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Effect of solvent fractions of kombucha tea on viability and invasiveness of cancer cells—Characterization of dimethyl 2-(2-hydroxy-2-methoxypropylidine) malonate and vitexin

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In the present study, cytotoxic and anti-invasive properties of solvent fractions of kombucha tea were studied. Kombucha tea was fractionated with chloroform, ethyl acetate and butanol. Three solvent extracts and the final aqueous phase were concentrated and used for anti-cancer study at 25, 50, 75 and 100 µg/mL concentrations. Results revealed that ethyl acetate fraction at a concentration of 100 µg/mL caused cytotoxic effect on 786-O and U2OS cells; reduced the activities of matrix metalloproteinase-2 (MMP-2) and -9 in 786-O cells and MMP-2 activity in A549 cells; and significantly reduced the cell invasion and cell motility in A549, U2OS and 786-O cells. Thus, ethyl acetate fraction was further purified by chromatographic studies and presence of dimethyl 2-(2-hydroxy-2-methoxypropylidene) malonate and vitexin were confirmed through IR, NMR and mass spectroscopic studies, which might be responsible for the observed anticancer property of kombucha tea.

Keywords: Anti-invasive, cancer, cytotoxicity, kombucha, vitexin, tea

Introduction

Kombucha tea is sugared black tea fermented for about 14 d with a symbiotic association of acetic acid bacteria and yeasts forming "tea fungus". Kombucha tea is composed of two portions, a floating cellulose pellicle layer and the sour liquid broth. This beverage has been consumed in Asia for over two millennia and is a popular beverage among traditional fermented foods across the world. The beverage has been claimed to be a prophylactic agent and to be beneficial to human health; however, this remains to be proved scientifically. The beneficial effects of kombucha tea are attributed to the presence of tea polyphenols, gluconic acid, glucuronic acid, lactic acid, vitamins, amino acids, antibiotics and a variety of micronutrients produced during fermentation. The US Food and Drug Administration has evaluated the practices of several commercial producers of the starter (Kombucha mushroom or tea fungus) and found no pathogenic organisms or other hygiene violations. By virtue of the numerous health-promoting aspects reported and the easy and safe preparation of this biomaterial at home, it has gained popularity as other traditional beverages[1].

Carcinogenesis is a multifactorial and multistage process, in which numerous genes are affected. Many of these genes are prime targets for chemopreventive agents because they regulate intracellular, cell-surface or extracellular functions. Several reports are available in the literature for the anticancer properties of black tea polyphenols[2]. Since kombucha tea is prepared from black tea, it is expected to have anticancer properties. There are several testimonials available in the internet for the anticancer properties of kombucha tea from kombucha drinkers throughout the world, but there are no scientific reports to validate the facts. Hence, in the present study, kombucha tea was fractionated using organic solvents and the solvent extracts were tested for their anticancerous properties. The potent fraction was also put to chromatographic study to purify and identify the constituents.

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Materials and Methods

Starter Culture

Starter culture or tea fungal mat of *Medusomyces gisevii*, obtained from the tribal people of Kolli hills, Tamil Nadu, was maintained in sugared black tea decoction. Bacterial component was identified as *Acetobacter aceti* MTCC 2945, and the yeast components were identified as *Zygosaccharomyces bailii* MTCC 8177 and *Brettanomyces claussenii* MTCC 7801 at Institute of Microbial Technology (IMTECH), Chandigarh, India. Identified cultures were deposited in Microbial Type Culture Collection, IMTECH, India.

Preparation of Kombucha Tea

Brooke Bond Red Label tea (Hindustan Lever Limited, Mumbai, India) was used as black tea source. Black tea (1.2%) was added to boiling water and allowed to infuse for about 5 min after which the infusion was filtered through sterile sieve. Sucrose (10%) was dissolved in hot tea and the preparation was left to cool. The cooled tea (200 mL) was poured into 500 mL glass jars that had been previously sterilized at 121°C for 20 min and inoculated with 3% (w/v) of freshly grown tea fungus that had been cultured in the same medium for 14 d and 10% (v/v) of previously fermented liquid tea broth aseptically. The jar was covered with a clean cloth and fastened properly. The fermentation was carried out in dark at 24±3°C for 14 d. After fermentation (14 d), it was centrifuged at 10,000 rpm and the supernatant was used for fractionation[1,3].

Fractionation of Kombucha Tea

Kombucha tea was subsequently fractionated using chloroform, ethyl acetate and butanol (1:2, v/v). The solvent extracts and the remaining aqueous phase were concentrated using a vacuum rotary evaporator. The concentrated extracts were then lyophilized and stored at -20ºC. Four fractions (KCF, kombucha tea chloroform fraction; KEAF, kombucha tea ethyl acetate fraction; KBF, kombucha tea butanol fraction; & KAP, kombucha tea aqueous phase) at four different concentrations (25, 50, 75, 100 µg/mL) were tested for their effects on cell viability, cell invasion and cell motility on A549, U2OS and 786-O cells. Since the KEAF was comparatively more effective, the compounds present in this fraction were purified by silica gel column chromatography and structure predictions were done by IR, NMR and mass spectroscopic studies.

Cell Culture

Cell lines, A549 (human lung carcinoma), U2OS (human osteosarcoma) and 786-O (human renal carcinoma) were obtained from American Type Culture Collection (Rockville, MD, USA). Cell lines A549 and U2OS were cultured in Dulbecco's modified Eagle medium (Life Technologies, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS), 2 m*M* glutamine, 100 U/mL of penicillin, and 100 µg/mL streptomycin sulfate. Cell line 786-O was cultured in Rosewell Park Memorial Institute (RPMI)-1640 medium (Life Technologies, Grand Island, NY, USA) supplemented with 10% FBS, 2 m*M* glutamine, 4.5 mg/mL glucose, 100 U/mL of penicillin, 100 µg/mL streptomycin sulfate, and 500 µg/mL non-essential amino acids (Sigma Chemical Co., St. Louis, MO, USA). All cell cultures were maintained at 37°C in a humidified atmosphere of 5% CO_2.

Determination of Cell Viability

To evaluate the effect of KCF, KEAF, KBF, and KAP on cell viability a 3-(4,5-dimethylthiazol-2-y1)-2,5-diphenyltetrazolium bromide (MTT) colorimetric assay was performed[4]. Cells were seeded onto 24-well plates at a density of 4×10^4 cells/well. In concentration-dependent assays, cells were treated with KCF, KEAF, KBF and KAP for 24 h. After an indicated exposure period, media were removed and cells were washed with phosphate-buffered saline (PBS), followed by an incubation with 0.5 mg/mL MTT in culture medium for an additional 4 h. Afterwards, 1 mL of lysis buffer (isopropyl alcohol containing 10% Triton X-100 and 0.1 *N* HCl) was added to dissolve the formazan formed and then measured spectrophotometrically (Beckman Spectrophotometer DU 640, Beckman Instruments, Fullerton, CA, USA) at 570 nm. All the fractions were tested in triplicates and the experiment was repeated at least three times.

Cell Invasion and Migration Assays

The cell invasion and motility were assayed according to the methods described by Chu *et al*[6]. After being treated with KCF, KEAF, KBF and KAP for 24 h, surviving cells were harvested and seeded to Boyden chamber (Neuro Probe, Cabin John, MD, USA) at 1.5×10^4 cells/well in serum free medium and then incubated for 6 h (A549), 3.5 h (786-O) or 24 h (U2OS) at 37°C (different types of cancer cells have distinct degree of invasiveness capability). For invasion assay, 10 µL Matrigel (25 mg/50 mL; BD Biosciences, MA, USA) was applied to polycarbonate membrane filters with a pore size of 8 mm and the

bottom chamber contained standard medium. Filters were then air-dried for 5 h in a laminar flow hood. The invaded cells were fixed with methanol and stained with Giemsa. Cell numbers were counted under a light microscope. To determine the cell motility, cells were seeded into Boyden chamber on membrane filters which were not coated with Matrigel. Migration of cells treated or untreated with extracts was measured as described in the invasion assay.

Determination of Matrix MMP-2 and -9 by Gelatin Zymography

The activities of MMP-2 and -9 were measured in a medium by gelatin-zymography protease assays[5]. Cells were treated with KCF, KEAF, KBF, and KAP for 24 h. Collected media were prepared with sodium dodecyl sulphate (SDS) sample buffer without boiling or reduction and subjected to 0.1% gelatin-8% SDS polyacrylamide gel electrophoresis (SDS-PAGE). After electrophoresis, gels were washed with 2.5% Triton X-100 and then incubated in reaction buffer (40 m*M* Tris-HCl, *p*H 8.0, 10 m*M* $CaCl_2$, 0.02% $NaNO_3$) for 16 h at 37°C. Then gel was stained with Coomassie brilliant blue R-250.

Purification of Compounds Present in Ethyl Acetate Fraction (KEAF)

Since KEAF shown better activity in anticancer experiment when compared to KCF, KBF and KAP, it was further fractionated by silica gel (60 X 120 mesh size) column chromatography. Petroleum ether and ethyl acetate in different ratios starting from 5:95 to 95:5 (v/v) were tried as mobile phase. Fractions were collected at the rate of 1 mL/min. Purity of the separated compounds was tested by TLC method. TLC was performed using glass plates coated with silica gel-G containing 13% calcium sulphate as binder. Petroleum ether and ethyl acetate were used as developing solvents (from 0:100 to 25:75 v/v). A chamber containing iodine vapour was used to locate the spots. Melting point of purified compounds was determined on Mettler FP5 and expressed in degree centigrade (°C).

IR, NMR and Mass Spectroscopic Studies

IR spectrum was recorded on Perkin Elmer 1600 spectrometer using KBr disc and the absorption frequencies are expressed in reciprocal centimetres (cm^{-1}). ^{1}H-NMR and ^{13}C-NMR spectra were recorded on AMX 400 (400 MHz) and AMX 500 (500 MHz) spectrometers using tetramethylsilane (TMS) as an internal reference. The chemical shifts are expressed in parts per million (ppm). Mass spectrum (MS), was recorded on AutoSpec EI+ mass spectrometer. Dimethyl 2-(2-hydroxy-2-methoxypropylidine) malonate (T-1) was obtained as white powder. EI-MS: m/z (%) 218 (M^+, 100), 201 (10), 186 (15), 143 (8), 88 (10); ^{1}H-NMR (DMSO-d_6, 400 MHz): δ 7.5 (1H, s, 3-OH), 4.57 (3H, s, 4-OCH_3), 3.99 (3H, s, 4-OCH_3), 3.59 (3H, s, 1′-$COOCH_3$), 3.41 (3H, s, 1-$COOCH_3$), 1.25 (3H, s, 4-CH_3); ^{13}C-NMR (DMSO-d_6, 100MHz): δ 163.5 (C-1), 163.0 (C-1′), 141.5 (C-2), 132.0 (C-3), 103.5 (C-4), 56.0 (1-OCH_3), 55.5 (1′-OCH_3), 51.5 (4-OCH_3), 29.0 (C-5). Vitexin (T-2) was obtained as yellow needles. EIMS: m/z 427 (M+1), 426 (M^+, 100), 306, 264. ^{1}H-NMR (DMSO-d6, 400MHz): δ 13.15 (1H, s, 5-OH), 8.01 (2H, d, *J*=8.5 Hz, 2′-H and 6′-H), 6.90 (2H, d, *J*=8.5 Hz, 3′-H and 5′-H), 6.77 (1H, s, 3-H), 6.26 (1H, s, 6-H), 4.68 (1H, d, *J*=10Hz, 1″-H). ^{13}C-NMR (DMSO-d_6, 100MHz): 182.5 (C-4), 163.5 (C-2), 162.5 (C-7), 161.5 (C-4′), 160.8 (C-9), 156.4 (C-5), 129.4 (C-2′ and C-6′), 122.1 (C-1′), 116.2 (C-3′ and C-5′), 105.0 and 104.4 (C-8 and C-10), 102.9 (C-3), 98.6 (C-6), 82.3 (C-5″), 79.1 (C-1″), 73.8 (C-2″), 71.2 (C-3″), 71.0 (C-4″), 61.7 (C-6″).

Results

Effect of Kombucha Tea Extracts on Viability of A549, U2OS and 786-O cells

The cytotoxic effect of various concentrations of solvent extracts of kombucha tea on A549, U2OS and 786-O cells are shown in Figs 1, 2 and 3. The cell viability was not significantly altered by KCF, KEAF, KBF and KAP on A549 and 786-O cells, while KEAF at high concentration (100 µg/mL) exerted slight inhibitory effect (21.56%) on 786-O cells. Further, the results showed that KEAF (100 µg/mL) significantly decreased the viable cells of U2OS in dose-dependent manner (93.45%), while no significant inhibitory effects were seen with KCF and KAP. KBF at 100 µg/mL concentration slightly reduced the cell viability (34%) of U2OS cells (Fig. 1).

Effect of Kombucha Tea Extracts on Cell Invasion and Motility

Since extracellular matrix (ECM) degradation is vital to cellular invasion, it was believed that matrix-degrading proteinases were required for cancer cell metastasis. Using a cell invasion and motility assay with Boyden chamber, it was shown that ethyl acetate extract (0, 25, 50, 75 and 100 µg/mL) significantly

reduced cell invasion and cell motility in A549, U2OS and 786-O cells (Fig. 1).

Effect of Kombucha Tea Extracts on Matrix Metalloproteinases (MMP) Activity

The tea polyphenols are believed to affect a number of molecular processes that include induction of tumour cell apoptosis and inhibition of tumour growth and invasion. Since matrix MMPs play an important role in these processes and have been linked to tumour cell invasion, the present study examined whether kombucha tea has inhibitory activity on MMPs. Via gelatin zymography assay (Figs 2-5), it was found that KEAF (0, 25, 50, 75 and 100 μg/mL) significantly reduced MMP-2 and MMP-9 activities in a concentration-dependent manner in

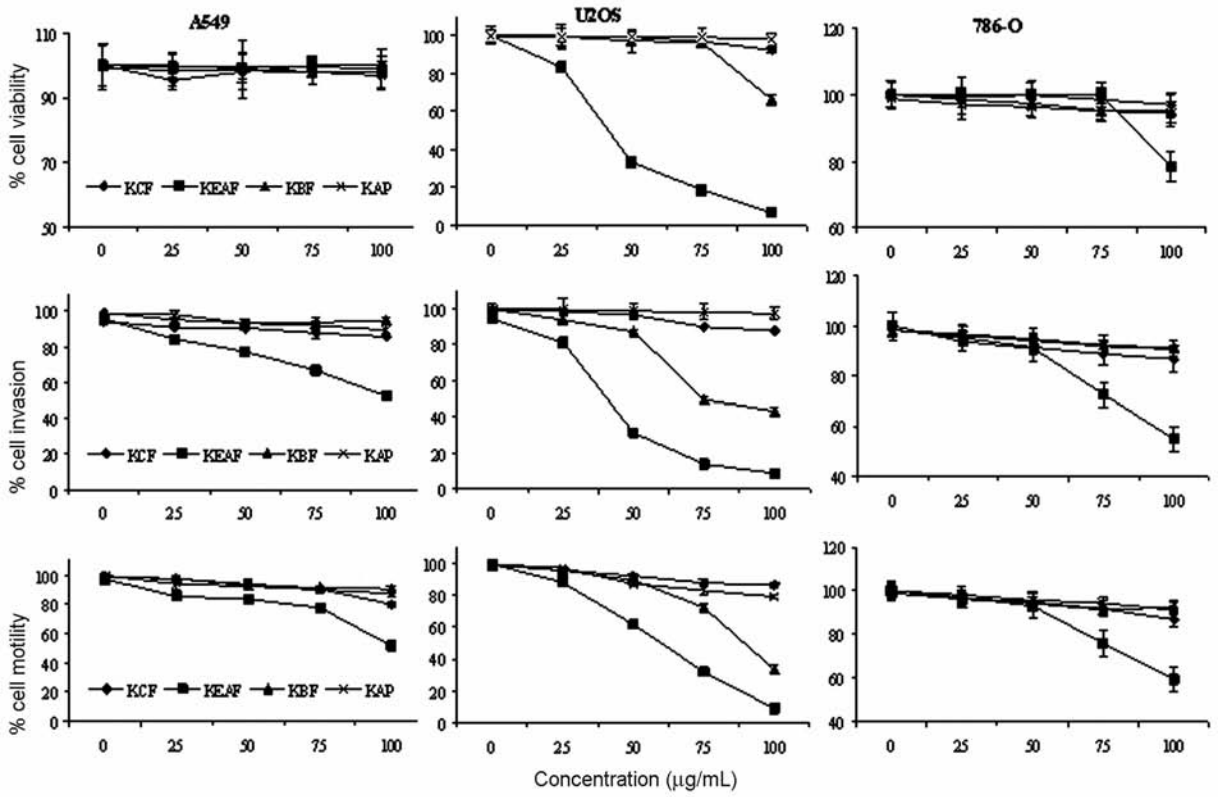

Fig. 1—Effect of kombucha tea extracts on cell viability, invasion and motility of A549, U2OS and 786-O cells.

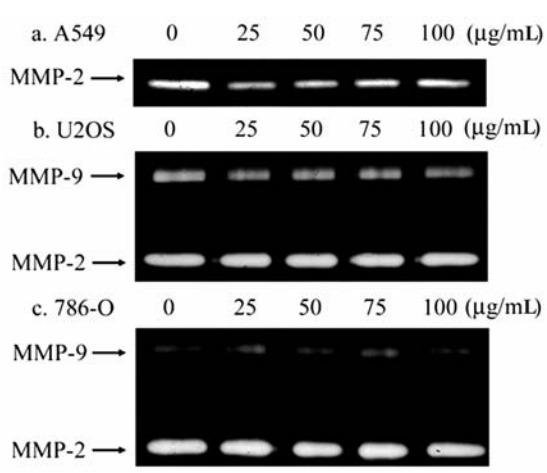

Fig. 2—Effect of KCF on MMP-2 of A549 and MMP2, 9 of U2OS and 786-O cells.

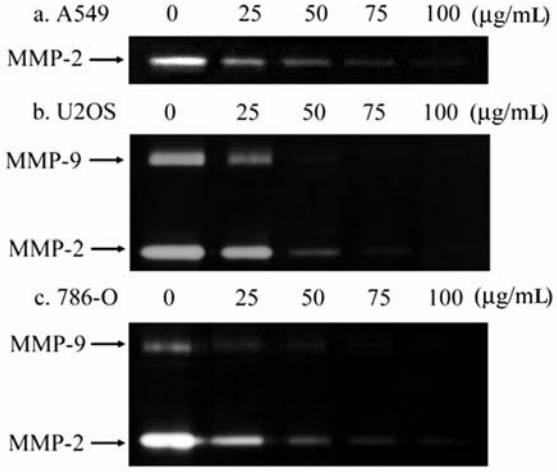

Fig. 3—Effect of KEAF on MMP-2 of A549, and MMP2 and -9 of U2OS and 786-O cells.

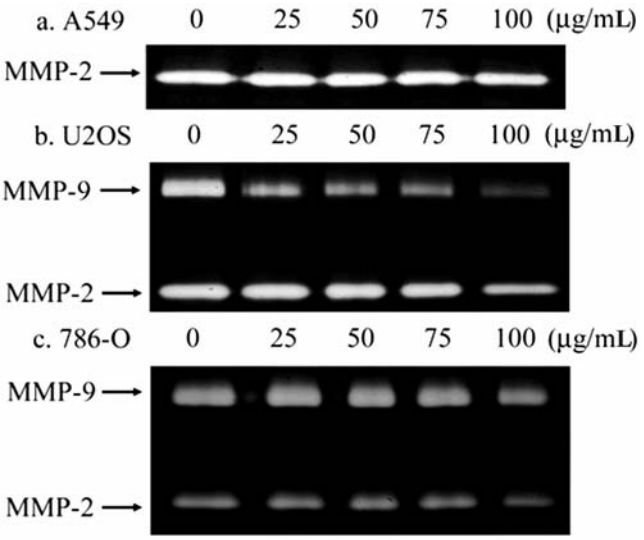

Fig. 4—Effect of KBF on MMP-2 of A549, and MMP-2 and -9 of U2OS and 786-O cells.

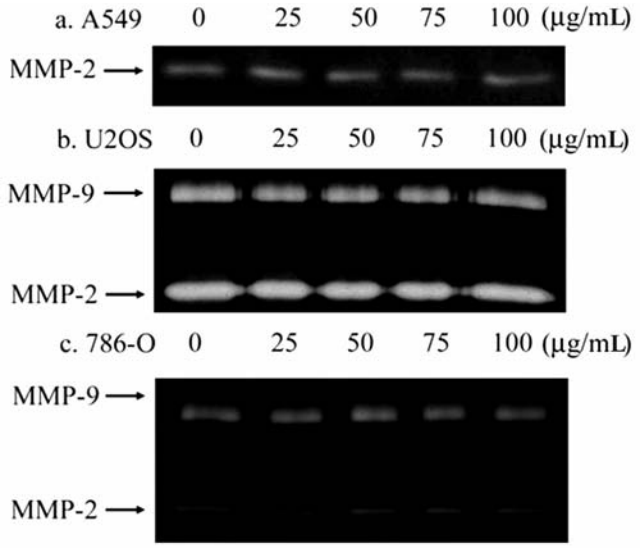

Fig. 5—Effect of KAP on MMP-2 of A549, and MMP-2 and -9 of U2OS and 786-O cells.

786-O cells, and MMP-2 activity was also inhibited by ethyl acetate extract in A549 cells in the absence of cytotoxicity (Fig. 3).

Characterization of Compounds From Ethyl Acetate Fraction of Kombucha Tea (KEAF)

KEAF was separated into two compounds, T-1 and T-2, using silica gel column chromatography.

Characterization of Compound T-1

Compound T-1 was eluted with petroleum ether and ethyl acetate (55:45 v/v) and subjected to repeat crystallization with ethanol. T-1 was obtained as a white powder and melting point was found to be 255ºC. CHN analysis associated with the molecular formula $C_9H_{14}O_6$ and has three degrees of unsaturation. The nature of oxygen in T-1 was shown to be hydroxyl group and ester group as indicated by IR spectrum. The high field region of ^{1}H-NMR showed four proton signals, each with 3 proton integration. Three proton singlets at δ1.25, δ3.41, δ3.59 and δ3.99 were due to CH_3 which attached with quaternary carbon, C_1-$COOCH_3$, C_1-$COOCH_3$, C_4-OCH_3 respectively. One proton singlet at δ4.57 indicates the presence of C_4-OH proton. One proton singlet at δ7.51 was due to the olifinic C_3-H proton. The ^{13}C-NMR spectrum indicated nine carbon signals. Based on the above data along with its mass spectrum the compound was identified as dimethyl 2-(2-hydroxy-2-methoxypropylidene) malonate (Fig. 6a).

Characterization of Compound T-2

The compound T-2 was eluted with petroleum ether and ethyl acetate (20:80 v/v) and obtained as

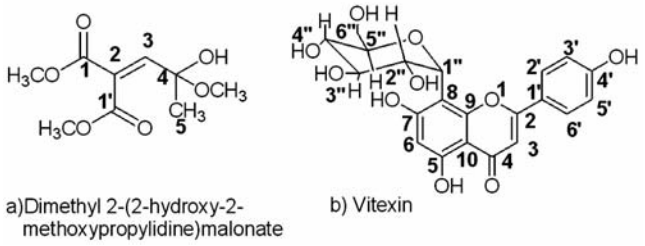

Fig. 6—Strucutre of dimethyl 2-(2-hydroxy-2-methoxypropylidine) malonate (a) and vitexin (b).

yellow solid. Melting temperature was found to be 272°C and analyzed as $C_{21}H_{14}O_{10}$. It produced a yellow fluorescence in UV light. It responded to Shimoda's test (Mg/HCl) for flavonoids giving red colour and also a brownish colour with alcoholic ferric chloride, indicating a chelated hydroxyl group. It showed UV absorptions at λmax (MeOH) (265, 335 nm) and IR (3380, 1654cm^{-1}) characteristics of a flavone (results not shown). The ^{1}H-NMR spectrum showed a downfield resonance at δ13.1, which was attributed to a chelated hydroxyl proton and it was exchangeable with D_2O. ^{1}H-NMR data indicated the presence of AA'BB' pattern in ring B, suggesting that the flavone moiety in T-2 was apigenin. ^{13}C-NMR spectrum revealed twenty one carbon signals, which suggested the presence of flavonoid and a saccharide moiety in T-2. The six carbon signals characteristic of sugar moiety were present at δ 82.30 (C-5''), 79.11 (C-1''), 73.83 (C-2''), 71.29 (C-3''), 70.98 (C-4''),

and 61.74 (C-6'') and thus suggesting that T-2 is a flavone C-glycoside. The sugar moiety was determined to be at the C-8 position of the aglycon moiety. The glycosidation position was unambiguously determined at the C-8 position by the appearance of glucosyl anomeric proton H-1'' at δ 4.68 (d, J = 10Hz). Based on these data, compound T-2 was identified as apigenin 8-C-β-D glucopyranoside (vitexin). The data was compared with reported values and it was found to be identical in all aspects (Fig. 6b).

Discussion

Growth inhibitory activities of tea and tea polyphenols have been demonstrated. It was observed that (–)-epigallocatechin gallate (EGCG) could trigger apoptosis and contribute to growth inhibition in human lung cancer cell line H-661[7]. EGCG and theaflavins have been shown to inhibit 12-O-tetradecanoylphorbol 13-acetate (TPA) and epidermal-growth-factor induced mouse JB-6 cell transformation; such effects are associated with inhibition of the transcription factor AP-1 activity. Park *et al*[8] reported that gallic acid inhibited the growth and proliferation of testicular cells in a dose-dependent manner. Babich *et al*[9] demonstrated that (–)-epicatechin gallate (ECG) was selectively cytotoxic to the human squamous carcinoma (HSC-2) cells causing inhibition of cell proliferation and apoptosis.

The chemoprevention of human cancers has long been the subject of research. The chemopreventive effects of tea polyphenols have been demonstrated in animal models of cancers of lung, skin, oesophagus, colon, and mammary gland, but the anticancer property of kombucha tea was not well characterized. Epidemiological studies suggest that tea may have a protective role against certain human cancers. Catechin polyphenols in green tea have been shown to inhibit the proliferation of cultured mammalian cells including colon carcinoma, lung carcinoma, breast carcinoma, melanoma, and leukemic cells. EGCG inhibits the growth of human tumor cells, including Caco-2 colorectal cancer cells, Hs578T breast cancer cells, ovarian cancer cells and SV40-transformed WI38 cells but has little or no inhibitory effect on the growth of their normal counterparts[10]. Black tea extract has been shown to be potent in inhibiting tumorigenesis in animal model systems, including skin, lung, colon, esophagus and mammary gland. Further, theaflavin-3-gallate has been shown to be as potent as EGCG in inhibiting the growth of human A431 carcinoma cells[11].

Cancer metastasis consists of several interdependent processes including cancer cell adhesion, cancer cell migration, and invasion of cancer cells. EGCG, ECG and TF (20 µg/mL) strongly suppressed the invasion of human fibrosarcoma HT1080 cells into the monolayer of human umbilical vein matrix endothelial cells/gelatin membrane and inhibited gelatin degradation mediated by MMP-2 and -9[12]. GCG also inhibited the activities of MMP-2 and -9 (IC_{50} 0.8-6 µM) as well as concanavalin A induced pro-MMP-2 activation in glioblastoma cells[13]. Yamakawa *et al*[14] observed that EGCG significantly inhibited the invasion of human umbilical vein endothelial cells (HUVECs) at the concentration of 10 µM. Therefore, it is clear that tea polyphenols have anti-metastatic activity on cancer cells. Anti-metastatic activity of KEAF observed on A549, U2OS and 786-O cells in the present study might be due to the presence of polyphenolic compounds as well as compounds produced during kombucha fermentation.

Kombucha tea is an ancient drink consumed worldwide since long. Based on personal observations and testimonials, it has been claimed to have anticancer properties and was proved by a population study conducted in Russia by the 'Central Oncological Research Unit' and 'Russian Academy of Sciences' in Moscow during 1951[1]. The results of the present study support the anticancer property of kombucha tea, which might be due to the inhibition of metastasis as confirmed by the inhibitory activity on MMP-2 and -9. The ethyl acetate fraction of kombucha tea was found to contain dimethyl 2-(2-hydroxy-2-methoxypropylidene) malonate and vitexin through silica gel column chromatographic procedure. Several malonated flavonol glycosides have been reported earlier from plants[15-17] and demonstrated to have strong DPPH radical scavenging activity[18].

Vitexin (8-β-D-glucopyranosyl-apigenin) in black tea has been previously reported[19]. Prabhakar *et al*[20] studied pharmacological effects of vitexin isolated from *Ochrocarpus longifolius* (L.) and *Arnebia hispidissima*. They reported hypotensive, anti-inflammatory and anti-spasmodic activities of vitexin and its anti-inflammatory activity were attributed to its anti-histaminic, anti-bradykinin and anti-serotonin properties. Choi *et al*[21] reported that vitexin inhibited hypoxia-inducible factor-1a (HIF-1a) and the migration of PC12 cells as well as their invasion rates, and also tube formation by human umbilical vein endothelium

cells (HUVECs). Ninfali *et al*[22] demonstrated its antioxidative and cytotoxic properties on MCF-7 breast cancer cells. They have also reported that vitexin strongly inhibited DNA synthesis in breast cancer cells. Vitexina, a product containing the flavonoid vitexin as the main component, has been derived from *Vigna radiata* (L.) and traditionally used in Vietnam for detoxification.

In the present study, dimethyl-2-(2-hydroxy-2-methoxy-propylidene) malonate was obtained from tea. Epicatechin isomers are well known gallate esters that can easily be split into smaller molecules. The same way, it could be expected that the glucosidemalonates might be dissociated into dimethyl-2-(2-hydroxy-2-methoxy-propylidene) malonate and thus dimethyl-2-(2-hydroxy-2-methoxy propylidene) malonate and vitexin might be responsible for the cytotoxic and anti-invasive effects of ethyl acetate fraction of kombucha tea.

Thus, cytotoxic and anti-invasive studies with solvent fractions of kombucha tea showed that ethyl acetate fraction at a concentration of 100 µg/mL can cause cytotoxic effect on 786-O and U2OS cells; can reduce MMP-2 and -9 activities in 786-O cells and MMP-2 activity in A549 cells; and can significantly reduce cell invasion and cell motility in A549, U2OS and 786-O cells. Dimethyl 2-(2-hydroxy-2-methoxypropylidene) malonate and vitexin were purified from ethyl acetate fraction of kombucha tea and these two compounds may be among the active compounds responsible for cytotoxic and anti-invasive effects.

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