Committee, 1988).

The present study has been focused on the compounds that could be obtained as pure compounds, especially those showing antiaggregating activity (Rasheed et al., 1984a). Gas chromatography on capillary columns with flame ionization detection was revealed to be a suitable technique to quantify the pharmacologically most active compounds. Our results correspond qualitatively and quantitatively with those published by Forrest and Heacock (1972). They found 0.1% for eugenol and 0.1% for isoeugenol.

It should be possible to make rough calculations as to the pharmacological activity of different oils using the concentrations of eugenol and isoeugenol in the oils as a basis. We calculated theoretical IC_{50} values as if the oils were only composed of eugenol and isoeugenol diluted with pharmacologically inert material. These calculations resulted in values that could be compared to the actual IC_{50} values obtained for the real oils tested using in vitro blood platelet aggregation. Both are compared in Table 5.

TABLE 5

COMPARISON BETWEEN EXPERIMENTALLY OBTAINED IC_{50} VALUES (g/ml) OF NUTMEG OIL AND CALCULATED ACTIVITY BASED ON EUGENOL AND ISOEUGENOL CONTENTS IN THE OILS

Oil source	Experimental IC_{50} ± S.E.M. ($\times 10^{-5}$)	Calculated IC_{50} (95% CL) ($\times 10^{-5}$)
Donck	1.71 ± 0.63	0.86 (0.67 – 1.17)
Liebig	1.35 ± 0.89	1.21 (1.17 – 1.39)
Universal Flavour	1.59 ± 1.16	0.90 (0.79 – 1.05)

Rasheed et al. (1984b) suggested the substitution of pure eugenol for nutmeg powder or oil. Our results give a real analytical/pharmacological basis for this suggestion. Our analytical approach combined with the in vitro bioassay data give evidence for the study of eugenol and/or isoeugenol as the nutmeg constituents responsible for antiaggregating activity. Since the experimental and theoretical data tend to overlap, one can consider the oil as a kind of matrix containing a limited number of active principles. In artificial mixtures, no potentiating action of one compound on another could be demonstrated.

Recently structure-activity studies on the carcinogenicity in mice and rats of naturally occurring and synthetic alkenylbenzene derivatives related to safrole have been carried out (Miller et al., 1983). Apparently no direct correlation could be found between the pharmacological activity of the aromatic ethers of nutmeg oil and their hepatocarcinogenicity in rats and mice. Beside safrole and methyleugenol, no other constituents had detectable activity for the initiation of hepatic tumors on administration to mice.

The question arises whether crude nutmeg products should be used medicinally. Eugenol is the major constituent of oil of clove obtained from *Syzygium aromaticum* (L.) Merril et Perry. It has been used in flavouring and in medicine (especially in dentistry) as an antiseptic and local anesthetic. In traditional medicine, eugenol is used to treat gastrointestinal upsets and chronic diarrhoea (List and Horhammer, 1973). Eugenol is approved by the Food and Drug Administration of the U.S.A., but its internal use has not been fully explored.

It has been noted that high doses of nutmeg can cause atropine-like side-effects (Payne, 1963). Nutmeg oil also decreased fertility in rodents (Pecevski et al., 1981) and some nutmeg constituents can induce tumors in rats and mice (Miller et al., 1983). As nutmeg oil contains a number of inactive compounds which can cause unwanted side-effects, it makes sense to investigate further pure eugenol as a medicinal substitute for nutmeg and nutmeg oil.

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