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Estimation of camptothecin and pharmacological evaluation of *Ophiorrhiza prostrata* D. Don and *Ophiorrhiza mungos* L.

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ABSTRACT

Objective: To carry out the qualitative and quantitative evaluation of camptothecin, estimation of total phenolic compounds and evaluation of *in vitro* antioxidant activity and cytotoxic activity of *Ophiorrhiza prostrata* and *Ophiorrhiza mungos*. **Methods:** Direct Analysis in Real Time- Mass Spectrometry (DART-MS) was employed for the detection of camptothecin in the *Ophiorrhiza* species, while high performance thin layer chromatography (HPTLC) was used for the estimation of camptothecin. Total phenolic compounds were estimated by modified Folins-Ciocalteu's reagent method. Antioxidant activity was evaluated through DPPH radical, hydroxyl radical, superoxide radical scavenging assays and reducing power assay. The cytotoxicity evaluation was performed using MTT assay on MCF-7 cell lines. **Results:** The presence of camptothecin was confirmed in both the species by the $[M_{++}H]$ peak at 349 by DART-MS analysis. Camptothecin content was estimated as 1.47 μg/gm (dry wt) in *O. prostrata* and 188.60 μg/gm (dry wt) in *O. mungos* using HPTLC method. The moderate *in vitro* antioxidant activities of the methanol extracts corroborates with the low content of phenolic compounds in *O. prostrata* (9.88 GAE mg/g) and *O. mungos* (12.73 GAE mg/g). The methanol extract of *O. prostrata* exhibited remarkable cytotoxicity on human breast cancer cell lines (MCF-7), with IC_{50} value 1.10 μg/mL compared to *O. mungos* (3.48 μg/mL) and standard camptothecin (3.51 μg/mL). **Conclusions:** The application of DART-MS proved to be a simple and rapid technique for the detection of camptothecin in *Ophiorrhiza* species. The higher cytotoxicity for *O. prostrata*, despite the low content of camptothecin suggests the presence of other potential cytotoxic compounds in *O. prostrata*.

1. Introduction

The genus *Ophiorrhiza* (Rubiaceae) is represented by 49 species in India and different species of the genus have been used in traditional medicines against snake bite, stomatitis, ulcers and wound healing[1]. *Ophiorrhiza* species, especially *O. mungos* has emerged as a source of the anticancer drug camptothecin, which has drawn great attention world wide as a potential inhibitor of DNA topoisomerase-1[2]. Topotecan and irinotecan, two semisynthetic derivatives of camptothecin, are currently being used for the treatment of colorectal and ovarian cancer[3]. Camptothecin also possesses activity against HIV-1[4], HSV-2[5], parasitic trypanosomas and leishmania[6]. The anticancer compound camptothecin has previously been reported from Camptotheca accuminata[7], Nothapodytes foetida[8], Merrilliodendron megacarpum[9], Ervatamia heyneana[10] and several *Ophiorrhiza* species including *O. mungos*[11] and *O. prostrata*[12].

O. mungos and *O. prostrata* are herbaceous species, distributed in the tropical Indo-Malaysian region[13].

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Though camptothecin has been estimated in Camptotheca accuminata[14] and Nothapodytes foetida[15], reliable estimation of camptothecin in *O. mungos* and *O. prostrata* are scarce[11]. Cytotoxicity of camptothecin has been tested in different cell lines and reported as an active compound[2], however, the cytotoxicity of the title plants have not been evaluated previously. Present study reports the detection of camptothecin by DART-MS and estimation of camptothecin by HPTLC together with estimation of total phenolic compounds, evaluation of *in vitro* antioxidant and cytotoxic activities of the methanol extracts of *O. mungos* and *O. prostrata*.

2. Materials and methods

2.1. Plant material

O. prostrata D. Don and *O. mungos* L. were collected from Thiruvananthapuram, Kerala, India in August 2010 and deposited in the herbarium of JNTBGRI (Herbarium voucher specimens nos.TBGT 69942 and TBGT 69945 for *O. prostrata* and *O. mungos* respectively).

2.2. Extraction

Shade dried whole plant samples were powdered (5g each) and defatted with petroleum ether using Soxhlet apparatus for 2h. The defatted samples were extracted with methanol using Soxhlet apparatus for 4 h. and the methanol extracts were used for further studies.

2.3. Direct analysis in real time- mass spectrometry (DART-MS)

The mass spectrometer used was a JMS-T100 LC (Accu ToF) atmospheric pressure ionization time-of-flight mass spectrometer (Jeol, Tokyo, Japan) fitted with a DART ion source. The mass spectrometer was operated in positive-ion mode. The DART ion source was operated with helium gas flowing at 4.0 L/min. The gas heater was set to 300 ℃. The potential on the discharge needle electrode of the DART source was set to 3000 V. Orifice 1 potential was set at 28V. The extracts were positioned in the gap between the DART source and mass spectrometer for measurements. Data acquisition was from m/z 10 to 1050.

2.4. HPTLC estimation of camptothecin

HPTLC was done on Camag HPTLC. 20 μL (7.2 mg/mL) and 1 μL (0.63 mg/mL) each of *O. prostrata* and *O. mungos* methanol extracts were applied on pre-coated silica gel plate 60F254 (E. Merk, Germany) using the Linomat V applicator. Camptothecin from Sigma Aldrich was used as the standard compound. 5.0 μL to 10.0 μL of standard camptothecin (0.1 μg/mL) was used for the estimation of *O. prostrata*, while 1.0 μL to 5.0 μL standard camptothecin (1.0 μg/mL) was used for the estimation of *O. mungos*. The separation was carried out in twin trough chamber using the solvent system toluene: acetonitrile: glacial acetic acid (65:35:1) as mobile phase. Quantitation was carried out using TLC scanner 3, in absorbance mode at 254 nm by comparing the peak area values of sample with that of standard using the Wincats software.

2.5. Total phenolic compounds estimation

Total phenolic compounds were estimated as gallic acid equivalent (GAE) mg/g dry weight using modified Folins-Ciocalteu's reagent colorimetric method[16].

2.6. 2, 2-diphenyl- 1-picrylhydrazyl (DPPH) radical scavenging assay

Radical scavenging activity was determined by spectrophotometric method based on the reduction of the stable free radical DPPH, using ascorbic acid as positive control[17].

2.7. Hydroxyl radical scavenging assay

The method depends on the competition between deoxyribose and the plant extract for hydroxyl radical generated from Fe^{3+}-EDTA-ascorbate system[18]. The hydroxyl radical scavenging activity of the extract was measured as percentage inhibition of deoxyribose degradation and ascorbic acid was used as positive control.

2.8. Superoxide scavenging assay

The method depends on the light induced superoxide generation by riboflavin and the corresponding reduction of nitroblue tetrazolium[19]. Quercetin was used as positive control.

2.9. Reducing power assay

The presence of reductants such as antioxidants substances in the samples causes reduction of the Fe^{3+}/ferricyanide complex to the green ferrous form. The extent of reduction can be monitored by measuring the formation of Perl's Prussian blue at 700 nm[20]. Ascorbic acid was used as positive control.

2.10. Cytotoxicity assay

Cytotoxicity was measured *in vitro* on human breast cancer cell line (MCF-7), using 3-(4,5-dimethythiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) colorimetric assay[21]. The 50% inhibitory concentration (IC_{50}) was derived from the dose-response curve.

2.11. Statistical analysis

The analyses were done in triplicate and the results are expressed as mean ± standard deviation. Results were analyzed by one-way analysis of variance (ANOVA). $P< 0.05$ was selected as statistically significant.

3. Results

3.1. Direct analysis in real time- mass spectrometry (DART-MS)

Camptothecin has been detected in the methanol extracts of both the species by the [M^{++}H] peak at m/z 349 (Figure1). The peak intensity was negligible for *O. prostrata* compared to *O. mungos*. DART-MS of *O. mungos* also revealed the presence of 9-methoxy camptothecin at m/z 379[22].

3.2. HPTLC estimation of camptothecin

The camptothecin content in *O. prostrata* was estimated as 1.465 ± 0.027 μg/g dry wt (0.0001465 ± 0.0000027%) (Figure 2) and that of *O. mungo* was 188.6 ± 12.3 μg/g dry wt (0.01886% ± 0.0012%) (Figure 3).

3.3. In vitro anti oxidant activity

Antioxidant activities of the methanol extracts of *O. mungos* and *O. prostrata* were tested in three *in vitro* free radical scavenging models, *viz.*, DPPH radical scavenging assay, superoxide radical scavenging assay and hydroxyl radical scavenging assay, along with reducing power assay. The results (Table 1) are expressed as IC_{50} values in μg/mL, the concentration of sample required to scavenge 50% free radical, except for reducing power assay. Reducing power increases with an increase in the absorbance at 700 nm and the results are expressed as absorbance of 50 μg/mL extract and standard solution at 700 nm. Both the extracts showed moderate activity, compared to the positive controls. The content of phenolic compounds were also low in both the species (Table 1).

3.4. Cytotoxicity assay

Both the extracts reduced the viability of the cell lines in a dose dependent manner. IC_{50} was derived from the dose-response curve (Figure 3). The methanol extract of *O. prostrata* exhibited remarkable cytotoxic activity on human breast cancer cell lines (MCF-7), with IC_{50} value 9.218±0.019 μg/mL, while *O. mungo* and standard camptothecin showed 34.81±0.03 μg/mL and 35.15±0.03 μg/mL respectively.

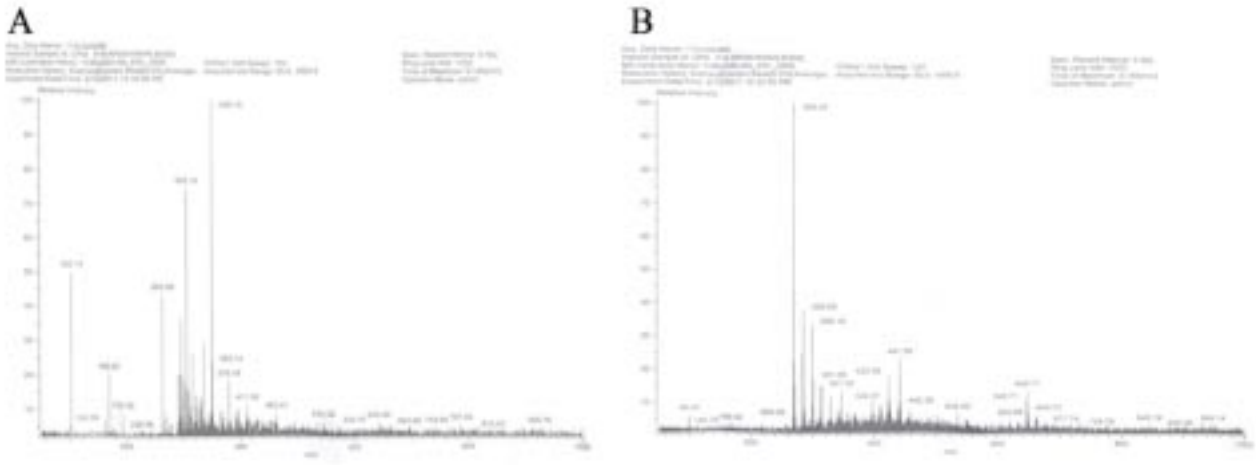

Figure 1. DART mass spectra.
A: *O. mungos*; B: *O. prostrata*.

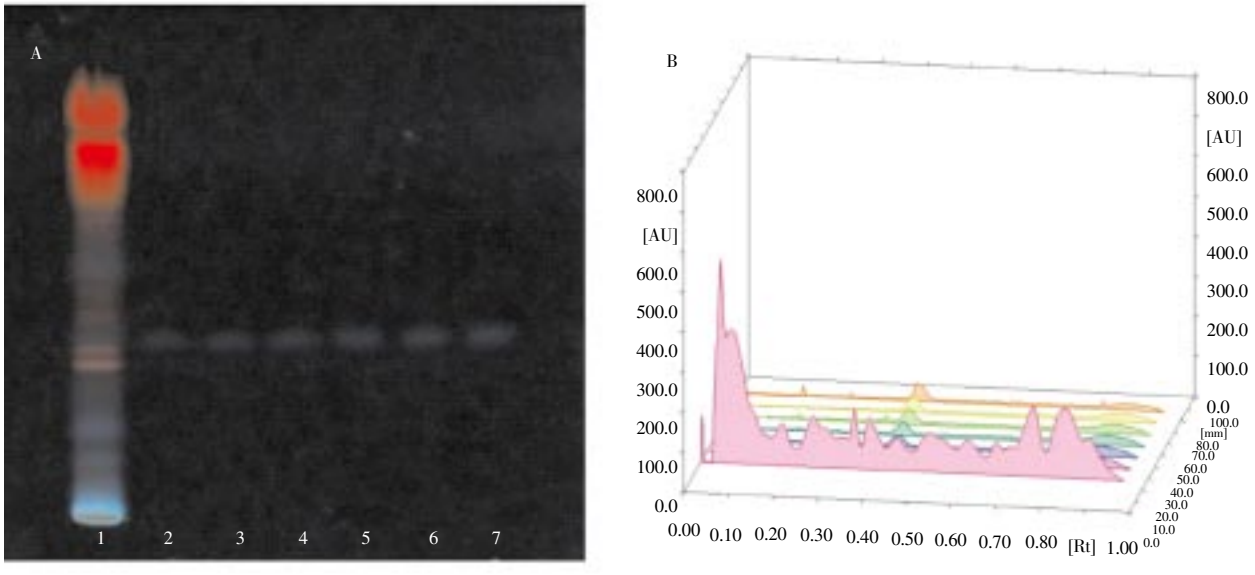

Figure 2. HPTLC of camptothecin from *O. prostrata* (Track1plant extract and track 2 -7 standard camptothecin).
A: chromatogram; B: three dimentional densitogram.

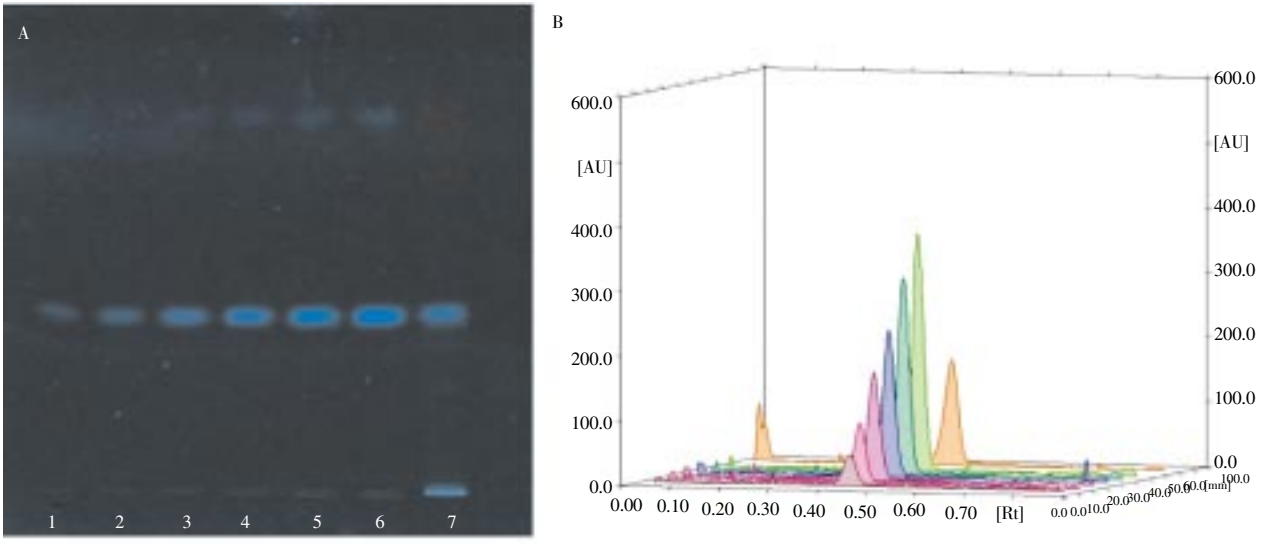

Figure 3. HPTLC of camptothecin from *O. mungo* (Track 1-6 standard camptothecin and track 7 plant extract).
A: chromatogram; B: three dimentional densitogram.

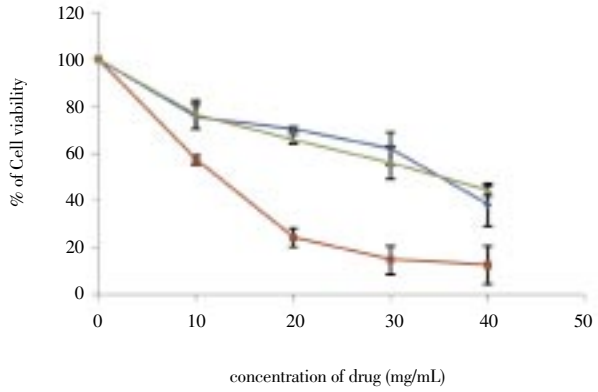

Figure 3. Dose-dependent cytotoxic activity of methanol extracts of *O. mungo*s (OM) and *O. prostrata* (OP) standard camptothecin (CPT), on MCF-7 using MTT assay.

4. Discussion

The ambient ionisation techniques such as direct analysis in real time (DART) and desorption electrospray ionisation mass spectrometry (DESI) gives the chemical profile with molecular weights without sample preparation or pre-separation as required in the case of liquid chromatography mass spectrometry analyses[22]. DART is a simple and rapid ambient ionization technique developed recently[23] and accepted widely in natural product research as a high-throughput tool for confirmation of chemical identity and metabolomic profiling[24]. The present investigation shows DART-MS as a reliable analytical technique for the detection of camptothecin in *Ophiorrhiza* species. The ion intensities shown by DART-MS suggests that the concentration found in *O. prostrata* is negligible than those found in *O. mungos* and the finding corroborates with the camptothecin content estimated by HPTLC. DART-MS of *O. mungos* also revealed the presence of 9-methoxy camptothecin at m/z 379[22] and the finding corroborates with previous report of camptothecin and 9-methoxy camptothecin in *O. mungos*[25].

HPTLC has become a potential tool for identification, authentication and quality control of phytochemicals[26]. HPTLC method has been successfully employed for the estimation of camptothecin from Nothapodytes foetida[27] and Camptotheca acuminata[28]. The present study showed the solvent system toluene-acetonitrile-glacial acetic acid (6.5:3.5:0.1) (v/v) giving the best resolution for the estimation of camptothecin in *Ophiorrhiza* species with varying concentration.

Phenolic compounds contribute significantly to the total antioxidant activity of plants[20] and the moderate *in vitro* antioxidant activities of *O. prostrata* and *O. mungos* were in corroboration with the low content of phenolic compounds in *O. mungos* and *O. prostrata*. Reactive oxygen species such as superoxide anion and hydroxyl radicals are known to exert

Table 1
Antioxidant activities of *O. prostrata* and *O. mungos* whole plant methanol extracts.

Ophiorrhiza species	IC_{50} values of free radical scavenging assays (μg/mL)			Reducing power assay*	Total phenolic compounds (GAE mg/g)
	DPPH radical	Hydroxyl radical	Suproxide radical		
O. mungo	64.57±2.42	80.30±6.71	55.59±5.14	0.16±0.02	12.72±0.57
O. prostrata	67.01±1.01	87.44±4.72	49.60±2.37	0.11±0.00	9.88±1.53
Standard compound#	03.30±0.15	08.73±0.70	27.32±1.06	1.11±0.02	–

*Absorbance of 50 μg/mL extract and standard solution at 700nm. #Ascorbic acid for DPPH radical, reducing power assay and for hydroxyl radical scavenging assay. Quercetin for superoxide radical scavenging assay. The analyses were done in triplicate and the results are expressed as mean ± standard deviation.

a mutagenic effect by oxidizing DNA bases and also cause DNA strand breakage, eventually increasing the risk of cancer development[29]. Plants rich in antioxidants such as phenolic compounds exert remarkable anticancer properties. However, the monoterpene indole alkaloids camptothecin and its analogues, reported widely in *Ophiorrhiza* species, induce cell death by blocking DNA replication enzyme topoisomerase-1[2] and this explains the higher cytotoxicity despite moderate antioxidant activity for the title plants. The difference in DART-MS profile as well as higher activity for *O. prostrata* suggests detailed phytochemical and biological investigation to isolate potential cytotoxic compounds leading to the discovery of new effective drugs against cancer.

Conflict of interest statement

We declare that we have no conflict of interest.

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