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Comparison of techniques for the extraction of the anti-cancer drug camptothecin from *Nothapodytes foetida*

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Abstract

Extraction methods using stirring extraction, Soxhlet extraction, ultrasonic extraction and microwave-assisted extraction (MAE) were evaluated for the percentage extraction of camptothecin (CPT) and 9-methoxycamptothecin (9-Me-CPT) from *Nothapodytes foetida*. The extracts were analyzed by high performance liquid chromatography (HPLC). Methanol (90%, v/v) extracted high percentage extraction of CPT and 9-Me-CPT compared to ethanol (90%, v/v). The results shows that the percentage extraction of CPT and 9-Me-CPT from *N. foetida* by MAE was more efficient in short time followed by Soxhlet extraction, ultrasonic and stirring extraction methods. Maximum percentage extraction of CPT (2.67%, w/w) was obtained by MAE technique. MAE has need of 3 min, whereas ultrasonic extraction, Soxhlet extraction and stirring extraction techniques require 30, 120 and 30 min, respectively to leach higher percentage extraction of CPT and 9-Me-CPT. The times taken by the microwave extraction process was 40 times less than the Soxhlet extraction for percentage extraction of alkaloids. The present results show that the extraction efficiency and considerable saving of time by MAE was more competent than the other extraction techniques.

Keywords: *Nothapodytes foetida*; Camptothecin; 9-Methoxycamptothecin; Extraction methods

1. Introduction

Nothapodytes foetida (Slemure) Wight [formerly, *Mappia foetida* (Miers)] is a rich source of potent alkaloid camptothecin (CPT) and 9-methoxycamptothecin [1,2]. Biological screenings have recognized that camptothecin and its derivative 9-methoxycamptothecin of *N. foetida* have promising anti-cancer activity [3]. The molecular target of camptothecin is inhibiting the nuclear enzymes topoisomerase I-DNA complex [4,5]. Li et al. [6] demonstrated that camptothecin inhibits Tat-mediated transactivation of human immunodeficiency virus type 1 (HIV-1) LTR and this important result offered a potential target for therapy of HIV-1 infection. Camptothecin itself is not used clinically due to its cytotoxicity, but its derivatives are most effective for the treatment of cancer throughout the world. Interest in camptothecin congeners was renewed when it was reported that the camptothecin derivative 9-aminocamptothecin exhibits curative activity against human colon adenocarcinoma xenografts grown nude mice [7]. Camptothecin and its derivatives inhibited the growth of human breast carcinoma cell in vitro and induced complete regression of breast tumors [8]. This stimulated the development of water-soluble analogues of camptothecin and two analogues irinotecan (CPT-11) and topotecan have been of major interest in the present decade and approved for the treatment of cancer [9,10]. These two semi-synthetic derivatives of camptothecin have the ability to decrease cytotoxicity and in-

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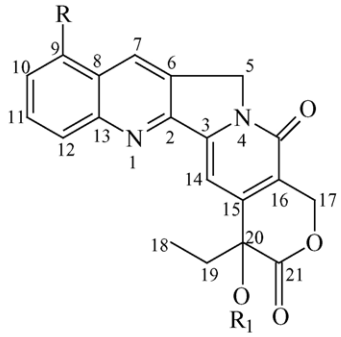
crease water solubility. Higher concentration of camptothecin alkaloid is present in *N. foetida* than other plants *Camptotheca acuminata*, *Ervatamia heyneana* and some species of the genus *Ophiorrhiza*, and thus it represent the most convenient and attractive source for large-scale production of this pharmacologically interesting biologically active compound.

Extraction and product recovery are the most imperative steps in the evaluation of target molecules from various plant parts. Most of the extraction processes are time consuming, laborious, involves lengthy operation techniques, bulk amount of solvents and ultimately thermal decomposition of the target molecules at continuous high temperature. Stirring extraction process using different concentration of solvents have also been used and it was found that the presence of water increase the extraction power of the solvents [11]. Stirring extraction methods have been applied for the extraction of biological active compounds [12,13]. The extraction of biological active compounds by Soxhlet has been used with several solvents. This method takes a few hours, even more than 24 h for extraction and large amount of solvents wasted. Soxhlet extraction has been applied for various compounds [14,15]. Ultrasonic extraction uses high frequency sound to disrupt the target compound from the plant materials. Ultrasonic process was used for the extraction of tobacco nicotine alkaloids [16]. Recently, there have been several reports on the application of ultrasonic methods in the extraction of various phytochemicals. Ultrasonic assisted extraction has been applied for essential oils [17], polysaccharides [18], aroma [19] and phytochemicals [20] from various parts of plants. Ganzler et al. [21] demonstrated first report on microwave-assisted extraction system for extraction of biologically active compounds has many advantages over other conventional extraction methods. Microwave-assisted extraction methods required shorter time, less solvents, higher extraction rate and better products with lower costs. Numerous biologically active compounds have been extracted with application of microwave-assisted extraction, such as extraction of taxanes from *Taxus brevifolia* needles [22], extraction of azadiractine related limonoids from *Azadirachta indica* seed kernel [23], extraction of glycyrrhizic acid from *Glycyrrhizia glaubra* root [24], extraction of tanshinones from *Salvia miltorrhiza bung* [12,25], extraction of artemisinin from *Artemisia annua* [13], and extraction of ginsenosider from *Panax ginseng* root [26].

Ultrasonic extraction CPT and 9-Me-CPT from the cell cultures of *N. foetida* and followed by analysis by high performance liquid chromatography has been reported [2,27]. However, no report has been done on the use of different extraction techniques for the extraction of CPT and 9-Me-CPT with a view to reduce the time and increase extraction percentage rate from the dried plant material of *N. foetida*. The aim of this work is to evaluate the extraction efficiencies of CPT and 9-Me-CPT (Fig. 1) using different extraction techniques.



CPT: R = R_1=H

9-Me-CPT: R = OMe, R_1 = H

Fig. 1. Chemical structure of camptothecin (CPT) and 9-methoxycamptothecin (9-Me-CPT).

2. Experimental

2.1. Plant materials and reagents

Plant material of *N. foetida* was provided by Medipla Biotech, Navi Mumbai, Maharashtra State, India. The material was dried at 50 °C in air dryer for 48 h. Dried material was powdered by Wiley mill (Model No. 4276, Thomas Scientific, USA). Methanol, ethanol, diammonium hydrogenorthophosphate (anhydrous) were all of analytical grade chemicals (SISCO Research Lab., Mumbai, India). Methanol and acetonitrile were used for HPLC of reagent of HPLC grade (Merck, Mumbai, India). Camptothecin was purchased from Sigma (USA) and 9-Me-camptothecin provided by Yakult Central Institute for Microbiological Research, Tokyo, Japan.

2.2. Stirring extraction

Stirring extraction was carried out on Schott magnetic stirrer (SLR, Schott Glass, Germany) equipped with temperature sensor. The stirring speed was 150 × *g* and suspension temperature controlled while stirring. Plant material was extracted with 90% methanol and ethanol. Material of *N. foetida* (5 g) was put into a 250 ml flask and added appropriate solvent (100 ml). The material was extracted at different temperature from 40 to 70 °C for 60 min and controlled by temperature sensor. After extraction the contents of the flasks were filtered through filter paper (Whatman No. 1, UK). The filtrates were centrifuged at 14,000 × *g* for 10 min and subsequently analyzed by HPLC. The stirring extraction procedure was repeated three times at different temperature with methanol and ethanol.

2.3. Ultrasonic extraction

Ultrasound assisted extraction was performed in a ultrasonic bath (Model No. TEC 40, Roop Telesonic Ultrasonix, Mumbai, India) and working frequency was 33 KHz. Five grams of *N. foetida* plant material extracted with 90% methanol (100 ml) in 100 ml volumetric flasks and kept for

sonication for 15, 30 and 60 min at room temperature. After extraction, the contents were filtered and evaporated to dryness and dissolved in methanol followed by centrifugation at 14,000 × *g* for 10 min. The procedure of ultrasonic extraction of plant material was repeated three times in the same manner.

2.4. Soxhlet extraction

For Soxhlet extraction, 5 g of dried plant material was put into 200 ml Soxhlet thimble. The apparatus was fitted with 250 ml round bottom flask containing 100 ml of 90% methanol. The extraction temperature was controlled at 70 °C with a regulator. The flask was heated for 60, 120 and 180 min and the solvent was refluxed until a given time was up.

2.5. Microwave extraction

N. foetida plant material (5 g) was put into a 100 ml Erlenmeyer flasks and added 100 ml 90% methanol. The flasks with suspension were exposed for 3 min in a microwave oven (MCG-LG, MG583, Mumbai, India) at 100 W. The suspensions were not allowed to super boil. Microwave irradiation stopped for a minute to avoid supper boil of suspension followed by the flasks were taken out and cooled under running water for 2 min. Same flasks were exposed to microwave irradiation after cooling. Above steps were repeated in order to complete 3 min microwave irradiation. Total 7 min required for each flask including cooling and microwave irradiation.

2.6. HPLC analysis and conditions

The suspensions from different extraction methods were filtered through filter paper followed by centrifugation at 14,000 × *g* (Eppendorff, Switzerland) for 10 min and aliquots of supernatant were transferred to glass vials. The HPLC analyses were carried out on a Jasco liquid chromatograph (Model 980, Japan) equipped with an autosampler injector (Model No. Jasco AS-950, Japan) with a 25 μl loop and a variable-wavelength detector (Model No. UV-975, Japan). Data collection and integration were accomplished using BORWIN software. Separations were performed on a Supelco C_{18} (250 mm × 4.6 mm i.d., Sigma, USA) column. The camptothecin was determined by using acetonitrile–5 mM diammonium hydrogen orthophosphate (anhydrous) (45:55, v/v) as a mobile phase. The flow rate was 1 ml/min and the elution was monitored at 254 nm. Retention times of CPT and 9-Me-CPT were 4.66 and 5.35 min, respectively (Fig. 2). This method is sensitive and accurate with good reproducibility. Validation of quantitative method was performed with samples for five times. The results of the five injections from the same samples at the five concentrations (0.01–0.5 μg) showed similar retention time. The analytical operation can be completed in 10 min. The percentage extraction efficiency of alkaloid was defined elsewhere [24].

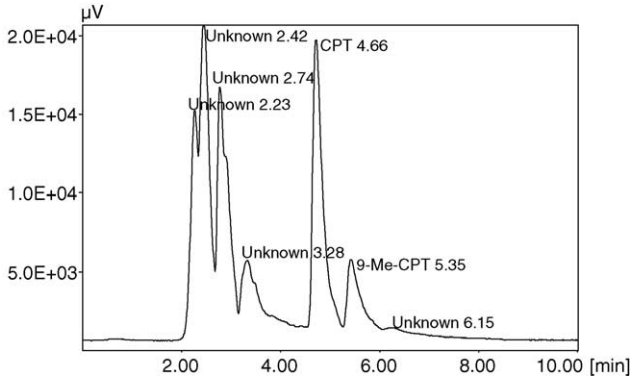


Fig. 2. Chromatogram of camptothecin and 9-methoxycamptothecin obtained by microwave extraction from plant material of *N. foetida*. Chromatographic conditions: mobile phase, acetonitrile–5 mM diammonium hydrogenorthophosphate (anhydrous) (45:55, v/v); flow rate 1 ml/min, column, Supelco C_{18} (250 mm × 4.6 mm i.d.); detection, UV at 254 nm; camptothecin (CPT-retention time 4.66) 9-methoxycamptothecin (9-Me-CPT-retention time 5.35).

3. Results and discussion

3.1. Effect of stirring extraction technique with solvents at different temperature on percentage extraction of CPT and 9-Me-CPT

Initially, preliminary experiments were performed in order to establish the suitable extraction solvent for CPT and 9-Me-CPT by using stirring extraction technique. Methanol and ethanol with similar concentration (90%) were studied. Fig. 3 shows the effect of methanol and ethanol at different temperature with application of stirring extraction technique on percentage extraction of CPT and 9-Me-CPT. The present results revealed that the highest percentage extraction was obtained when the material extracted with methanol (90%)

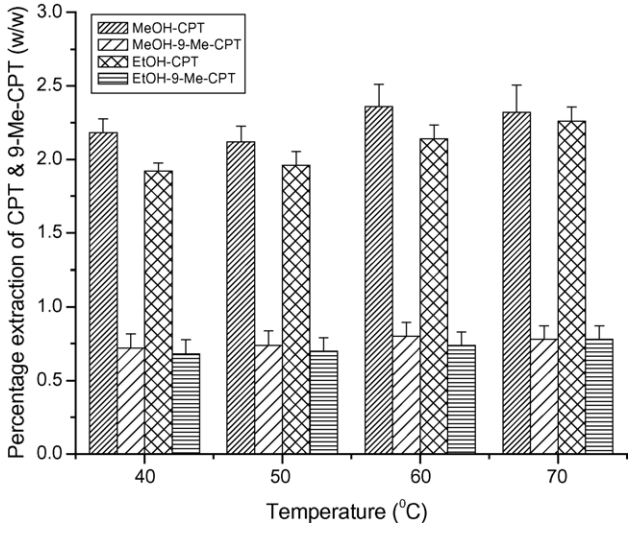


Fig. 3. The effect of stirring extraction with methanol and ethanol (90%, v/v) solvents at different temperature on percentage extraction of CPT and 9-Me-CPT. Values expressed as means ± S.D. ($n = 3$).

at 60 °C and achieved 2.36% extraction of CPT. The percentages extraction of compounds steadily increased with the increase of temperature. Similarly, 90% (v/v) aqueous ethanol shows the increase of percentage extraction efficiency increased with the temperature. Highest percentage of extraction of CPT and 9-Me-CPT was achieved at 70 °C (Fig. 3). Pan et al. [25] reported that the percentage extraction of tanshinones increased with increase of temperature. The present data indicated that the highest percentage extraction of CPT and 9-Me-CPT were obtained by 90% aqueous methanol as compared with ethanol by using stirring extraction technique. Therefore, 90% aqueous methanol was preferred to use for ultrasonic, Soxhlet and MAE to evaluate the percentage extraction of CPT and 9-Me-CPT from the plant material of *N. foetida*.

3.2. Effect of ultrasonic extraction time on percentage extraction of CPT and 9-Me-CPT

The dried material was extracted in sonicator for period of 15, 30 and 60 min with 90% methanol in order to determine the contact time required to achieve the maximum yield of percentage extraction of CPT and 9-Me-CPT. Fig. 4 shows the percentage extraction of alkaloids from *N. foetida* against sonication time period. The percentage extraction of CPT and 9-Me-CPT slight increased with sonication period extended from 15 to 30 min (Fig. 4). Period of sonication time increased the percentage extraction efficiency of tobacco alkaloids [14]. In contrast, longer sonication extraction time was decreased the percentage extraction of tanshinones from *Salvia miltiorrhiza bunge* [25]. Present results also indicated that decreased the percentage extraction of CPT and 9-Me-CPT when dried materials were extracted for longer sonication extraction time (60 min).

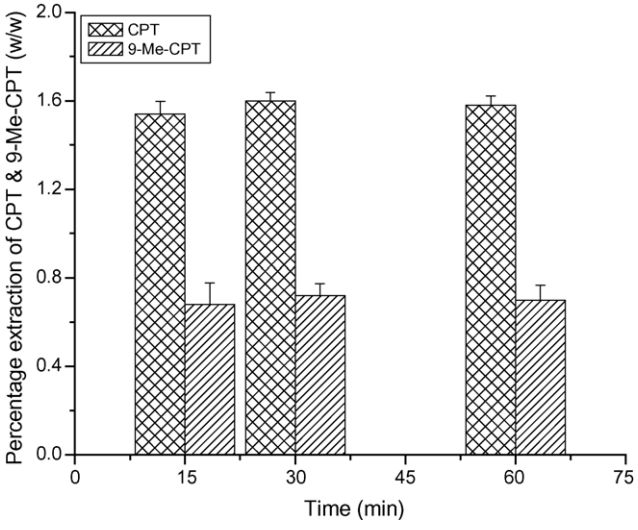


Fig. 4. The effect of ultrasonic extraction time on percentage extraction of CPT and 9-Me-CPT. Values expressed as means ± S.D. ($n = 3$).

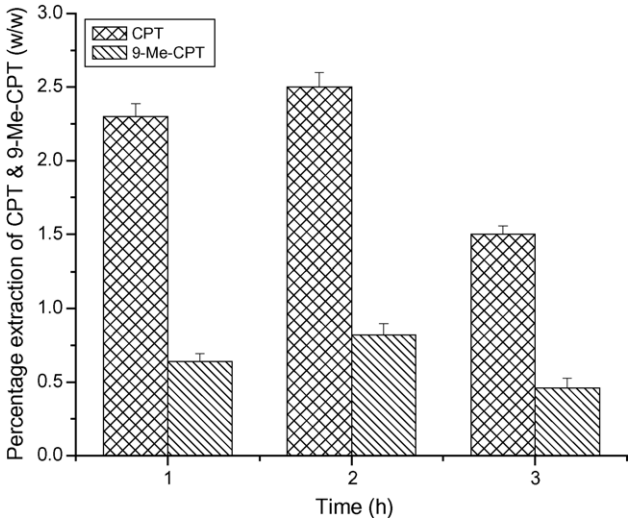


Fig. 5. The effect of Soxhlet extraction time on percentage extraction of CPT and 9-Me-CPT. Values expressed as means ± S.D. ($n = 3$).

3.3. Effect of Soxhlet extraction time on percentage extraction of CPT and 9-Me-CPT

Fig. 5 shows the effect of Soxhlet extraction time on percentage extraction of CPT and 9-Me-CPT. The results show that the percentage extraction of alkaloid increased with the increase of Soxhlet extraction time period from 1 to 2 h. When the Soxhlet extraction period was increased to 3 h the percentage extraction of alkaloids decreased due to partly to the decomposition of compounds. Soxhlet extraction provides better reproducibility and percentage extraction higher than that of ultrasonic extraction techniques [28–30]. Similarly, our results with plant material of *N. foetida* shows that the Soxhlet extraction techniques achieved a 1.5-fold higher extraction than the ultrasonic extraction technique.

3.4. Comparison of microwave-assisted extraction and conventional extraction techniques

The percentage extraction of CPT and 9-Me-CPT obtained from different extraction techniques in order to achieve high extraction rate with less consumption of solvent and saving extraction time. Fig. 6 depicts that percentage extraction of CPT and 9-Me-CPT was reached after 30, 30, 120 and 3 min when stirring extraction, ultrasonic extraction, Soxhlet extraction and MAE techniques were used respectively. The results show that the MAE reduces the extraction time of period as compare to conventional techniques. MAE technique obtained maximum percentage extraction of alkaloids in short time compared to the more time consuming Soxhlet, ultrasonic and stirring extraction techniques. Similar results have been reported on comparing conventional extraction techniques with MAE for the extract of tanshinones from *Salvia miltiorrhiza bunge* [25,28], for extraction of tobacco alkaloids from tobacco [14], artemisinin from *Artemisia annua*

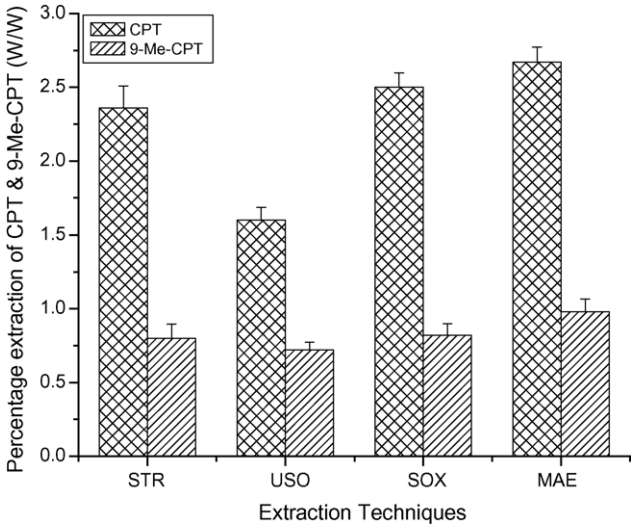


Fig. 6. Comparison of percentage extraction of CPT and 9-Me-CPT from plant material of *N. foetida* by different extraction techniques. STR: stirrer extraction; USO: ultrasonic extraction; SOX: Soxhlet extraction; MAE: microwave-assisted extraction. Values expressed as means ± S.D. ($n = 3$).

[13], saponins from cultured cells of *Panax notoginsend* [31], and furanocoumarins from *Pastinaca sativa* fruits [32].

4. Conclusion

By comparing various extraction methods for camptothecin and 9-methoxycamptothecin, the microwave-assisted extraction was more efficient by other time consuming techniques. In order to product recovery with solvents of methanol and ethanol, the methanol showed higher percentage of extraction of CPT and 9-Me-CPT. The extraction temperature was found to be one of the important factors. The percentage extraction of alkaloids was minimum by ultrasonic extraction techniques. The MAE reduce the extraction time and required 3 min to extract similar percentage of extraction of alkaloids whereas Soxhlet extraction techniques need 2 h which is 40 times more. When compared with stirring extraction, ultrasonic extraction and Soxhlet extraction, and the MAE system showed extraction efficiency in short time. Therefore, MAE is an alternative extraction technique for speedy extraction of anti-cancer drug CPT and 9-Me-CPT from plant material of *N. foetida*. The results indicated MAE for large-scale efficient extraction of camptothecin from plant materials.

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