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Journal of Pharmaceutical and Biomedical Analysis 43 (2007) 151–157
JOURNAL OF PHARMACEUTICAL AND BIOMEDICAL ANALYSIS
www.elsevier.com/locate/jpba

Screening and analysis of bioactive compounds in traditional Chinese medicines using cell extract and gas chromatography–mass spectrometry

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Received 23 March 2006; received in revised form 15 June 2006; accepted 20 June 2006
Available online 28 July 2006

Abstract

As the cost of drug development is always many times more than that of drug discovery, predictive methods aiding in the screening of bioavailable drug candidates are of profound significance. In this paper, a novel method for screening bioactive compounds from traditional Chinese medicines (TCMs) was developed by using living cell extract and gas chromatography (GC)–mass spectrometer (MS). The method was validated by using elemene emulsion injection (EEI), a typical TCM with known active compound, to interact with murine ascites hepatocarcinoma cell strain with high metastatic potential (HCa-F). Finally, the method was applied to screen the bioactive compounds from multi-component zedoary turmeric oil and glucose injection (ZTOGI). After HCa-F cells was incubated in ZTOGI, ethyl acetate (EtOAc) was used to extract the compounds in the cells for GC–MS analysis. Fourteen compounds were detected in the desorption eluate of HCa-F cell extract of ZTOGI, and further identified by MS. Curzerene and β-elemene were found to be two major bioactive compounds in ZTOGI. These results show that the method developed may be applied to quickly screen the potential bioactive components in TCMs interacting with the target cells.

Keywords: Bioactive compounds; Traditional Chinese medicines; Cell extract; Gas chromatography–mass spectrometer; Murine ascites hepatocarcinoma cell; Elemene emulsion injection; Zedoary turmeric oil and glucose injection

1. Introduction

There has been considerable interest among basic and clinical researchers in traditional Chinese medicines (TCMs) in recent years for their long history of clinical practice and reliable therapeutic efficacy. However, the components of TCMs are complex mixtures, almost consisting of hundreds or even thousands of different compounds but only partial compounds are responsible for the pharmaceutical and/or toxic effects. Further more, the effective components in TCMs are generally in low level. Therefore, how to screen bioactive compounds from the complex mixtures of TCMs has been a great challenge for many investigators. The conventional procedure for finding bioactive components is to extract the effective compound groups or purify compounds in TCMs with the method of phytochemistry followed by animal pharmacological experiments. However, this method is time-consuming, arduous, expensive and low efficient for directly screening bioactive compounds from TCMs.

Several years ago, cell membrane transport assays were considered to be critical for deciding which new compounds were drug leads [1–5]. From then on, many relevant techniques have been exploited to engage in these studies. For example, biochromatography techniques especially of immobilized artificial membrane and immobilized liposome chromatography techniques developed in recent years [6–13] are considered as a powerful tool to investigate drug–membrane, drug–receptor or drug–enzyme interactions in vitro. The core of the technique is that active cell membrane, receptor or specific enzyme was noncovalently or covalently immobilized on soft gel particles or silica particles as HPLC stationary phase to screen bioactive compounds from TCMs according to their different combination ability with stationary phase. The method can be used to provide some essential information for guiding drug discovery, but it may be of some drawbacks in the practical performance. For example, strongly strict restriction is required for the mobile phase in this method to avoid damaging the stationary phase, namely, physiological saline buffer or organic solvents with extremely low

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doi:10.1016/j.jpba.2006.06.033

concentration are specially required for serving as the mobile phase, which may result in poor separation due to less changeable mobile phases. In addition, the interaction of compounds in TCMs with biomembrane can hardly perceive all these complex interactions clearly [14].

Pharmacokinetics-based in vitro high throughput screening approaches using genes, receptors or channels on cellular membrane as targets acted by TCMs, have ever been extensively developed [15–19]. These studies have shown that combination with receptors or channels on cell membrane is the first step of drug action, which can directly disclose the interaction mechanisms between bioactive compounds and targets at the level of molecule and cell. However, the proposed method is very laborious. Recently, a method using human red cell membrane extraction and HPLC was developed for screening active components in TCMs and successfully hypothesized four potential active potential candidates in *Angelica sinensis* [20], but the biomembrane used was prepared with modification. Therefore, some special characteristics of the cellular membrane may be changed during the preparation.

Zedoariae rhizoma (ZR) is a traditional Chinese medicine that has been used in removing blood stasis and alleviating pain for over a thousand years. Essential oils are considered as active constituents in ZR and have been used for treatment of liver cancer and fibrosis and showed inhibitory effect on cytotoxicity in cells [21]. ZR inhibits proliferation of human hepatic myofibroblasts and block PDGF-BB binding to its receptor. Eighty percent aqueous acetone extract from ZR induces growth arrest in G_0/G_1 phase, indicating that the 80% aqueous acetone extract from ZR includes acting compounds [22]. Many sesquitepenes have been identificated from Curcuma rhizomes by GC–MS and pressurized liquid extraction (PLE) [23]. Elemene, isolated from ZR, is well-known to exhibit antitumor activity in human and murine tumor cells including murine ascites hepatocarcinoma cells (H22), the parent line of murine ascites hepatocarcinoma cell strain with high metastatic potential (HCa-F) [24–28]. These reports indicate that both ZR and elemene could enter into and interact with cells, and potential active compounds could be screened from the interacted compounds with cells.

In the present work, we developed a new method for in vitro screening bioactive compounds from TCMs by use of cell extract and gas chromatography–mass spectrometry (GC–MS). This rationale of the experiment is based on the observation that most of drugs must enter cells and combine with cell membrane or components within cells to elicit activity and intracellular availability of drugs requires drug transport across the target cell membrane [29]. Therefore, it can be concluded that cell is the target for most of the drugs to produce pharmaceutical effect and can be applied to rapidly and accurately predict the bioactivities of multiple compounds in TCMs.

2. Experimental

2.1. Chemicals and materials

Elemene emulsion injection (EEI) was purchased from Dalian JinGang Pharmacy Ltd. (Dalian, China). Zedoary turmeric oil glucose injection (ZTOGI) was purchased from Lizhu Group Limin Pharmacy Ltd. (Shaoguan, China). Ethyl acetate (EtOAc) and ethanol (EtOH) of analytical grade were from Shenyang Lianbang Chemical Reagent Plant (Shenyang, China). Dimethylsulfoxide (DMSO) was from Tianjin Bodi Chemicals Co., Ltd. (Tianjin, China). RPMI 1640 medium and D-Hank's balanced salt solution (DHBSS) were from Gibco. BRL was from Gibco BRL (Grand Island, NY). New born bovine serum (NBBS) was from Hangzhou Sijiqing Biological Engineering Materials Co., Ltd. (Hangzhou, China).

EEI medium with 32 μg/ml elemene was prepared from EEI (5000 μg/ml), which was diluted by RPMI 1640 medium supplemented with 10% (v/v) NBBS and 80 U/ml gentamycin sulfate (GS). ZTOGI medium with 360 μg/ml zedoary turmeric oil was prepared from ZTOGI (400 μg/ml) by addition of RPMI 1640 medium powder and 10% (v/v) NBBS.

2.2. Cell culture

HCa-F cells were injected to 615 mice to incubate in duplicate for two passages in order to harvest a great amount of cells in less than 2 weeks, which was described in our previous report [30]. The cells in ascites were drawn and seeded into bottles for culture at a density of 1×10^5 cells/ml in RPMI 1640 medium with 10% NBBS in a 5% CO_2 atmosphere at 37 °C for 48 h, and all experiments were performed with cells in the logarithmic growth phase. Then aliquots of 8×10^5 cells/ml were seeded into10 ml EEI medium with 32 μg/ml elemene in tubes of 10 ml in a 5% CO_2 atmosphere at 37 °C for 12 h. Aliquots of 1×10^6 cells/ml of the same cells were seeded into 40 ml ZTOGI medium with 360 μg/ml zedoary turmeric oil in bottles under the above condition for 4 h. Phase-contrast microscope and Tyrpan blue exclusion test were used to determine the cell viability.

2.3. Extraction of active components in cells

Sample 1. After being pretreated with elemene medium in living state at 37 °C for 12 h, the mixture of the cells and elemene medium was centrifuged at 1500 rpm for 5 min. The cell precipitate was reserved and washed with DHBBS for five times by the use of oscillating to scour off the unbound components. Each share of the cell suspending was centrifuged at 1500 rpm for 5 min. Finally, the cell precipitate was digested by addition of 200 μl DMSO and was put under ultrasonic vibrations for 10 min when cells were disintegrated by observing microscopically the cells, then the bound components to cells were released. The desorption eluate of the cells from this step was extracted by 2 ml EtOAc and then centrifuged at 3500 rpm for 20 min before analysis. The blank desorption eluate control was obtained from the cells incubated in RPMI 1640 medium without elemene, and then the blank control for GC–MS analysis was performed using the method described above.

Sample 2. Considering the higher cytotoxicity of zedoary turmeric oil at high concentration to cells, the time for the cells incubated in ZTOGI medium was confined within 6 h. After HCa-F cells were pretreated with ZTOGI medium with 360 μg/ml zedoary turmeric oil for 4 h, the incubation mixture

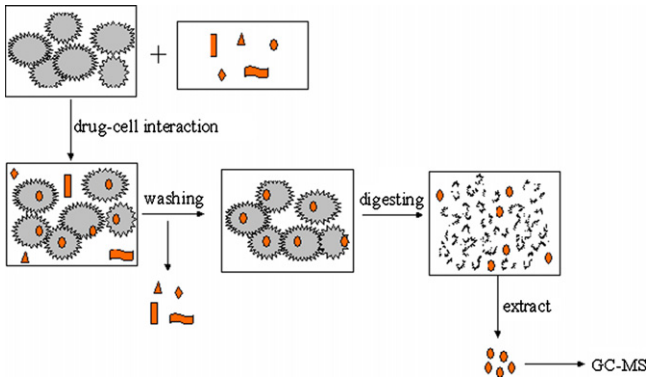

Fig. 1. Flow scheme of screening bioactive components in TCMs based on cell extract and GC–MS.

was centrifuged at 1500 rpm for 5 min. The rest of procedures were analogous to those of sample 1.

2.4. GC–MS analysis

GC–MS was performed with a SHIMADZU QP2010 mass spectrometer and a SHIMADZU ChemStation software (SHIMADZU corporation analytical and measuring instruments division, Kyoto, Japan). A DB-5 capillary column (30 m × 0.25 mm i.d.) coated with 0.25 μm film 5% phenyl methyl siloxane was used for separation. The GC injector was maintained at 240 °C, the GC transfer line at 260 °C, the ion source at 200 °C and the vacuum manifold at 70 °C. The column temperature started with 70 °C for injection, then programmed at 5 °C/min to 120 °C, at 8 °C/min to 160 °C, then from 160 to 185 °C at 4 °C/min, and finally at 8 °C/min to 240 °C, held isothermally 8 min at 240 °C. Manual injection (0.6 μl) in a purged splitless mode was conducted and high-purity helium was used as carried gas at 0.68 ml/min flow rate. The mass spectrometer was operated in electron-impact ionization (EI) mode with 70 eV energy. The scan range was 40–400 amu and the scan rate was 0.2 s per scan. The NIST library was the 174,948 entry edition (Version 2.0, 2002).

3. Results and discussion

The flow scheme of screening bioactive components in TCMs based on cell extract and GC–MS was shown in Fig. 1. The target cells are incubated together with the drug in RPMI 1640 medium with 10% (v/v) NBBS for 4–12 h (depending on the kind of TCMs). The potential bioactive components of the drug might interact with target cells by combining with them. The unbound compounds on the cell surface are washed away by DHHBS. Afterwards, the potential active components in the target cells are released by cell digestion solvent and then extracted with organic solvent. Finally, GC–MS is performed to acquire the structural information of the bioactive compounds.

3.1. Establishment of the experimental conditions

Due to its extensive availability, sufficient drug contact, high drug sensitivity, HCa-F cells were selected as target cells. HCa-F cells can first duplicate in the abdominal cavity of 615 mice and then in RPMI 1640 medium with a freely suspending pattern of growth. Elemene has been reported to be able to interact with cells [24–28], and EEI is a typical TCM with elemene as active component. So, EEI was chosen to treat HCa-F cells to verify the method for screening and analyzing bioactive compounds in TCMs using cell extract and GC–MS.

3.1.1. Conditions of cell culture

It is well known that the drug absorption of the cells plays a very important role in drug–cell interaction. Too low drug concentration will render a missing detection while too high one may result in cell death due to cytotoxicity of drug. The cell quantity is another significant factor to be considered, and the time of drug–cell interaction should not be neglected, either.

In the preliminary experiments, the above three factors were investigated. EEI medium with 16, 32 and 50 μg/ml elemene were tested. For cell quantity, aliquots of cells of 1×10^6 and 4×10^6 (at a seeded density of 4×10^5 cells/ml) as well as cells of 8×10^6 and 1×10^7 (at a seeded density 8×10^5 cells/ml) were considered. Toward incubated time of drug–cell, five time points (4, 8, 12, 24 and 36 h) were tested. Taking the results of optimization (data not shown) into account, the conditions of the cell culture proposed were: concentration of elemene, 32 μg/ml; the seeded cells for elemene-cell treatment, 8×10^6; time for elemene-cell treatment, 12 h.

3.1.2. Conditions of cell digesting

Aliquots of elemene-cell incubation mixture were washed five times with DHBBS to remove the unbound compounds from the cell surface before digesting. The remaining components inside of the target cells would be of potential bioactivities. As most of the active compounds were in low level, it would be very important to ensure these compounds binding with cells were well released. In the present experiment, three organic solvents of EDTA, 75% EtOH and DMSO were used to digest cells, respectively. DMSO was found to be the best digestion solvent by observing the treated cells microscopically. While EDTA and 75% EtOH were not observed to digest cells completely and were not considered.

3.2. Validation of the method

EEI, a wide spectrum antitumor drug with the main active ingredient of β-elemene, was first used to interact with the target cell in order to validate the method. As we have well known that all drugs must be absorbed by cells before they can have an effect on the body. The process of absorption brings the drug from any site of exposure into the circulatory system. However, a drug must traverse several semipermeable cell membranes before reaching the systemic circulation, that is to say, the absorption of a chemical substance from any site of exposure involves its passage across cellular membranes [31]. Therefore, cells are the target for most of the drugs to produce pharmaceutical effect and accordingly, it is an optimal selection to use cell extract for screening of active candidates from TCMs. In addition, GC–MS is the preferred identification method for volatile components of

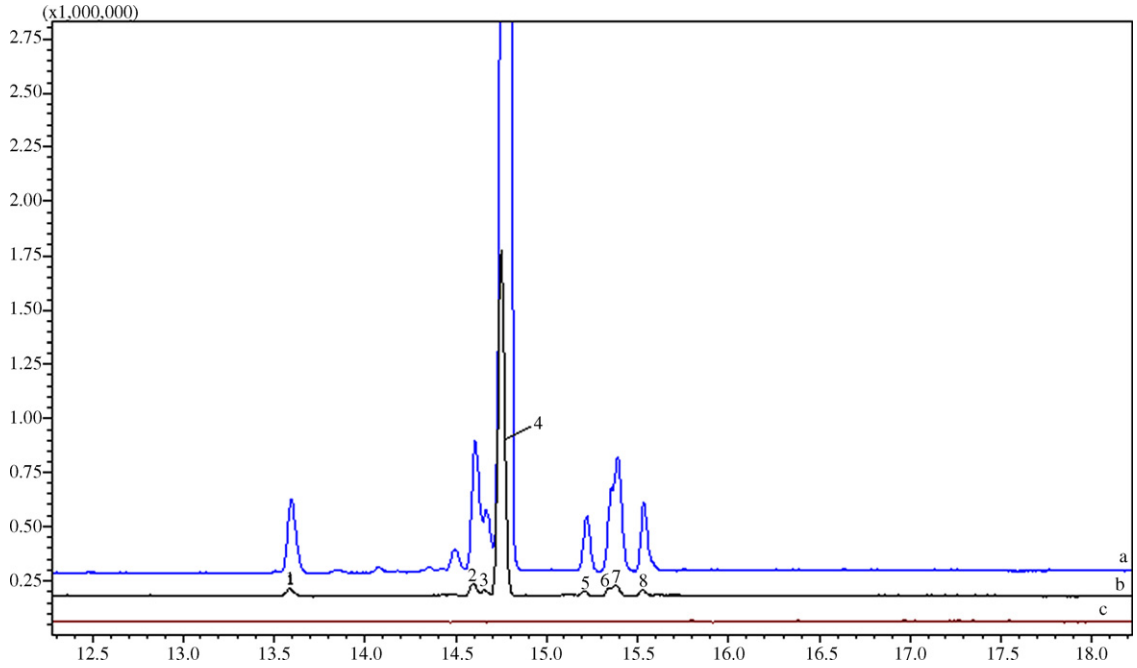

Fig. 2. GC chromatograms of EtOAc extract: (a) EEI; (b) desorption eluate of cells incubated with EEI; (c) desorption eluate of cells cultured in RPMI 1640 medium without EEI (blank).

TCMs and it also provides a convenient tool to directly acquire structural information of the bioactive compounds.

Based on the experimental conditions as mentioned above, GC chromatograms of EtOAc extract of EEI and desorption eluate of cells incubated with EEI as well as the blank control (desorption eluate of cells cultured in RPMI 1640 medium without EEI) were shown in Fig. 2. Eight peaks were detected in desorption eluate of cell extract. And no peak was found in the blank control. According to the mass spectra, peaks 1–8 were identified as δ-elemene, 8-isopropenyl-1,5-dimethyl-cyclodeca-1,5-diene, β-bourbonene, β-elemene, junipene, β-cubebene, β-caryophyllen, and γ-elemene, respectively. It can be seen from Table 1 that the molecular masses (M_w) of components for all detected peaks are determined as 204. And the chemical structures given by MS indicate those compounds are all isomers of sesquiterpenes.

β-elemene is the main bioactive component of EEI. It has been extensively validated to have broad anticancer effect by experimental and clinical pharmacology [24–28]. In the present experiment, β-elemene is observed to be a main peak in target cells (see Fig. 2b), which implies that it is the main active component of EEI for HCa-F cells. Particularly, the main peak of β-elemene and other minor detected peaks all belong to sesquiterpenes. In fact, many volatile oils with bioactivities reveal the occurrence of unusual sesquiterpenes as the major compound. The anticancer activity of some sesquiterpenes has

Table 1
Fragment mass of eight components identified from EtOAc desorption eluate of elemene-cell incubated mixture

No.	Compound	t_R (min)	Simila. (%)	Mass data[a]
1	δ-Elemene	13.61	93	204(M^+, 2), 189(5), 161(28), 136(60), 121(100), 119(10), 105(21), 93(80), 91(29)
2	8-Isopropenyl-1,5-dimethyl-cyclodeca-1,5-diene	14.62	91	204(M^+, 2), 189(19), 161(27), 147(19), 133(24), 121(34), 107(53), 93(100), 91(37), 81(85), 79(52), 77(25), 67(59), 55(35)
3	β-Bourbonene	14.68	94	204(M^+, 3), 161(21), 123(78), 119(9), 105(15), 91(16), 81(100), 79(27), 69(5)
4	β-Elemene	14.77	97	204(M^+, 2), 189(22), 161(27), 147(42), 133(34), 121(45), 93(100), 91(41), 81(93)
5	Junipene	15.23	93	204(M^+, 35), 189(38), 161(92), 147(32), 133(67), 119(66), 107(71), 105(82), 91(100), 79(69), 77(40), 67(46), 65(24)
6	β-Cubebene	15.36	86	204(M^+, 10), 161(85), 120(100), 119(41), 105(81), 93(43), 91(67), 79(38), 67(20)
7	β-Caryophyllen	15.41	93	204(M^+, 7), 189(14), 161(35), 147(26), 133(87), 120(53), 107(47), 105(63), 93(100), 91(92), 79(71), 77(41), 69(80), 67(41)
8	γ-Elemene	15.55	95	204(M^+, 6), 189(10), 161(20), 133(22),121(100), 119(30),107(44), 105(43), 93(69), 91(38), 79(33), 77(22), 67(30), 65(10)

[a] (m/z) Relative intensity shown in parenthesis, and the ion of relative intensity 100 was used for the quantification.

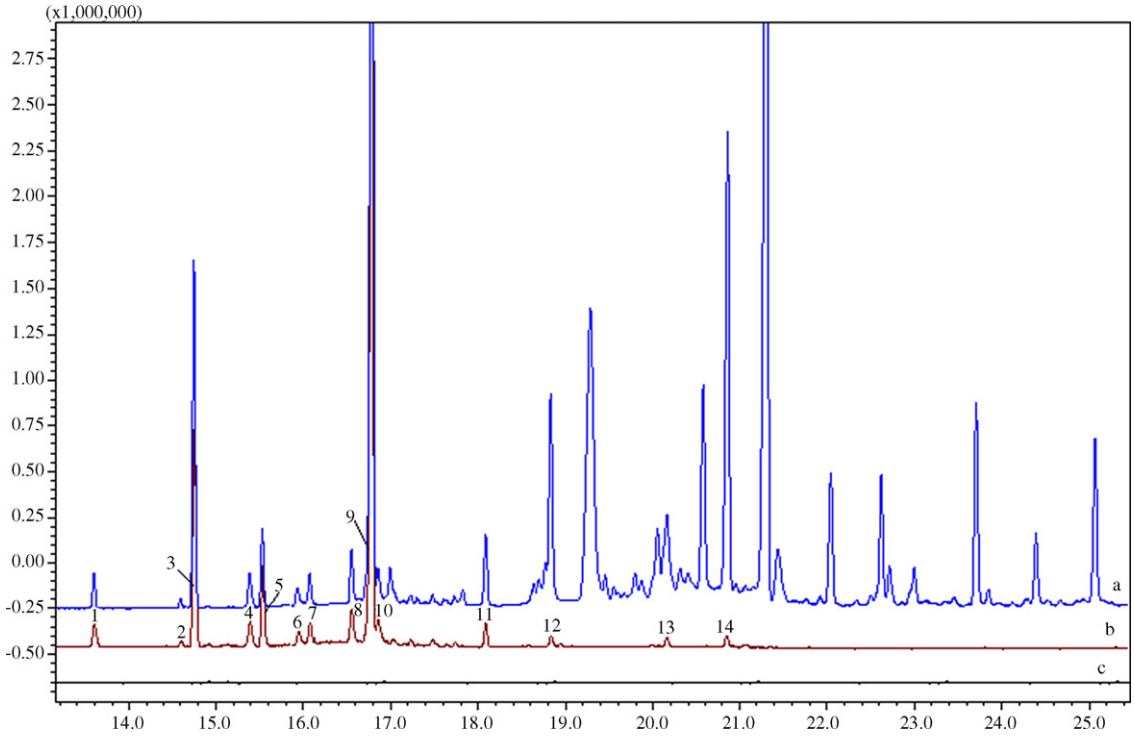

Fig. 3. GC chromatograms of EtOAc extract: (a) ZTOGI; (b) desorption eluate of cells incubated with ZTOGI; (c) desorption eluate of cells cultured in RPMI 1640 medium without ZTOGI (blank).

been reported [32,33]. Therefore, we can conclude that the established method is effective.

3.3. Application of the method in screening bioactive components from ZTOGI

Zedoariae rhizoma (ZR) is a traditional Chinese medicine which essential oils are considered as active constituents. Sesquitepenes are chemical constituents of ZR, some of which were reported to exhibit antihepatotoxic effect [22,23]. ZTOGI is a kind of antivirus medicine in China and has been widely used in clinical trials for its good therapeutic effect on early cervical cancer and has been used to treat malignant lymphoma as well as idiopathic liver cancer.

For this ZTOGI-cells treatment, the selection of conditions of cell culture is analogous to that described in Section 3.1.1 (procedures omitted here). The condition is that aliquots of cells of 4×10^7 (at a seeded density of 1×10^6 cells/ml) were incubated in ZTOGI medium with 360 μg/ml zedoary turmeric oil for 4 h.

According to the experimental method as described in Section 2.3, sample 2, EtOAc extract of ZTOGI, the desorption eluate of cells interacted with ZTOGI and the blank desorption eluate were obtained. GC–MS was performed for the analyses (Fig. 3). It can be seen most of the main components in chromatogram of ZTOGI were separated well. Nearly 50 peaks were found in chromatogram of ZTOGI, including some minor peaks (Fig. 3a). Fourteen principal peaks were detected in the cell desorption eluate, excluding several minor peaks. Qualitative identification of the different constituents was performed by their relative retention time and mass spectra [34–38]. In chromatogram of ZTOGI (Fig. 3a), components for peaks with the retention times between 13.5 and 16.6 min belong to sesquiterpenes; components for peaks with the retention time between 16.9 and 20 min are mainly compounds of hydroxy sesquiterpenoids with the M_w of 222, and peaks after retention time of 20 min are mainly keto-sesquiterpenes and diketo-sesquiterpenes. Components for peaks 1–14 except peak 10 in the desorption eluate of cell extract (Fig. 3b) were identified as δ-elemene, 8-isopropenyl-1, 5-dimethyl-2, 5-cyclodecadiene, β-elemene, β-caryophyllene, γ-elemene, seychellene, α-caryophyllene, germacrene D, curzerene, germacrene B, β-elemenone, neocurdione, and germacrone, respectively. Peak 10 was partially overlapped with peak 9 and several minor peaks (unmarked) failed to be identified. Curzerene and β-elemene were observed to be two main bioactive compounds in cell extract of ZTOGI. The chemical structures of these identified bioactive compounds were displayed in Fig. 4.

Comparing Fig. 3a and b, two important points deserve to be noted. The first one is there are more than 50 peaks in the original ZTGOI, while only 14 principal peaks in the cell extract. The second one is some high content components in ZTOGI have no binding with the target cell, while some components with very low content are detected in the ZTOGI-cells extraction. This indicates that these components with low content have significant interactions with the target cell, that is to say, the interaction between cell and drug is selective. Further more, one can find that some bioactive compounds, such as δ-elemene, 8-isopropenyl-1, 5-dimethyl-2, 5-cyclodecadiene, β-elemene, β-caryophyllene as well as γ-elemene were all detected both in elemene-cells and ZTOGI-cells when comparing the identified results of detected bioactive components from EEI and those from ZTOGI, which firmly validated the reliability of our methodology.

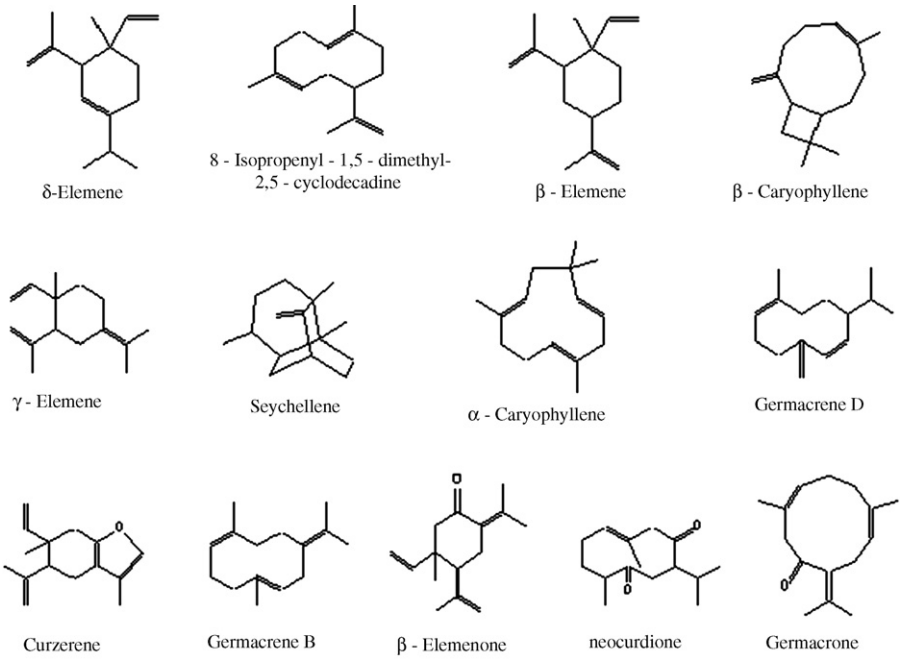

Fig. 4. Chemical structures of compounds identified in cell extract for ZTOGI.

Besides β-elemene, curzerene and neocurdione as well as germacrone are thought to be the main biological active ingredients in zedoary turmeric oil. Ogunwande et al. studied an essential oil with abundance of curzerene and germacrone and found it exhibited potent cytotoxic activity and varying antibacterial effects [39]. Matsuda et al. [22,40] isolated several sesquiterpenes from turmeric rhizomes and made a few studies by in vitro experiments. Germacrone and neocurdione were found to show potent protective effects on D-galactosamine/lipopolysaccharide-induced liver injury in mice and potent inhibitory effects on contractions induced by high concentration of K^+ in isolated rat aortic strips. In addition, β-caryophyllene has been found to exhibit cytotoxic activity against several solid tumor cell lines [23,41]. Farag et al. reported the finding of a receptor neuron type in females of *Helicoverpa armigera* with high sensitivity to and selectivity for the germacrene D [42]. However, most of the biological activity researches on essential oils extracted from turmeric rhizomes so far mainly focused on the biological efficacy of the formula that is composed of a few bioactive components. Therefore, further studies aimed at determining the anticancer properties of the other major constituents, as well as identifying the unknown compounds of oils, are necessary to fully understand its bioactivity.

4. Conclusions

The organic solvent extraction of cell that was acted by TCMs in this study could be employed in screening of bioactive compounds against different varieties of cells from TCMs. As there are no reports in the literatures on screening of bioactive compounds using cell extract, and hence direct comparison with other reports seemed to be difficult. Available in vitro screenings so far mainly focused on immobilized artificial membrane technique such as immobilized liposome chromatography and immobilized plasma proteins biochromatography. But in this paper, we reported a facilitated method for predicting the bioactivities of multiple compounds in TCMs simultaneously using cell extract integrated with GC–MS, which greatly improve the resolution and structural identification of potential active compounds in TCMs. Further more, it took into account the integrative characteristic of the pharmaceutical activity of TCMs. Apart from that, the cell strain that was described could perform various TCMs with very low content in a comparatively short time. This application can be extended to the non-volatile samples of TCMs, if combined with other chromatographic techniques, such as HPLC, as well as other complementary techniques, such as MS and NMR. Quantitative characterization of the method will be performed in the future.

Acknowledgements

The studies have been supported by the foundation (No. 20425516) for Distinguished Young Scholars and foundation (No. 90209048) from National Natural Science Foundation of China and the Knowledge Innovation Program of the Chinese Academy of Sciences (KSCX2-SW-329).

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