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Isolation of an antibacterial component from roasted coffee[1]

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Abstract

A coffee beverage obtained from instant dark coffee that had been previously shown to possess high antibacterial activity, was acidified (pH 2) and extracted with ethyl acetate. After alkalinization (pH 12) the aqueous phase was re-extracted with the organic solvent. The acidic and basic extracts were evaporated to dryness and the aqueous phase freeze-dried. Residues were dissolved in sterile water and assayed for antibacterial activity against two reference bacteria (*Staphylococcus aureus* ATCC 25923 and *Streptococcus mutans* 9102). The acidic extract was found to be highly active and was separated by gel permeation chromatography (GPC) into five fractions. Fractions GPC_4 and GPC_5 were found to possess antibacterial activity: most of the activity was evident in fraction GPC_5. These fractions were separated by RP-HPLC using a gradient elution with methanol–water as mobile phase. Both GPC fractions gave an active subfraction with methanol–water (70:30, v/v). The experimental conditions used to separate the antibacterial compound that originates during the roasting process, indicate that it possesses low molecular mass (probably no more than 200 Da), weak acidic properties and an λ_{max} at 205 nm. The very small amount of this compound isolated from roasted coffee, indicates that it may be a very strong antibacterial agent.

Keywords: Roasted coffee; Antibacterial activity; Antimicrobial agent; Anticariogenic agent

1. Introduction

Coffee is one of the most widely consumed beverages in the world, because of its pleasant aroma and taste, its pharmacological effects and, above all, its stimulatory effects on mental and physical activity.

The beverage is a very complex mixture of several hundred chemicals which are either naturally occurring or else induced by the roasting process. The chemistry of these substances has not yet been completely elucidated.

Most literature reports concern caffeine and its pharmacological effects but recently other bioactivities of coffee have been investigated. Coffee has been found to act as a potent antioxidant and to inhibit peroxidation in model systems [1]. It was shown that coffee possesses antibacterial properties against a number of bacteria such as

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Staphylococcus aureus and *Streptococcus mutans* that has been implicated as primary causative agent of dental caries [2–4]. Since green coffee does not show antibacterial activity, the activity is clearly induced by the roasting process and has been shown to depend on the degree to which the coffee beans are roasted. The antibacterial activity of roasted coffee must therefore be attributed to compounds generated during the Maillard reaction, carbohydrate caramelization, thermal decomposition and pyrolysis of organic compounds. To date, the compounds responsible for the antibacterial activity have not been isolated.

The aim of this study was to isolate the active antibacterial components from the coffee brew. The results would thus be useful for defining the chemical composition of the beverage and the changes occurring during roasting. Furthermore, considering coffee as a possible source of non traditional, natural antimicrobial agents, the active isolated components might have potential use as anticariogenic drugs and as food additives for prolonging the shelf-life of food.

2. Experimental

2.1. Coffee beverage preparation and organic extraction

Instant coffee (12.5 g) was prepared using boiling Millipore grade water (500 ml). The beverage obtained was filtered and a 50 ml aliquot was extracted with ethyl acetate. The extract was evaporated to dryness under reduced pressure at 40°C and the residue was freeze dried and the residue dissolved in 5 ml of sterile water; the aqueous phase was dissolved in 5 ml of sterile water and then both were assayed for antibacterial activity. Moreover, filtered coffee beverages (50 ml) were acidified with HCl (10% w/v; pH 2) and extracted with either ethyl acetate, ethyl ether or dichloromethane. The aqueous phases were adjusted to pH 12 with NaOH (10% w/v) and re-extracted with the organic solvents. The extracts were evaporated to dryness and the residues were dissolved in 5 ml of sterile water. The aqueous phases were freeze-dried, the residues

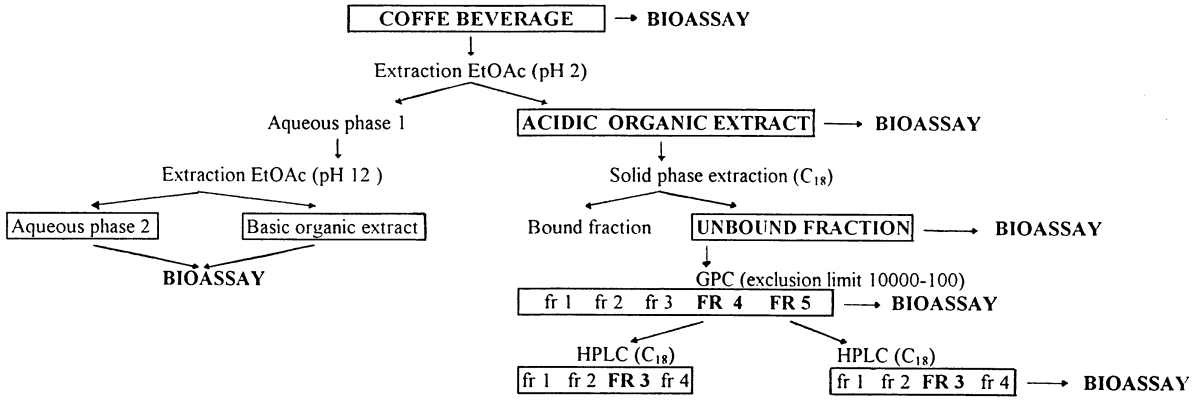

Fig. 1. Scheme of extraction and isolation of the component with antibacterial activity.

Table 1
Antibacterial activity (MIC) of coffee beverage and coffee fractions obtained by organic extraction

Bacterium	MIC (mg ml^{-1})			
	Coffee beverage 2500 mg	Acidic extract 288 mg	Basic extract 45 mg	Aqueous phase 2150 mg
S. aureus ATCC 25924	1.56	0.84	—	13.5
S. mutans 9102	<1.56	0.76	—	12.7

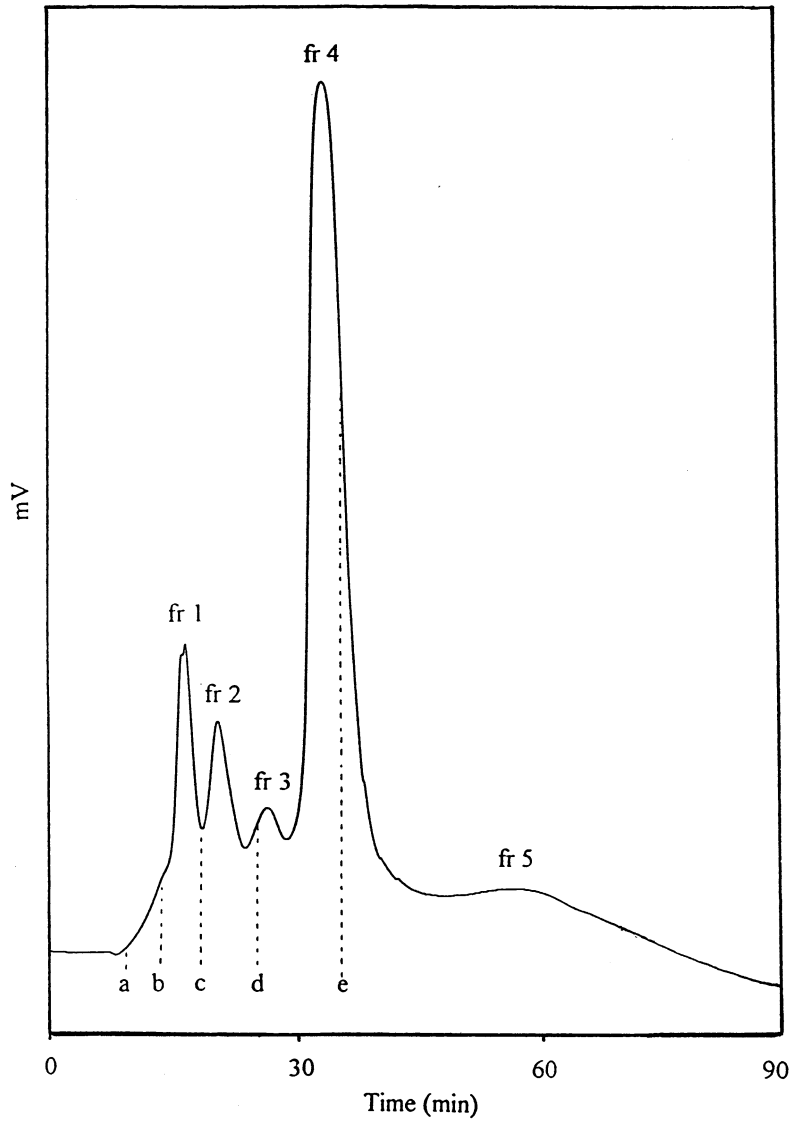

Fig. 2. GPC of coffee extract. Operative conditions were as described in the text. (a) Dextran T 10 (MW 10000); (b) Polystyrene (MW 5970); (c) Melizitose (MW 594); (d) Chlorogenic acid (MW 354); (e) Caffeine (MW 194).

were dissolved in 5 ml of sterile water and all fractions obtained were assayed for antibacterial activity.

2.2. Fractionation of ethyl acetate acidic extract

The water soluble active acidic extract (obtained with ethyl acetate) was separated into two fractions using a Bakerbond C_{18} solid phase extraction cartridge. The C_{18} cartridge was preconditioned with 5 ml of methanol–Millipore grade distilled water (30:70, v/v). After sample loading (1 ml), C_{18} cartridge was washed with 2 ml of water and C_{18} bound compounds were eluted with 3 ml methanol–water (50:50, v/v). After evaporation of the solvent, each fraction was weighed, dissolved in a 1 ml aliquot of sterile water and tested for microbiological activity. The active un-

bound fraction was separated into five fractions by preparative gel permeation chromatography (GPC) using a Merck Superformance universal glass-cartridge system 300 × 10 mm i.d. column with Toyopearl HW 40 (F) packing (Tosohaas, Tokyo). The system was equipped with a Waters 490E UV-Vis detector (Waters Chromatography Division, Milan) and a Hitachi-Merk D 2500 integrator. Mobile phase was Millipore grade distilled water and flow rate was 1 ml min^{-1}. UV detection was at 205 nm.

After evaporation of the solvent, each fraction was weighed, dissolved in a 500 μl aliquot of sterile water and tested for microbiological activity.

The active fractions obtained from preparative GPC were further divided into four subfractions by preparative RP-HPLC using a Merck LiChrosphere 100RP-18 300 × 10 mm i.d. column. The mobile phase was methanol (A)–orthophosphoric acid (B) (pH 4; 0.25 mM). Mobile phase composition was 40% A and 60% B at time 0, composition increased toward 80% A and 20% B for the next 85 min and thereafter it decreased to 40% A and 60% B for the next 10 min. Flow rate was 1 ml min^{-1} and UV detection was at 205, 254 and 270 nm. Samples were injected via a Rheodyne 7125 injection valve with a 1000 μl loop. The HPLC eluates were evaporated to dryness, the residues dissolved in 500 μl of sterile water and submitted to the microbiological assay.

2.3. Antibacterial activity assay

Antibacterial activity was assayed against *S. aureus* ATCC 25923 and *S. mutans* 9102. The minimum inhibitory concentration (MIC) was determined using the broth dilution method. Desired concentrations were achieved by the addition of appropriate volumes of coffee fractions to 1 ml of Iso-Sensitest broth (ISB, Oxoid) in 15 × 150 mm test tubes.

Overnight cultures, diluted with sterile broth, were added to the test tubes to bring inoculum size to $\sim 10^4$–10^5 CFU ml^{-1}. The MIC value was evaluated after 18 h of incubation at 37°C, as the lowest concentration that completely inhibited the formation of visible microbial growth. MIC was expressed as mg ml^{-1} (w/v) of the obtained fraction residue or as μl ml^{-1} (v/v) of the obtained fraction.

3. Results and discussion

The extraction and isolation scheme for the antibacterial active component is reported in Fig. 1. Instant dark roasted coffee is a highly complex mixture of several hundred compounds. The coffee beverage, obtained by the addition of boiling water to instant dark roasted coffee, was submitted to organic extractions. The results obtained by the extractions conducted at the pH of the beverage or after acidification and alkalinization with dichloromethane or ethyl ether or ethyl acetate,

Table 2
Molecular masses and antibacterial activity (MIC) of coffee fractions obtained by GPC of acidic extract

Coffee fractions	Molecular mass	MIC (mg ml^{-1})	
		S. aureus ATCC 25923	*S. mutans* 9102
GPC_1	<6000	N.A.[a]	N.A.
GPC_2	<550	N.A.	N.A.
GPC_3	350<MW<550	N.A.	N.A.
GPC_4	200<MW<350	1.86	1.95
GPC_5	<200	0.34	0.34

[a] N.A., No antibacterial activity found.

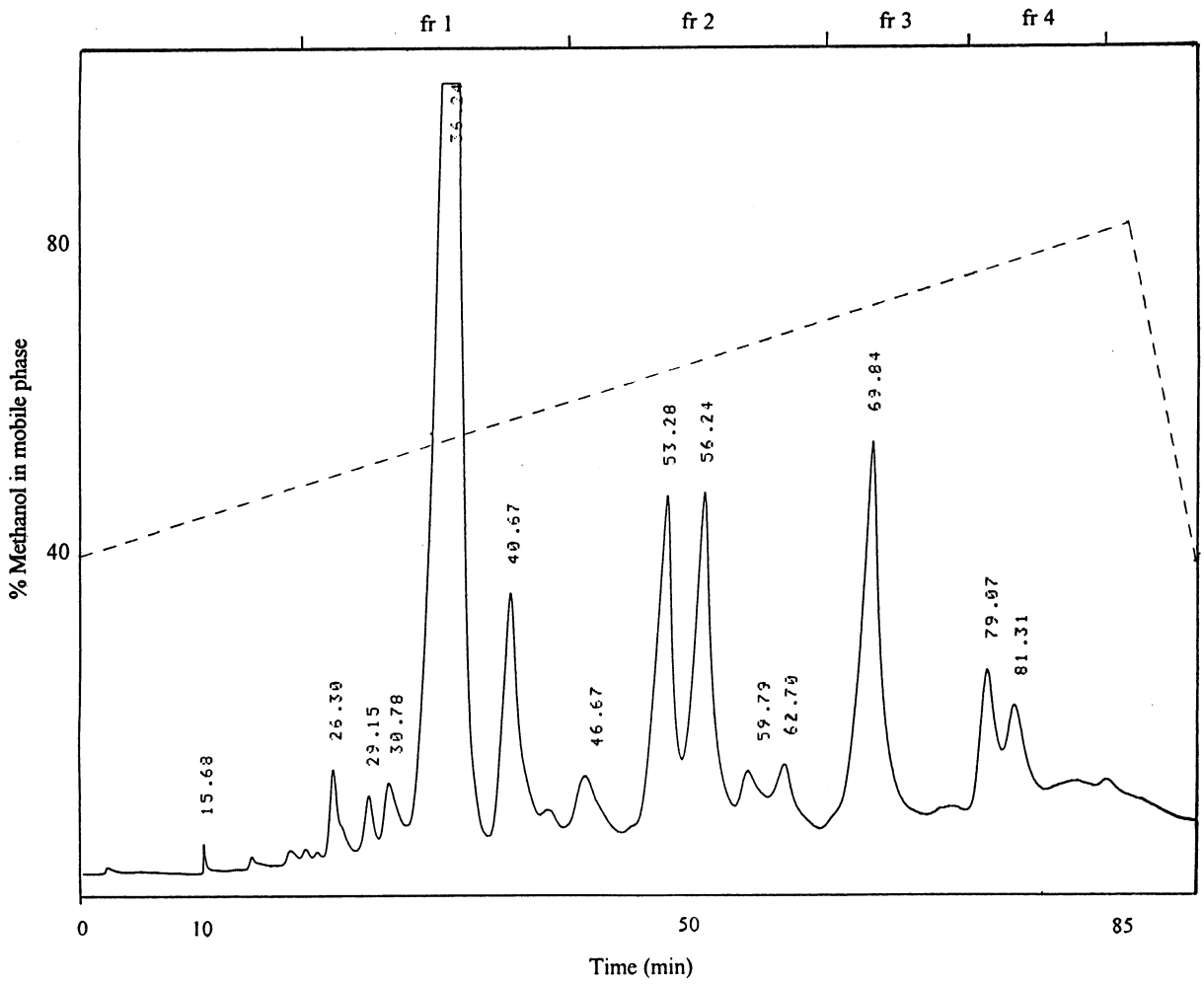

Fig. 3. RP-HPLC of GPC_5 fraction. Solvent and gradient conditions were as described in the text. The dashed line indicates variations in mobile phase compositions during the analysis.

showed that almost complete extraction of the active components was only obtained when ethyl acetate was used on the acidified beverage. After extraction in fact the beverage had a very low antibacterial activity as shown by the MIC value (w/v) of the aqueous phase (aqueous phase 2). All basic extracts obtained by extraction with the different organic solvents never showed activity (Table 1).

The water soluble fractions (7.9% of the instant coffee) which occurred in yellow-brown ethyl acetate acidic extract was still very complex and the less polar unactive compounds were removed by Baker C_{18} cartridge. The more polar unbound active compounds were separated by gel-permeation chromatography (Fig. 2) into five fractions. Each was freeze-dried, the residues were weighed, dissolved in sterile water and tested for antibacterial activity. The MIC values (w/v) and the molecular masses for each fraction are shown in Table 2. The pale-yellow GPC_4 and GPC_5 fractions, that contained the components with lower molecular masses, were found to be active. These fractions represented about 0.21 and 0.09% of instant coffee, respectively.

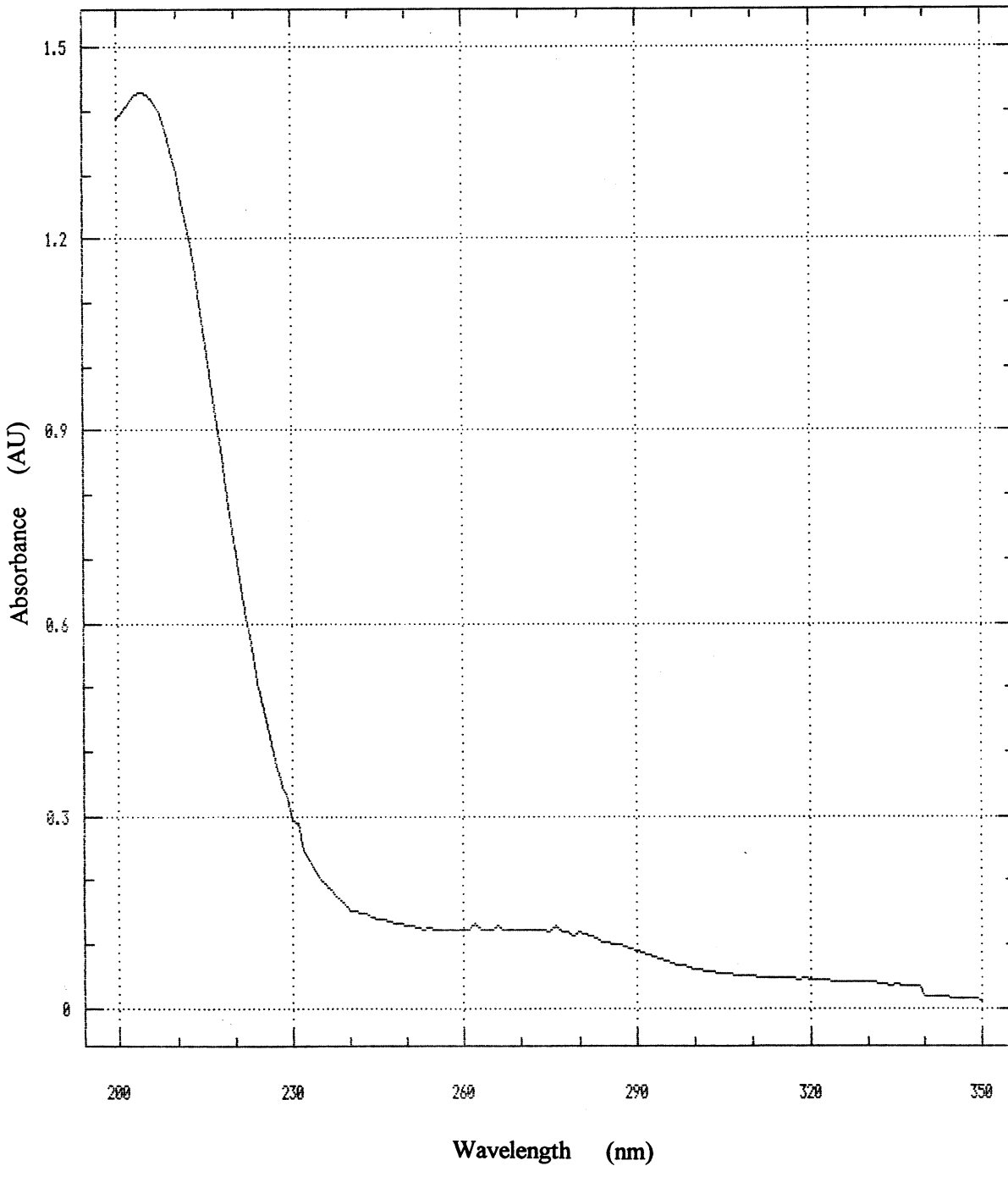

Fig. 4. UV spectrum of active antibacterial HPLC fraction.

Further separation of GPC_4 and GPC_5 by RP-HPLC resulted in quite complex HPLC profiles (Fig. 3). The eluates obtained from HPLC analysis of the GPC_4 and GPC_5 fractions, were both

subdivided to give four subfractions (see Fig. 3). Both colorless eluates (70% A–30% B; RT about 70 min) obtained from HPLC analysis of the GPC_4 and GPC_5 fractions were found to be active and both showed maximum absorption at λ 205 nm (Fig. 4). However, the finding that the eluate obtained from fraction GPC_4 had much less antibacterial activity and lower absorbance (MIC = 6% v/v; $AU_{\lambda 205} = 0.200$) than the eluate obtained from fraction GPC_5 (MIC = 2% v/v; $AU_{\lambda 205} = 1.430$) would appear to indicate that only one component with strong antibacterial activity had been isolated. The fact that activity was evident in both fractions GPC_4 and GPC_5 is because of their incomplete resolution in the actual experimental conditions.

4. Conclusions

The results obtained thus far, seemed to demonstrated that the coffee roasting process probably generated only one compound with strong antibacterial properties against both Gram-positive and Gram-negative bacteria. The very small amount of this compound which occurred in the roasted coffee indicates that it must be a very strong antibacterial agent. Furthermore, the fact that this compound was extracted with an organic solvent in acidic media, was eluted from a C_{18} column with a high percentage of organic solvent, and was slowly eluted from the GPC column used, indicates that it possesses weak acidic properties and a small molecular mass, probably no more than 200 Da.

Investigations are currently under way in our laboratory to better isolate the highly active compound and to characterize its chemical structure.

Acknowledgements

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