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Multidrug resistance reversal in mouse lymphoma cells by indian tea leaves, indian coffee seeds and chicory

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SUMMARY

Systematic analysis of caffeine from the commercial samples of Indian tea leaves was performed by a routine method and the content of caffeine was found to be 19.0-37.4 mg/100 g leaves. The caffeine contents from coffee seeds and chicory from Indian origin were analyzed and found to be 0.6540-1.4920 g/100 g seeds. Caffeine contents of roasted Indian chicory roots were lower than either those of Indian tea leaves or Indian coffee seeds. The multidrug resistance (MDR) reversing effects were tested on a mouse leukemia cell line of L-5178 cells by methanol extracts [**M1-M15**] of Indian tea leaves and coffee seeds, comparing to a control of (±)-verapamil. The effects were measured by fluorescence ratio between treated and untreated group cells. Among fifteen methanol extracts, a Gemini tea [**M6**] (fluorescence activity ratio 5.26) had the most potent effect for L-5178 cells. The extract **M6** was 0.63-fold of (±)-verapamil. We suggest that one of mechanisms of reversal by **M6** might have strong affinity to dopamine D_1 and D_2 receptors. Further studies with many more tumor and normal cell lines are necessary to confirm the MDR reversal specificity of coffee methanol extracts.

Key words: Indian teas; Caffeine; Chicory; Methanol extracts; MDR reversal

INTRODUCTION

Indian teas of *Orthosiphon stamineus* Benth (Labiaceae) are widely cultured in Southeast Asia to Australia. Decoction of the leaves is also called Java tea. Indian tea is used as a diuretic, for ailments of the bladder and kidneys as a natural folklore medicine, especially in The Netherlands and Java (Uphof, 1968a). Coffee trees of *Coffea arabica* L. (Rubiaceae) (Arabian coffee), *Coffea liberica* Hiern (Rubiaceae) (Liberian coffee) and *Coffea stenophylla* G. Don. (Rubiaceae) (Highland coffee of Sierra Leone) are mainly cultured to get coffee beans. Roasted coffee beans are source of a beverage, being prepared in different ways, also used for flavoring ice cream, candies, soft drinks and pastries. Roasted coffee beans are also an important source of caffeine (3,7-dihydro-1,3,7-trimethyl-1*H*-purine-2,6-dione), which is one of the most widely consumed xanthine derivatives. The dried ripe seeds are used medicinally as stimulant, nervine, diuretic and can act on the central nervous system (CNS), kidneys, heart and muscles (Uphof, 1968b). Roasted roots of chicory of *Cichorium intybus* L. (Compositae) are a well-known substitute for coffee seeds. The dried roots are also medicinally used in diuretic, tonic, stomach, depurative and also in homeopathy for liver and gall ailments (Uphof, 1968c).

Caffeine has been confirmed to enhance the cytotoxic effect of antitumor agents on tumor cells by either necrosis, enzyme inhibitions or programmed cell death (apoptosis).

Concerning the effect on necrosis of cancer cells, a combination of caffeine with a phenothiazine enhanced significantly the antitumor effect of an

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alkylating agent of 1,3-*bis* (2-chloroethyl)-1-nitrosourea in murine leukemia L1210. It is proposed that there is possibility of charge transfer (CT) reaction between the free radical formation of phenothiazine and one or more intracellular constituents is responsible for the effect (Cohen, 1973; Cohen, 1975). Caffeine enhanced the antitumor activity of adriamycin (ADR), in terms of prolonging survival of Ehrlich ascites carcinoma-bearing mice. The effect of caffeine depends on its effect on the tumor distribution of ADR (Sadzuka *et al.*, 1995). Caffeine had a high enhancer effect on L929 cells exposed to tumor necrosis factor (TNF) for 24 hours. L929 cells stimulated with TNF died *via* apoptosis, as judged by both morphological criteria and the occurence of internucleosomal DNA cleavage. Exposure of TNF-treated cells to caffeine caused a greater increase in the proportion of apoptotic cells as well as the extent of internucleosomal DNA fragmentation (Belizaro *et al.*, 1993). In experiments using methylxanthines, adozelesin was synergistic with nontoxic doses of caffeine (Smith *et al.*, 1995).

In the antimutagenic effect of caffeine as enzyme inhibitors, caffeine acts by partially inhibiting some repair processes acting in the oocytes, processes that are required for the repair or misrepair of chromosome breaks induced in the spermatozoa (Mendelson and Sobels, 1974). Caffeine reduces the efficiency of a system in *Drosophila* oocytes that repairs X-ray-produced chromosome breaks in both the paternal and maternal genomes (Mendelson D, 1974; Sobels and Mendelson, 1975; Semenov *et al.*, 1994). Caffeine with seeds of *Crepis capillaris* could modify the rate of mutagenesis induced, as well as to affect the content of endogenous cAMP. Then, sometimes, higher concentrations of caffeine could apparently decrease the cyclicnucleotide content in the seeds (Semenov *et al.*, 1994). In antimutagenic mechanism of coffee extraction against tryptophan-P-1 (Trp-P-1) using of *Salmonella typhimurium* TA98 under the presence of S-9 mixture, low molecular weight fraction of coffee extracts showed strong antimutagenicity. Coffee extracts exhibit its antimutagenicity by reacting with activated Trp-P-1 rather than S-9. Coffee extracts showed a strong scavenging activity against hydoxyl radical and superoxide anion (Nguyen *et al.*, 1995). Anti-mutagenic effects of caffeic acid on by *Salmonella typhimurium* were studied. Apparently, caffeic acid was effective on the mutagenicity of Trp-P-1 (Yamada and Tomita, 1996).

Third, on apoptosis of caffeine, coffee cherry could enhance the differentiation of thymocytes and the activation of peripheral T lymphocytes (Kobayashi *et al.*, 1996). Isobologram analysis of the 50% inhibitory concentration (IC_{50}) revealed that caffeine displays their potentiating effect when combined with cisplatin. Caffeine could enhance the cytotoxic effect of antitumor agents by shortening the duration of the S phase and relieving the cell cycle arrest in the G2 phase, thereby limiting the repair process of damaged DNA. In this process, caffeine could reduce the dose of antitumor agent needed and antitumor activity of cisplatin is attributable to apoptosis, and a combined treatment with caffeine enhances the induction of apoptosis (Shudo *et al.*, 1997). In the enhancement of the antitumor effect and apoptosis induction of the *cis*-diaminedichloroplatinum (CDDP) and caffeine combination, when the concentration of CDDP was low, CDDP significantly decreased the proliferation of STKM-1 human stomach cancer cells, thus the synergistic effect of the CDDP and caffeine group is evident. The apoptosis index of the CDDP plus caffeine combination was significantly higher than that of the CDDP group (Takahashi *et al.*, 1998).

Indian teas and coffees might be the most important source of caffeine as an enhancer of natural products. The purpose of this paper is to search the increasing effects for Indian tea and coffee with higher contents of caffeine in India. For this study, Indian teas and coffees were collected from the main product places in India and the isolation and crystallization of compounds including caffeine were performed.

MATERIALS AND METHODS

Materials

All materials in Table 1 were collected from the market in India.

Isolation of caffeine from Indian tea and Indian coffee seeds

A dried Indian tea or ground Indian coffee seed (regular grind) (100 g) and water (363 mL) were refluxed for 20 min (Fig. 1) (Pavia *et al.*, 1976). The

mixture was filtered. 10% Lead acetate (73 mL) was added to the dark brownish water filtrate, a brown precipitate was removed by filtration. A dark brown filtrate (363 mL) was extracted with chloroform (2 ×73 mL) and 10% NaOH (30 mL). The combined extracts (146 mL) were washed with H_2O (60 mL), dried over anhydrous Na_2SO_4, and concentrated under reduced pressure. The crude caffeine was weighed and determine m.p. of this crude caffeine (sublimate) (Pavia *et al.*, 1976). The isolation of caffeine from roasted chicory roots was also adopted by the method of Indian tea and Indian coffee seeds.

Crystallization of caffeine

The crude caffeine could be crystallized by a mixed solvent method as follows: the crude solid was dissolved in a few amount of hot benzene and adds petroleum (60-80°C) to turn the solution faintly cloudy. The solution was cooled and collected the caffeine crystals by filtration. The m.p. of pure caffeine was determined (Pavia *et al.*, 1976). The crystallization of caffeine from Indian tea leaves and roasted chicory roots were also followed by the method of Indian coffee seeds.

Preparation of extracts

Methanol extracts: Dried leaves of Indian tea or seeds of coffee (100 g) were extracted with methanol (363 mL) under reflux for 20 min. The mixture was filtered and the filtrate was evaporated using a rotary evaporator under reduced pressure (Fig. 1).

Aqueous extracts: Dried leaves of Indian tea or seeds of coffee (100 g) were extracted with water (363 mL) under reflux for 20 min. The mixture was filtered and the filtrate was evaporated under reduced pressure (Fig. 1).

Cell and fluorescence uptake, multidrug reversal (MDR) effect: The L5178 mouse T cell lymphoma cell line was infected with the pHa *MDR1/A* retrovirus as previously described (Aszalos *et al.*, 1995). *MDR1* expressing cell lines were selected by culturing the infected cells with 60 ng/mL colchicine to maintain expression of the MDR phenotype. The L5178 MDR cell line and the L5178Y parent cell line were grown in McCoy's 5A tissue culture medium with 10% heat-inactivated horse serum, L-glutamine and 15 methanol extracts **[M1-M15].** The cells were adjusted to a concentration of 2×10^6/mL and resuspended in serum-free McCoy's 5A medium and the cells were distributed into 0.5 mL aliquote to Eppendorf centrifuge tubes. Then, the tested compounds were added in various concentrations (0.2-20.0 μL) of the 1.0 mg/mL stock solutions and the samples were incubated for 10 min at room temperature. Next, 10 μL (5.2 μM final concentration) of rhodamine 123 (R123) were added to the samples and the cells were incubated for further 20 min at 37°C, washed twice and resuspended on 0.5 mL phosphate buffered saline (PBS) for analysis. The fluorescence of cell population was measured by flow cytometry using Beckton Dickinson FACScan instrument. (±)-

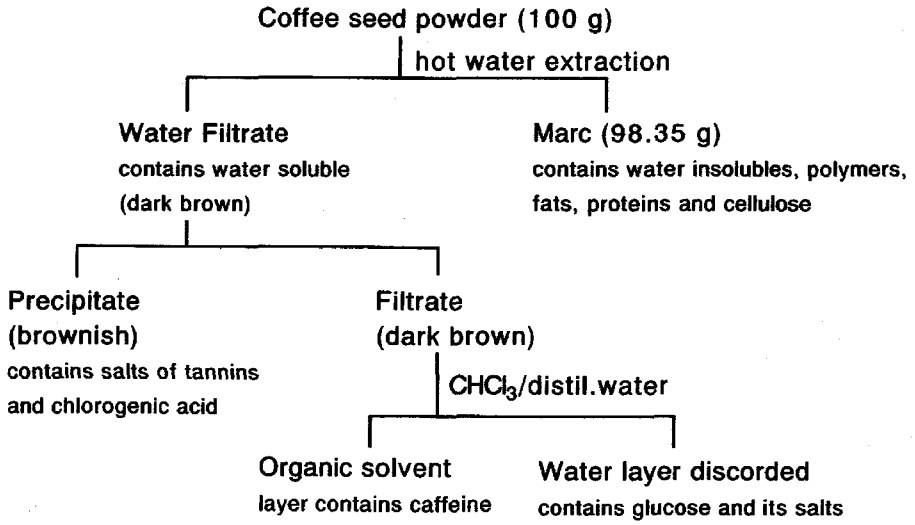

Fig. 1. Systematic analysis of caffeine from coffee seeds.

Table 1. Caffeine from commercial samples of Indian tea leaves, dusts, seeds and chicory and the effect of methanol extracts [**M1-M15**] on lymphoma 5178 cells with multidrug resistance

Extract's No. (origin)	Caffeine				MDR test				
	Content (%)[a]		m.p. (°C)	Caffeine (mg)/100g leaf or dust tea[b]	Concentration (μg/mL)[c]	Forward scatter height	Side scatter height	Fluorescence[d]	Fluorescence activity ratio[e]
	Coffee	Chicory							
par (control)[f]						582.22	142.22	839.68	30.25
MDR+R123 (control)[g]						629.12	212.82	27.76	1.00
(±)-verapamil[h]					5	626.09	228.71	233.50	8.41
Methanol extracts:									
M1(Taj Mahal Tea Brooke Bond Mumbai-400 020)			220	37.4/l	20	623.94	237.32	46.09	1.77
M2(Society Tea Hasmukhrai & Co. Mumbai-400 012)			235	36.6/l	20	623.43	237.32	137.88	4.97
M3(Nestea Nestle New Delhi-110 001 Mumbai-400 020)			237	26.5/d	20	631.23	233.95	41.61	1.50
M4(Top Star Tea, Lipton Mumbai-400 020)			210	25.0/d	20	615.06	227.18	41.96	1.51
M5(Chakra Gold Tea, Tata Calcutta-700 020)			220	24.0/l	20	627.76	229.03	65.83	2.37
M6(Gemini Tea, Tata Calcutta-700 020)			225	23.0/l	20	638.74	236.71	146.14	5.26
M7(Three Roses Tea, Brooke Bond, Mumbai-400 020)			214	19.0/l	20	644.15	236.17	65.48	2.36
M8(Pure Coffee Seeds, Mangalagiri, A. P.)	100	0	238	1040.0/p	20	654.11	237.01	21.13	0.76
M9(Nestle, Sunrise Premium, New Delhi-110 001)	70	30	236	1200.0/g	20	654.65	234.47	17.27	0.62
M10(BRU, Brooke Bond, Filter Coffee, Mumbai)	70	30	228	1070.0/fp	20	647.24	230.74	23.58	0.85
M11(Tata Kaapi, Instant coffee. Karnataka)	70	30	241	900.0/fp	20	663.75	243.94	19.45	0.70
M12(Family Blend Trinadha, Coffee Powder, Guntur)	60	40	233	650.0/p	20	660.63	227.63	16.64	0.60
M13(Brooke Bond Cafe Gold, Hindustan Liver Ltd., Mumbai-400 020)	53	47	235	1490.0/fp	20	634.70	232.08	23.79	0.86
M14(Tata Coorg Double Roast, Consolidated Coffee Ltd., Coorg Karnataka)	53	47	235	1230.0/p	20	623.08	228.88	22.71	0.82
M15(Green Label, Hindusthan Lever Ltd., Mumbai-400 020)	53	47	234	1040.0/g	20	636.77	233.48	22.38	0.81
DMSO					20	642.49	234.00	21.47	0.77

a) Sample [**M8**]: pure coffee seeds powdered (no chicory is mixed). All commercial samples [**M9-M15**] from different companies available in the market. In these coffee seeds were mixed with chicory in different amounts and powdered for rich in taste, flavour and concentration. The general coffee seeds and chicory mixed ratios are to 70:30, 60:40 and 53:47. b) l: leaf; d: dust; p: powder; g: granule; fp: fine powder. c) The hot water extracts and caffeine were not completely dissolved in DMSO. d) Ref: Weaver et al., 1993; Kessel et al., 1989. e) Ref: Motohashi et al., 2001. f) par: parental without multidrug resistant gene. g) MDR: parental with multidrug resistant gene. h) (±)-verapamil: a control for MDR reversal.

Verapamil was used as a positive control in R123 exclusion experiments (Weaver *et al.*, 1993). The percentage of control means fluorescence intensity was calculated for the following equation on the basis of measured fluorescence values (Kessel, 1989).

MDR reversal activity=

$$\frac{\text{(MDR treated/MDR control)}}{\text{(parental treated/parental control)}}$$

RESULTS AND DISCUSSION

Caffeine in Indian tea leaves

Commercial Indian tea leaves or dusts of seven places in India were collected and analyzed their caffeine amount (Table 1). Caffeine contents of seven teas, Taj Mahal Tea **[1]** had the highest content (37.4 mg) of caffeine, comparing to those of other six teas. Six are follows: Society tea **[2]**, 36.6 mg; Nestea Nestle **[3]**, 26.5 mg; Top Star Tea, Lipton **[4]**, 25.0 mg; Chakra Gold Tea, Tata **[5]**, 24.0 mg; Gemini Tea, Tata **[6]**, 23.0 mg and Three Roses Tea **[7]**, 19.0 mg. Interestingly, caffeine contents (37.4 mg) of Taj Mahal Tea **[1]** was around two time rate of Gemini Tea, Tata **[6]**, (23.0 mg). When the m.p.s of caffeines isolated from seven tea leaves **[1-7]** were measured, the Nestea Nestle **[3]** (26.5 mg) showed the highest m.p. (237°C) and Top Star Tea, Lipton **[4]** had the lowest m.p. (210°C) values (Table 1).

Caffeine in Indian coffee seeds and chicory

Contents of eight commercial Indian coffee seeds (beans) mixed with different amounts of chicory in India were collected and analyzed their caffeine amount (Table 1). Among eight Indian coffee seeds **[8-15]**, the Brooke Bond Cafe Gold **[13]** had the highest content of caffeine (1.49 g). Contents of other seven Indian coffee seeds are follows: Pure Coffee Seeds **[8]** (1.40 g); Nestle, Sunrise Premium **[9]**, 1.20 g; BRU, Brooke Bond **[10]**, 1.07 g; Tata Kaapi, Instant Coffee **[11]**, 0.90 g; Family Blend Trinadha **[12]**, 0.65 g; Tata Coorg Double Roast **[14]**, 1.23 g and Green Label **[15]**, 1.04 g. Brooke Bond Cafe Gold **[13]** (1.49 g) contains 1.4 times higher caffeine content than that of Green Label **[15]** (1.04 g). There is a good relationship of m.p. with caffeine contents, except two Indian coffee seeds of Brooke Bond **[10]** (m.p. 228°C) and Tata Kaapi, Instant Coffee **[11]** (m.p. 241°C). Caffeine contents of eight Indian coffee seeds (100 g) are 1.04-1.49 g (Table 1). Moreover, caffeine contents of different materials are as follows: tea, 1.0-4.8 g; cola nuts, 2.7-3.6 g; coffee, 1.0-1.5 g; leaves of a species of *Ilex cassine* (1.0-1.6 g); mate (*Ilex paraguariensis*), 1.25-2.0 g and guarna (*Paullinin cupana*), 3.1-5.0 g. Cocoa waste contains theobromine from which caffeine can be obtained by methylation (Sastri, 1951). Based on these data, Indian coffee seeds showed average values on caffeine content (0.65-1.49 g).

From above caffeine analysis, caffeine contents of Indian tea leaves was 19.0-37.4 mg/100 g (Table 1). Caffeine contents in Indian coffee seeds and chicory were 0.65-1.49 g/100 g (Table 1). Coffee plants might be medicinally one of the most important plants in the future of the region. In view of medicinal drugs, caffeine will be essential in materials and applications of many different type's drugs, softdrinks for nutritions and very safely natural medicines.

MDR reversal on tumor cells

Fifteen methanol extracts **[M1-M15]** of Indian tea leaves **[1-7]** and coffee seeds **[8-15]** were tested on the P-glycoprotein mediated MDR efflux-pump of tumor cells. Because fifteen hot water extracts, however, were not sufficiently dissolved in dimethylsulfoxide (DMSO), their MDR activities could not be measured.

The MDR reversing effects of methanol extracts **[M1-M15]** of coffee by different origin were compared to that of (±)-verapamil, using a mouse leukaemia cell line of L-5178 cells. The effects were measured by fluorescence ratio activity between treated and untreated cells (control cells). When the effect of extract **M6** (fluorescence activity ratio 5.26) was compared to (±)-verapamil (fluorescence activity ratio 8.41) as a control, the extract **M6** was 0.63-fold of (±)-verapamil. Interestingly, the affinity of various compounds including some benzazepines to two dopamine D_1 and D_2 receptors was high. It is possible that some of the tea components had the MDR reversal effect on this mechanism (Kawase *et al.*, 1998; Motohashi *et al.*, 2001). This suggests that **M6** also might have strong affinity to dopamine D_1 and D_2 receptors. Further studies with many more tumor and normal cell lines are neccessary to confirm their MDR reversal specificity.

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