Sains Malaysiana 51(5)(2022): 1487-1504
http://doi.org/10.17576/jsm-2022-5105-18

Bioassay-guided Isolation of Triterpene Compounds from *Dillenia suffruticosa* and Their Cytotoxic Activities against Cancer Cells

(Pengasingan Bioasai-Terarah oleh Sebatian Triterpena daripada *Dillenia suffruticosa* dan Aktiviti Sitotoksik Melawan Sel Kanser)

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Received: 30 April 2020/Accepted: 8 October 2021

ABSTRACT

Previous studies showed the ability of *Dillenia suffruticosa* to inhibit the growth of cancer cells by means of cell cycle arrest and apoptosis, thus validating the traditional use of the plant in treating cancer. Therefore, the present study was designed to isolate and elucidate the bioactive compounds responsible for the anticancer properties of *D. suffruticosa* extract. Bioassay-guided fractionation of the most potent fractions of DCM and EtOAc extract of *D. suffruticosa* was performed via column chromatography followed by purification using preparative HPLC. The structures of the isolated compounds were elucidated by NMR spectroscopy. Biological experiment by MTT assay and a series of column chromatography resulted in the isolation of three triterpene compounds. However, only the structure of compound (**3**) was confirmed as 1-isopropenyl-4α, 4β, 8, 10, 14-pentamethyl-icosahydro-cyclopenta[a]chrysene-3, 17-diol. Another two compounds were labelled as (**1**) and (**2**), which currently are unidentified due to unsuccessfulness in the full analysis of the spectroscopic data that enable the assignment of all protons, carbons, and confirmation of the structure. Compound (**2**) isolated from DCM extract of *D. suffruticosa* was most cytotoxic towards the selected cancer cells compared to the other two compounds and selected chemotherapeutic drugs, tamoxifen, and cisplatin. The ability of the isolated compounds to inhibit the growth of cancer cells indicates that these compounds are the bioactive constituents in *D. suffruticosa* that is mainly responsible for the cytotoxic activities. For this reason, these isolated compounds could be used as a means for the standardisation of herbal product from *D. suffruticosa*.

Keywords: Active constituents; bioassay-guided fractionation; cancer cells; cytotoxicity; *Dillenia suffruticosa*; triterpenes

ABSTRAK

Kajian terdahulu mendedahkan keupayaan *Dillenia suffruticosa* untuk menghalang pertumbuhan sel kanser melalui tangkapan kitaran sel dan apoptosis dan membuktikan penggunaan tumbuhan tersebut secara tradisi dalam mengubati kanser. Oleh itu, kajian ini dirancang untuk memencilkan dan menguraikan sebatian bioaktif yang berperanan terhadap sifat antikanser ekstrak *D. suffruticosa*. Fraksinasi berpandukan bioasai terhadap fraksi yang paling poten daripada ekstrak DCM dan EtOAc tumbuhan *D. suffruticosa* dilakukan menggunakan kromatografi turus diikuti dengan penulenan menggunakan HPLC preparatif. Struktur sebatian yang dipencilkan telah diterbitkan menggunakan spektroskopi NMR. Uji kaji biologi menggunakan asai MTT dan siri kromatografi turus menghasilkan tiga sebatian triterpenes. Walau bagaimanapun, hanya struktur sebatian (**3**) telah disahkan sebagai 1-isopropenyl-4α, 4β, 8, 10, 14-pentamethyl-

icosahydro-cyclopenta [a] chrysene-3, 17-diol. Dua lagi sebatian yang dilabelkan sebagai (**1**) dan (**2**), dikelaskan sebagai belum dapat dikenal pasti kerana analisis penuh data spektroskopik yang membolehkan pengenalpastian semua proton, karbon dan pengesahan struktur, tidak dapat dilaksanakan. Sebatian (**2**) yang telah diasingkan daripada ekstrak DCM tumbuhan *D. suffruticosa* adalah yang paling sitotoksik terhadap sel kanser yang dipilih berbanding dengan dua sebatian lain dan drug kemoterapi terpilih, iaitu tamoksifen dan cisplatin. Keupayaan sebatian yang dipencilkan untuk menghalang pertumbuhan sel kanser menunjukkan bahawa sebatian ini adalah unsur-unsur bioaktif dalam *D. suffruticosa* yang berperanan terhadap ciri sitotoksik tumbuhan tersebut. Atas sebab ini, sebatian yang dipencilkan ini dapat digunakan untuk tujuan pempiawaian produk herba daripada *D. suffruticosa.*

Kata kunci: *Dillenia suffruticosa*; kandungan aktif; kesitotoksikan; pemecahan bioasai-terarah; sel kanser; triterpena

Introduction

Recent global cancer statistics estimated to have risen to 18.1 million new cases and 9.6 million deaths in 2018, with breast and lung cancer, were the most frequently diagnosed among women and men, respectively (Bray et al. 2018). In Malaysia, a total of 103,507 new cases were identified from 2007 until 2011, with breast and colorectal cancer being the highest detected among females and males, respectively (Manan et al. 2016).

Many chemotherapeutic drugs have been used intensively in the clinic as a standard treatment for the management of cancer, such as tamoxifen (for estrogen receptor (ER)-dependent growth breast cancer) (El Saghir et al. 2011), trastuzumab (for human epidermal growth factor receptor 2 (HER-2) positive breast cancer) (Perez 2011), gefitinib (for non-small lung carcinoma in epidermal growth factor receptor mutated patients) (de Marinis et al. 2011) and cisplatin (for cervical cancer) (Randal-Whitis & Monk 2007). The chemotherapeutic drugs are used either to alleviate advanced unresectable cancer or in an attempt to reduce the risk of recurrence after radical surgery (Aschele et al. 2009).

Despite the success of these chemotherapeutic drugs, they may cause adverse side effects such as increased incidence of endometrial cancer associated with tamoxifen (Wysowski et al. 2002), cardiac dysfunction related to trastuzumab (Chen et al. 2011; Shaffer et al. 2009), arthralgia linked with aromatase inhibitors (Din et al. 2011), and hematologic toxicity and myelosuppression resulting from topotecan (Lee et al. 2010). Consequently, developments of new therapeutic medicine from natural product-derived medications are highly in demand and become a promising candidate for the drug lead.

Dillenia suffruticosa (Family: Dilleniaceae) is one of the medium-sized trees found in the perennial forests of Malaysia. Locally known as '*Simpoh air'* (Malay), *D. suffruticosa* has been used ethno-medically to treat cancerous growth (Ahmad & Holdsworth 1995), manage rheumatism (Hanum & Hamzah 1999), promote wound healing and to relieve fever (Mat-Salleh & Latiff 2002). *D. suffruticosa* also demonstrates antimicrobial (Wiart et al. 2004) and antivirus properties against dengue (Muliawan 2008).

Findings from our previous *in vitro* studies shown that the dichloromethane (DCM) and ethyl acetate (EtOAc) extract from the roots of *D. suffruticosa* exhibited significant cytotoxicity towards various human cancer cell lines, including cervical adenocarcinoma cell (HeLa), breast adenocarcinoma cell (MCF-7 and MDA-MB-231), lung carcinoma cell (A549), colon carcinoma (HT29) and ovarian adenocarcinoma (CaOV3) cell lines. Phytochemical analysis of DCM and EtOAc extract showed the presence of saponins, triterpenes, and sterols along with polyphenolic compounds, which are believed to contribute to their cytotoxic properties (Armania et al. 2013a). Further study shows that the cytotoxicity of the active fractions derived from chromatographic fractionation of DCM and EtOAc of *D. suffruticosa* was due to G_2/M cell cycle arrest and induction of apoptosis (Armania et al. 2013b). All the above results suggest the potential use of *D. suffruticosa* extract for the treatment of cancer.

The utilisation of crude extract from the medicinal plant for therapeutic purposes has major drawbacks concerning discrepancy due to variation in the amount of the active constituents owing to ecological conditions, geographical areas, variability in the collection, storage, and preparations of the raw materials (batch-to-batch variation) (Colegate & Molyneux 2008). As such, the uniformity of a natural-based product is crucial. One of the essential keys towards the standardisation of natural-based product is by identification and quantification of the target active compound(s) that reflects the efficacy of the herbal product.

Recognition of the active compound(s) could be achieved through bioassay-guided fractionation (Alsayari et al. 2018; Chen et al. 2007; Colegate & Molyneux 2008;

Mfotie Njoya et al. 2014). Considered as a valuable approach in drug discovery (Pieters & Vlietinck 2005), the bioassay-guided fractionation technique is one of the methods used in isolating the bioactive components from medicinal herbs which elicit particular bioactive characteristics, including compounds with anticancer properties (Alsayari et al. 2018; Lai et al. 2010).

Isolation and purification of the bioactive compounds that induce a particular pharmacological response required a simple, specific and rapid *in vitro* test such as 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay to screen the source material and monitor the chromatographic fractionation (such as column chromatography, preparative thin-layer chromatography (Prep TLC) and preparative HPLC) (Colegate & Molyneux 2008). Therefore, the present study aims to elucidate the bioactive compound(s) responsible for the cytotoxicity of *D. suffruticosa* by using bioassay-guided fractionation techniques.

Materials and Methods

PLANT MATERIAL

Roots of *D. suffruticosa* was collected from the state of Terengganu, Malaysia. Botanical identification of the plant was carried out, and a voucher specimen (SK1937/11) has been deposited in the herbarium of the Biodiversity Unit of Institute of Bioscience, Universiti Putra Malaysia.

CHEMICAL

Analytical grade purity solvents including hexane, dichloromethane (DCM), ethyl acetate (EtOAc), methanol (MeOH), toluene, acetone, chloroform ($CHCl_3$), acetonitrile, sulfuric acid and dimethyl sulfoxide (DMSO) were purchased from Fisher Scientific (Loughborough, Leicestershire, UK); silica gel 60, 0.063-0.200 mm, thin layer chromatography (TLC) silica gel 60 F254 and MeOH (HPLC grade purity) were purchased from Merck (Darmstadt, Germany); 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT), ferric (III) chloride, deuterated chloroform ($CDCl_3$) and deuterated methanol (CD_3OD) were purchased from Sigma–Aldrich Co. (St. Louis, MO, USA); Roswell Park Memorial Institute (RPMI) 1640, Mycoplex™ fetal bovine serum (FBS), penicillin and streptomycin (100x), and trypsin EDTA (1x) were purchased from PAA Laboratories GmBH (Pasching, Austria).

BIOASSAY-GUIDED FRACTIONATION AND ISOLATION OF THE ACTIVE COMPOUNDS

DCM and EtOAc extract from roots of *D. suffruticosa* were prepared by sequential solvent extraction as reported elsewhere (Armania et al. 2013a). In the present study, active fractions from DCM and EtOAc extract were prepared by column chromatography and solvent-solvent extraction, respectively, as previously reported (Armania et al. 2013b). Our previous findings reported that fraction 4 and 5 from DCM extract of *D. suffruticosa* (designated as D/F4 and D/F5, respectively) eluted with hexane/EtOAc (70:30 v/v and 60:40 v/v, respectively) were found to be the most cytotoxic towards selected cancer cells compared to other fractions (Armania et al. 2013b). Subsequently, purification of compounds from D/F4 and D/F5 was carried out as simplified in Figure 1. Briefly, 300 mg of D/F4 was subjected to re-chromatography by silica gel column chromatography (20 × 2 cm) and eluted with a mobile phase consisting combination of DCM/ EtOAc (100:0-0:100 v/v, 100 mL) and finally changed to MeOH (100% v, 100 mL). The polarity of the mobile phase was increased by a 10% increment. The eluent was collected in a fraction of 50 mL for each solvent composition. The TLC profile for each fraction was evaluated and performed using $CHCl_3$/EtOAc as the mobile phase in various proportions. A total of 12 combined fractions were pooled together according to the similarity in their TLC pattern. The TLC was developed by spotting each fraction on TLC aluminium-backed plates pre-coated with silica gel 60 F254 using a fine glass capillary tube in a chromatographic chamber saturated with the mobile phase at room temperature. The spots were visualised using UV light (254 and 365 nm) and stained using 10% ethanolic sulfuric acid and 1% Ferric (III) chloride. The TLC profile and yield of these fractions are summarised in Supplementary Table S1.

These 12 fractions were subjected to cytotoxicity assay using MTT towards different human cancer cell lines. From the bioassay, fraction 5 and fraction 10 (designated as D/F4/5 and D/F4/10) were the most cytotoxic towards the selected cancer cell lines, as shown in Table 1(A). D/F4/10 (130 mg) was then further re-chromatographed through a column chromatography (20 × 1 cm) packed with silica gel 60, 0.063-0.200 mm. The mobile phases comprising combinations of toluene, acetone, and MeOH were used by gradually increasing the polarity of solvent (5% increment). The initial solvent composition was toluene (100% v; 100 mL), then toluene-acetone (increased the polarity of solvent gradually by 2% v/v; 100 mL), to acetone (100% v; 100 mL), and finally to MeOH (100% v; 100 mL). The eluent was collected in a fraction of 10 mL

for each solvent composition. Purification of D/F4/10 delivered a total of 29 combined fractions. The TLC profile and yield of these fractions are summarised in Supplementary Table S2. From the bioassay results, fraction D/F4/10/17, D/F4/10/18 and D/F4/10/23 were found to be the most cytotoxic towards the selected cancer cell lines (Table 1(B)). Therefore, these fractions were combined and further purified using preparative HPLC, resulting in the isolation of compound **1**. D/F4/5 was not further purified due to the minimal amount present (Supplementary Table S1).

Re-chromatography of D/F5 (300 mg) was performed through column chromatography (20 × 3 cm) packed with silica gel 60, 0.063-0.200 mm (Merck, Darmstadt, Germany) and eluted with mobile phase including the combination of DCM, EtOAc, and MeOH by gradually increasing the polarity. The initial solvent composition was DCM (100% v; 100 mL), then changed to DCM-EtOAc (increased the polarity of solvent by 5% v/v; 100 mL), to EtOAc (100% v; 100 mL) and finally to MeOH (100% v; 100 mL). The eluent was collected in a fraction of 50 mL for each solvent composition. Fractionation of D/F5 produced a total of 17 combined fractions (Table 1(C)). As illustrated in Table 1(C), D/F5/2 and D/F5/4 were substantially cytotoxic compared to other fractions. D/F5/4 was then further purified using preparative HPLC and successfully isolated compound **2**. However, due to the limited amount present, D/F5/2 was not further purified (Supplementary Table S3).

For EtOAc extract, our previous findings reported that polar fraction that was partitioned with hexane (200 mL) and EtOAc/MeOH (1:1 v/v, 200 mL) was found to be significantly cytotoxic towards several cancer cell lines as compared to a non-polar fraction (Armania et al. 2013b). Thus, purification of compounds from the polar fraction of the EtOAc extract was then carried out as simplified in Figure 2. Briefly, 5 g of the polar fraction was passed through column chromatography (20 × 3 cm) packed with silica gel 60, 0.063-0.200 mm and eluted with mobile phase comprising the combination of hexane/acetone (100:0-0:100 v/v, 500 mL) and finally eluted with MeOH (100 % v, 500 mL). The polarity of the mobile phase was increased by a 10% increment. The eluent was collected in a fraction of 50 mL for each solvent composition. The various chemical components present in the fraction were evaluated using toluene/acetone as the mobile phase in various proportions and producing 44 fractions. The TLC profile and yield of these fractions are summarised in Supplementary Table S4. From the bioassay results, fraction 4 and fraction 13 (designated as E/F4 and E/F13, respectively), eluted with hexane/acetone (80:20 v/v and 50:50 v/v, respectively), were found to be persistently cytotoxic towards the selected cancer cell lines (Table 2(A)).

E/F13 (190 mg) was then further re-chromatographed by silica gel column chromatography (20 × 1 cm) and eluted with mobile phase containing a combination of toluene/acetone (100:0-0:100 v/v, 100 mL, increased the polarity of solvent gradually by 2%) and finally with MeOH (100% v, 100 mL). The eluent was collected in a fraction of 10 mL for each solvent composition and producing a total of 18 fractions (Supplementary Table S5). From the bioassay results, fraction 12, 13, 14, 15, 16, and 18 (designated as E/F13/12, E/F13/13, E/F13/14, E/F13/15, E/F13/16, and E/F13/18, respectively), eluted with toluene/acetone

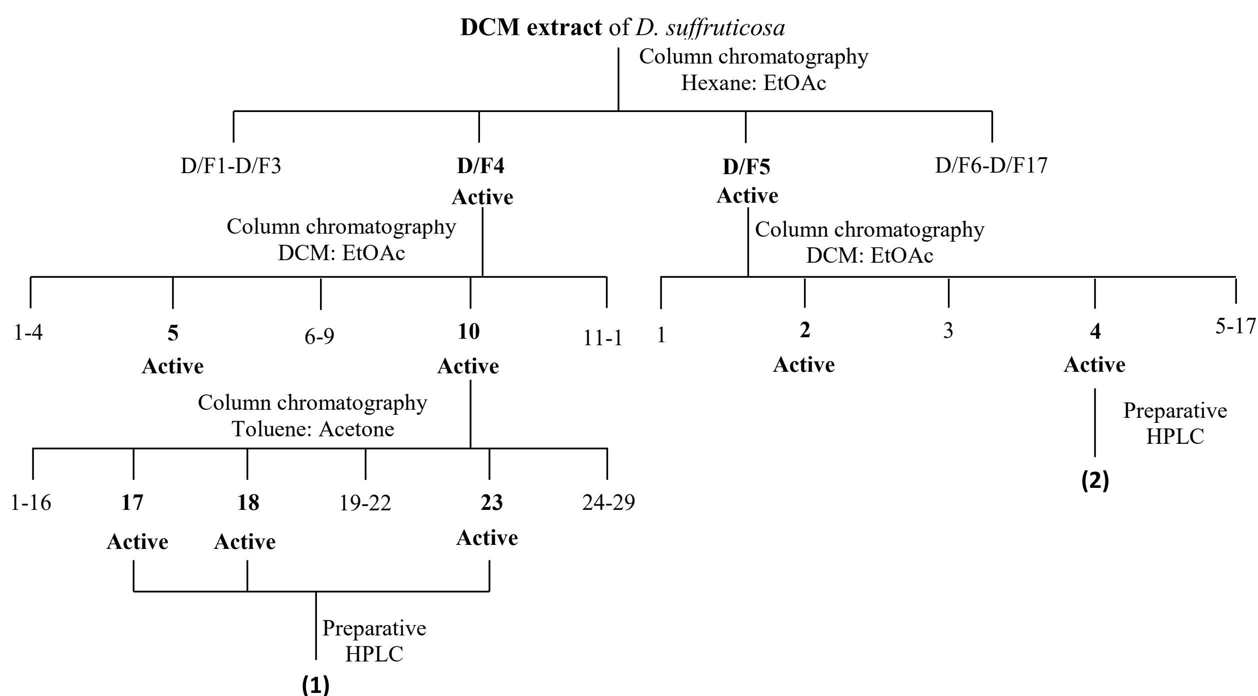

FIGURE 1. Fractionation and isolation of bioactive compounds from DCM extract of *D. suffruticosa* roots

(86:14 v/v, 84:16 v/v, 84:16 v/v, 82:18 v/v, and 82:18 v/v) and MeOH (100% v), respectively, were found to be consistently cytotoxic toward MCF-7 (Table 2(B)). However, due to the limited amount of active fractions, further purification from these active fractions was not performed (Supplementary Table S5).

Meanwhile, E/F4 was further purified using preparative HPLC and successfully isolated compound **3**. In this fraction, purification by preparative HPLC was carried out due to the limited amount of E/F4 as compared to E/F13 (Supplementary Table S4).

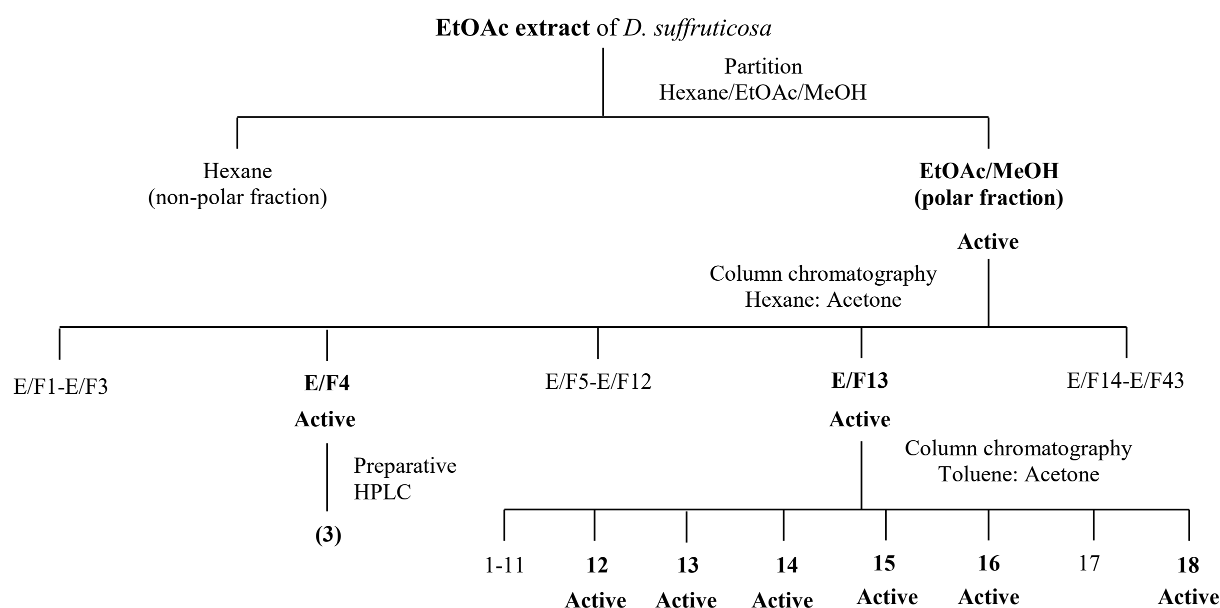

FIGURE 2. Fractionation and isolation of bioactive compounds from EtOAc extract of *D. suffruticosa* roots

TABLE 1. Cytotoxicity of (A) D/F4 (B) D/F4/10 and (C) D/F5 fractions of DCM extract on selected cancer cell lines after 72 h of incubation

(A)

Fraction / Cell line	IC_{50} (μg/mL)					
	MDA-MB-231	MCF-7	HeLa	CaOV3	A549	3T3
D/F4/1	*NA*	*NA*	*NA*	*NA*	*NA*	*NA*
D/F4/2	>30	>30	>30	>30	>30	*NA*
D/F4/3	15.00 ± 1.32	>30	>30	>30	13.33 ± 0.58	9.00 ± 0.50
D/F4/4	12.50 ± 0.50	29.00 ± 1.00	>30	>30	18.17 ± 4.04	11.67 ± 0.58
D/F4/5	11.33 ± 0.29	>30	>30	>30	8.00 ± 0.58	12.67 ± 0.58
D/F4/6	>30	>30	>30	>30	>30	*NA*
D/F4/7	>30	>30	>30	>30	>30	*NA*
D/F4/8	24.50 ± 1.32	>30	>30	>30	>30	128.67 ± 5.03
D/F4/9	>30	>30	>30	>30	>30	*NA*
D/F4/10	8.17 ± 0.29	10.67 ± 1.04	>30	14.83 ± 1.26	>30	85.67 ± 1.52
D/F4/11	>30	>30	>30	>30	>30	*NA*
D/F4/12	>30	>30	>30	>30	>30	*NA*

(B)

Fraction / Cell line	IC_{50} (μg/mL)			
	MDA-MB-231	MCF-7	CaOV3	3T3
D/F4/10/17	16.17 ± 1.26	>30	>30	*NA*
D/F4/10/18	**11.17 ± 0.76**	>30	29.17 ± 0.29	>150
D/F4/10/23	14.67 ± 1.15	>30	>30	*NA*

(C)

Fraction / Cell line	IC$_{50}$ (μg/mL)					
	MDA-MB-231	MCF-7	HeLa	CaOV3	A549	3T3
D/F5/1	23.67 ± 1.04	>30	>30	>30	>30	42.33 ± 1.53
D/F5/2	**10.5 ± 0.50**	>30	>30	**8±2.18**	>30	**23.33 ± 2.31**
D/F5/3	>30	20.50 ± 2.50	19.17 ± 2.84	22.33 ± 1.15	20.33 ± 0.58	15.33 ± 3.06
D/F5/4	25.50 ± 0.50	22.67 ± 2.08	**12.50 ± 0.50**	26.33 ± 0.76	>30	19.00 ± 1.00
D/F5/5	>30	>30	>30	28.00 ± 2.65	>30	37.33 ± 1.15
D/F5/6	>30	>30	>30	>30	>30	*NA*
D/F5/7	22.00 ± 0.50	>30	29.33 ± 0.29	>30	>30	32.67 ± 1.15
D/F5/8	>30	>30	18.83 ± 2.02	28.33 ± 0.76	>30	18.33 ± 0.58
D/F5/9	19.17 ± 2.36	>30	>30	25.67 ± 0.29	>30	14.67 ± 4.93
D/F5/10	20.00 ± 1.80	>30	>30	>30	>30	*NA*
D/F5/11	>30	>30	>30	>30	>30	*NA*
D/F5/12	>30	>30	>30	>30	>30	*NA*
D/F5/13	>30	>30	>30	>30	>30	*NA*
D/F5/14	>30	>30	>30	>30	>30	*NA*
D/F5/15	>30	>30	>30	>30	>30	*NA*
D/F5/16	>30	>30	>30	>30	>30	*NA*
D/F5/17	>30	>30	>30	>30	>30	*NA*

Data are expressed as mean ± SD of triplicates experiments.
NA: Not applicable. The MTT assay was not performed for this fraction due to the limited amount present. Cytotoxicity of the fraction that demonstrated an IC$_{50}$ value of more than 30 ug/mL in cancer cells was not further determined in normal cells.

TABLE 2. Cytotoxicity of (A) polar and (B) E/F13 fractions of EtOAc on selected cancer cell lines after 72 h of incubation
(A)

Fraction / Cell line	IC$_{50}$ (μg/mL)			Fraction / Cell line	IC$_{50}$ (μg/mL)		
	MCF-7	CaOV3	3T3		MCF-7	CaOV3	3T3
E/F1	>150	>150	*NA*	E/F22	>150	>150	*NA*
E/F2	43.67±2.31	39.33±2.08	*NA*	E/F23	>150	>150	*NA*
E/F3	58.67±8.34	35.33±2.31	*NA*	E/F24	>150	>150	*NA*
E/F4	**14.33±0.58**	**21.67±2.52**	**12.17±0.29**	E/F25	95.33±4.16	>150	*NA*
E/F5	59.67±2.08	55.33±1.15	*NA*	E/F26	112.67±1.15	>150	*NA*
E/F6	77.67±0.58	76.33±0.58	*NA*	E/F27	86.67±7.02	>150	*NA*
E/F7	65.33±0.58	64.00±2.00	*NA*	E/F28	>150	>150	*NA*
E/F8	>150	103.33±4.16	*NA*	E/F29	>150	>150	*NA*
E/F9	142.67±5.03	>150	*NA*	E/F30	>150	>150	*NA*
E/F10	>150	146.67±1.15	*NA*	E/F31	>150	>150	*NA*
E/F11	>150	>150	*NA*	E/F32	>150	>150	*NA*
E/F12	146.67±1.15	142.67±4.62	*NA*	E/F33	>150	>150	*NA*
E/F13	**12.33±0.58**	**52.00±2.00**	**47.67±2.08**	E/F34	>150	>150	*NA*
E/F14	19.33±1.15	129.33±1.15	76.33±0.58	E/F35	>150	>150	*NA*
E/F15	25.67±2.52	132.00±4.00	*NA*	E/F36	>150	>150	*NA*
E/F16	24.00±2.00	41.33±1.15	70.67±1.53	E/F37	>150	>150	*NA*
E/F17	85.33±5.03	>150	*NA*	E/F38	>150	>150	*NA*
E/F18	>150	>150	*NA*	E/F39	>150	>150	*NA*
E/F19	49.33±2.31	>150	*NA*	E/F40	>150	>150	*NA*
E/F20	35.33±5.03	>150	*NA*	E/F41	>150	>150	*NA*
E/F21	42.67±5.03	>150	*NA*	E/F42	>150	>150	*NA*
				E/F43	>150	>150	*NA*

(B)

Fraction / Cell line	IC_{50} (µg/mL)	
	MCF-7	3T3
E/F13/1	>30	NA
E/F13/2	>30	NA
E/F13/3	>30	NA
E/F13/4	>30	NA
E/F13/5	>30	NA
E/F13/6	>30	NA
E/F13/7	>30	NA
E/F13/8	>30	NA
E/F13/9	>30	NA
E/F13/10	>30	NA
E/F13/11	>30	NA
E/F13/12	**8.58±0.58**	**22.33±0.58**
E/F13/13	**5.08±1.39**	**14.67±1.15**
E/F13/14	**4.00±1.10**	**12.17±0.29**
E/F13/15	**4.08±0.49**	**14.00±1.00**
E/F13/16	**6.00±0.84**	**13.83±0.29**
E/F13/17	>30	NA
E/F13/18	**6.83±0.26**	**20.67±1.15**

Data are expressed as mean ± SD of triplicates experiments.
NA: Not applicable. The MTT assay was not performed for this fraction due to the limited amount present. Cytotoxicity of the fraction that demonstrated an IC_{50} value of more than 30 ug/mL in cancer cells was not further determined in normal cells

PURIFICATION OF BIOACTIVE COMPOUNDS BY PREPARATIVE HPLC

The chromatographic separation was performed on a Jasco HPLC system equipped with Jasco UV 2077 Plus 4λ Intelligent UV/VIS detector, Jasco RV 2080-02 Recycle Valve unit, Jasco DG 2080-53 three-line degasser, Jasco Mx 2080-31 solvent mixing module and Jasco PU 2086 Plus intelligent prep pump (Jasco, Japan). The analysis was performed on X Bridge Prep C18 5 µm 10 × 150 mm column (Water Corporation, Massachusetts, USA) using MilliQ water (solvent A) and MeOH (solvent B) as a mobile phase. Gradient elution was used for preparative separation as follows: Method 1: 70% B (5 min), 85% B (10 min), and 100% B (40 min) with the flow rates of 2 mL/min; and Method 2: 50% B (2 min), 70% B (10 min), 85% B (15 min), and 100% B (40 min) with the flow rates of 3 mL/min. Method 1 was used to isolate compounds **1** and **2** (Supplementary Figure 1(A) and 1(B)), while Method 2 was used to isolate compound **3** (Supplementary Figure 1(C)). The injection volume was 50 µL, and the detection was performed at 210, 254, 280, and 360 nm using a UV detector. The preparative HPLC equipment was controlled by ChromNav Chromatography Software (Jasco, Japan). The peaks of the isolated compounds (Supplementary Figure 1(A)-(C)) were collected manually.

STRUCTURAL ELUCIDATION BY NMR ANALYSIS

The proton nuclear magnetic resonance (^{1}H NMR) and carbon nuclear magnetic resonance (^{13}C NMR) spectra were recorded on a Varian Unity Inova 500MHz spectrometer in deuterated chloroform ($CDCl_3$) or deuterated methanol (CD_3OD). The tetramethylsilane (TMS) was used as the internal standard, and the chemical shifts are recorded in δ values. The ^{1}H signals were described in abbreviations, including multiplicities (s=singlet, d=doublet, t=triplet, q=quartet, m=multiplet), coupling constants (*J*), and the number of

protons. The ^{13}C NMR was recorded with off-resonance decoupling, and 1H-1H COSY, HSQC, and HMBC spectra were obtained from the usual pulse sequences.

DETERMINATION OF CYTOTOXICITY CANCER CELL LINES

Human cancerous cells such as breast adenocarcinoma (MDA-MB-231 and MCF-7), ovarian adenocarcinoma (CaOV3), lung carcinoma (A549) and human cervical adenocarcinoma (HeLa) were purchased from the American Type Culture Collection (ATCC) (Manassas, VA, USA). A non-cancerous Swiss mouse embryo fibroblast (3T3 F442A) cell lines was purchased from the European Collection of Authenticated Cell Cultures (ECACC) (Salisbury, United Kingdom). RPMI 1640 medium supplemented with 10% FBS, penicillin (100 µg/mL) and streptomycin (100 µg/mL) was used as culture medium. The cells were grown and maintained in a humidified atmosphere with 5% CO_2 at 37 °C.

MTT ASSAY

The MTT assay was performed as reported elsewhere (Armania et al. 2013b). Briefly, the cells (1×10^5 cells/mL) were seeded in a 96-well plate and incubated for 24 h. The cells were then treated with different concentrations of fractions of *D. suffruticosa* (3-30 µg/mL and 2.34-150 µg/mL for human cancer cell lines and non-cancerous cell line, respectively) for 72 h. Control or untreated cells was also included. At the end of incubation, 20 µL of MTT solution (5 mg/mL) was added to each well and incubated for 4 h at 37 °C in the dark. The medium was aspirated out, and 100 µL of DMSO was added into each well to solubilise the insoluble formazan blue crystal. The absorbance was measured at 570 nm using ELx800™ Absorbance Microplate Reader (BioTek Instruments, Inc., Vermont, United States). The percentage of cell viability for each treatment concentration was calculated using equation (1).

$$\text{Cell viability (\%)} = \frac{Absorbance\ sample}{Absorbance\ \ control} \times 100 \quad (1)$$

STATISTICAL ANALYSIS

Data are presented as mean ± S.D (standard deviation). The statistical significance was established using Statistical Program for Social Sciences (SPSS for Windows, Version 20) (International Business Machines Corp. IBM, New York, USA). A *p*-value of less than 0.05 ($p < 0.05$) is considered statistically significance.

RESULTS AND DISCUSSION

The present research was designed to identify the bioactive constituents that is responsible for the cytotoxicity of *D. suffruticosa* root extracts against cancer cells by a bioassay-guided fractionation approach. Our previous finding showed that DCM and EtOAc extract of *D. suffruticosa* were found to be significantly cytotoxic towards selected cancer cell lines (MDA-MB-231, MCF-7, A549, CaOV3 and A549) (Armania et al. 2013a). Further study showed that the cytotoxicity of the active fraction in these extracts towards cancer cells was due to G_2/M cell cycle arrest and induction of apoptosis (Armania et al. 2013b). Thus, further isolation and purification of the said bioactive compounds from the active fractions were carried out in this study.

IDENTIFICATION AND CHARACTERISATION OF THE ISOLATED COMPOUNDS

After a series of column chromatography, three compounds were isolated from the roots of *D. suffruticosa*. These compounds were classified as triterpenes according to their ^{13}C NMR spectrum. In the phytochemical analysis, the presence of triterpenes was only detected in the EtOAc extract (Armania et al. 2013a). However, further isolation of the active compounds also showed the presence of triterpenes in the DCM extract (compounds **1** and **2**). This indicates that phytochemical constituents, particularly those present in trace or minor amount, were not able to be detected by the phytochemical assay used in the previous study (Armania et al. 2013a). Therefore, a more sensitive and quantitative measurement such as high-performance liquid chromatography coupled with mass spectrometry (HPLC/MS or LCMS) is needed for the identification of the active compounds (Yang et al. 2009).

Compound **1** was obtained as a white amorphous powder (2.8 mg) with a chemical formula of $C_{30}H_{48}O_3$. The ^1H NMR spectrum (500 MHz CD_3OD) of compound **1** showed the existence of six tertiary methyl and displayed dishielded proton signals at δ_H 4.70, 4.55, and 3.14, representing the exomethylene protons and hydroxyl group, respectively. The ^{13}C NMR spectrum (125 MHz CD_3OD) of compound **1** showed thirty carbon signals suggesting this compound to be a triterpene. However, the structure of the compound is still unidentified. As such, further studies need to be carried out involving a full analysis of the spectroscopic data to enable the assignment of all protons and carbons. The spectral identification of compound **1** is as followed: ^1H NMR (CD_3OD), 500 MHz: ^1H NMR: δ 4.70, 4.55, 3.14, 2.54, 2.26, 1.97, 1.90, 1.69, 1.55, 1.41, 1.32, 1.13, 1.00, 0.96,

0.86, 0.75, 0.71, ^{13}C NMR (CD_3OD), 125 MHz: 181.6, 151.5, 108.2, 78.3, 57.1, 55.5, 50.8, 49.4, 48.2, 42.2, 40.6, 38.7, 38.5, 38.0, 37.5, 36.9, 34.3, 32.9, 30.7, 29.7, 27.2, 26.6, 25.7, 20.8, 18.3, 18.1, 15.4, 15.3, 14.7, 13.7.

Compound **2** was obtained as a white amorphous powder (8.1 mg) with a chemical formula of $C_{30}H_{48}O_3$. The ^1H NMR spectrum (500 MHz $CDCl_3$) of compound **2** demonstrated the presence of six tertiary methyl and displayed dishielded proton signals at δ_H 4.74, 4.61, and 3.21, representing the exomethylene protons and hydroxyl group, respectively. The ^{13}C NMR spectrum (125 MHz $CDCl_3$) of compound **2** showed thirty carbon signals suggesting this compound to be a triterpene. The downfield signals at δ_C 179.8 and 79.0 displayed a carbonyl group whereby signals at δ_C 150.4 and 108.2 indicated the alkene carbons. Since the compound structure is still unidentified, further studies need to be carried out involving a full analysis of the spectroscopic data to enable the assignment of all protons and carbons. The spectral identification of compound **2** is as followed: ^1H NMR ($CDCl_3$), 500 MHz: δ 4.74, 4.61, 3.21, 3.01, 2.28, 2.20, 1.98, 1.69, 1.61, 1.52, 1.42, 1.38, 1.30, 1.21, 0.98, 0.93, 0.83, 0.76, 0.69, ^{13}C NMR ($CDCl_3$), 125 MHz: 179.8, 150.4, 109.7, 79.0, 56.3, 55.4, 50.5, 49.2, 46.9, 42.4, 40.7, 38.9, 38.7, 38.4, 37.2, 37.0, 34.3, 32.2, 30.5, 29.7, 28.0, 27.4, 25.5, 20.8, 19.4, 18.3, 16.1, 16.0, 15.3, 14.7.

Compound **3** (Figure 3) was collected as white needles (14.3 mg) and exhibited a molecular weight of 428.70 with a chemical formula of $C_{29}H_{48}O_2$. Based on these spectral interpretations, compound **3** is characterized as 1-Isopropenyl-4α, 4β, 8, 10, 14-pentamethyl-icosahydro-cyclopenta[a]chrysene-3, 17-diol. The ^1H NMR spectrum (500 MHz $CDCl_3$) of compound **3** showed the presence of six tertiary methyl and dishielded proton signals at δ_H 4.75, 4.62, and 3.22, representing the exomethylene protons and hydroxyl group, respectively. The ^{13}C NMR spectrum (125 MHz $CDCl_3$) of compound **3** showed twenty-nine carbon signals suggesting this compound to be a triterpene. With the aid of HSQC experiment, it disclosed the presence of resonance for six methyls (CH_3) at δ_C 28.0(C-23), 19.4 (C-28), 16.1 (C-25), 16.0 (C-26), 15.3 (C-24), 15.0 (C-27), ten methylene (CH_2), six methyne (CH) and seven quaternary carbons at δ_C 150.4 (C-19), 56.2 (C-17), 42.4 (C-14), 37.2 (C-10), 40.7 (C-8) and 38.9 (C-4). The downfield signals at δ_C 79.0 (C-3) showed a hydroxyl group, whereby signals at δ_C 150.4 (C-19) and 108.2 (C-30) showed the alkene carbons.

The COSY spectrum showed the ^1H-^1H correlation between H-1 and H-2, H-2 and H-3, H-5 and H-6, H-11 and H-12, H-12 and H-13, H-15 and H-16, H-13 and H-18, H-18 and H-20, H-20 and H-21, H-21 and H-22 (Table 3), which confirmed their relative positions in the respective carbon. A singlet proton at δ_H 4.75 (H-29a) was directly correlated with 4.62(H-29b), suggesting that the double bond was located at C-29. Besides that, there was a ^1H-^1H correlation between H-29a/H-29b and H-28 which confirmed the position of C-28.

The HMBC spectrum showed the ^3J correlation between 109.7 (C-29) and 19.4 (C-28), which confirmed that the double bond was located between C-29 and C-28 (Figure 3). The full analysis of the spectroscopic data of compound **3** comprised of the ^1H-^1H COSY, DEPT, HSQC, and HMBC enabled the assignment of all protons and carbons (Table 3) and to confirm the structure (Figure 3). The comparison of the spectroscopic data for compound **3** with the previously reported literature values was depicted in Table 4 (Hess & Monache 1999).

The spectral identification of compound **3** is as followed: ^1H NMR ($CDCl_3$), 400 MHz: δ 4.75 (1H, s),

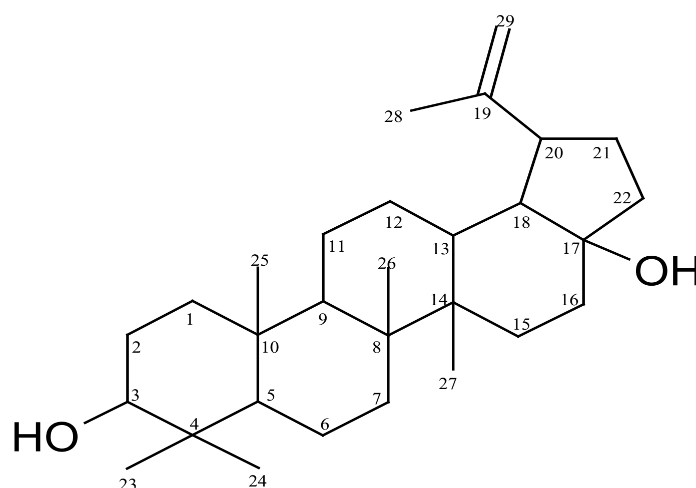

FIGURE 3. Structure and numbering of compound **3**

4.62 (1H, s), 3.22 (1H, dd, J=7 Hz), 3.01 (1H, m), 2.27 (3H, m), 1.98 (3H, m), 1.70 (5H, t, J=15.5 Hz), 1.62 (3H, m), 1.53 (4H, m), 1.45 (4H, m), 1.39 (3H, s), 1.31 (2H, t, J=12.5 Hz), 1.22 (1H, d, J=14 Hz), 1.06 (1H, d, J=12.5 Hz), 0.99 (6H, d, J=5.5 Hz), 0.95 (3H, s), 0.92 (1H, dd, J=13 Hz), 0.84 (3H, s), 0.77 (3H, s), 0.71 (1H, d, J=10 Hz), ^{13}C NMR ($CDCl_3$), 125 MHz: δ 150.4 (C-19), 109.7 (C-29), 79.0 (C-3), 56.2 (C-17), 55.3 (C-5), 50.5 (C-9), 49.3 (C-18), 46.9 (C-20), 42.4 (C-14), 40.7 (C-8), 38.9 (C-4), 38.7 (C-1), 38.4 (C-13), 37.2 (C-10), 37.0 (C-22), 34.3 (C-7), 32.2 (C-16), 30.5 (C-21), 29.7 (C-2), 28.0 (C-23), 27.4 (C-15), 25.5 (C-12), 20.8 (C-11), 19.4 (C-28), 18.3 (C-6), 16.1 (C-25), 16.0 (C-26), 15.3 (C-24), 15.0 (C-27).

TABLE 3. ^{1}H (500 MHz) and ^{13}C NMR (125 MHz) data for 1-isopropenyl-4α, 4β, 8, 10, 14-pentamethyl-icosahydro-cyclopenta[a]chrysene-3, 17-diol (3)

Position	δ C	HSQC (^{13}C-^{1}H)	COSY (^{1}H-^{1}H)	HMBC
C-1	38.7	0.99, 1.70	0.92, 1.06, 1.22, 1.98, 2.27, 4.62, 4.75	0.84
C-2	29.7	1.22, 1.53	1.53, 1.70, 2.27 1.06, 1.22, 1.98, 2.27, 3.22	0.99
C-3	79.0	3.22	1.53	0.77, 0.99
C-4	38.9	-	1.70	0.77, 0.99
C-5	55.3	0.71	1.39	0.77, 0.84, 0.99
C-6	18.3	1.53, 1.39	1.06, 1.22, 1.98, 2.27, 3.22 0.71	1.39
C-7	34.3	1.39	0.71	0.95
C-8	40.7	-	-	0.84, 0.95, 0.99, 1.22, 1.53
C-9	50.5	1.31	1.06	0.84, 0.95, 1.22
C-10	37.2	-	-	0.84
C-11	20.8	131, 1.45	1.06 1.98, 2.27, 3.01	1.31
C-12	25.5	1.06, 1.70	1.31, 1.53, 1.62, 1.70, 2.27 1.39	0.99
C-13	38.4	2.27	1.06, 1.22, 1.45, 1.53, 1.62, 1.70	1.62
C-14	42.4	-	-	0.95, 0.99
C-15	27.4	1.53, 1.62	1.06, 1.22, 1.98, 2.27, 3.22 1.06, 1.98, 2.27, 3.01	1.70
C-16	32.2	1.45, 2.27	1.98, 2.27, 3.01 1.06, 1.22, 1.45, 1.53, 1.62, 1.70	-
C-17	56.2	-	-	1.62, 1.98
C-18	49.3	1.62	1.06, 1.98, 2.27, 3.01	1.98
C-19	150.4	-	-	1.70, 1.62
C-20	46.9	3.01	1.45, 1.62, 1.70, 1.98	1.62, 1.70, 1.98, 4.62, 4.75
C-21	30.5	1.45, 1.98	1.98, 2.27, 3.01 1.45, 1.53, 1.62, 1.70, 3.01	3.01
C-22	37.0	1.45, 1.98	1.98, 2.27, 3.01 1.45, 1.53, 1.62, 1.70, 3.01	-
C-23	28.0	0.99	-	0.84, 3.22
C-24	15.3	0.77	-	0.71, 0.99, 3.22
C-25	16.1	0.84	-	1.06, 1.22
C-26	16.0	0.95	-	0.71, 1.39
C-27	15.0	0.99	-	1.06
C-28	19.4	1.70	0.92, 1.06, 1.22, 1.98, 2.27, 3.01, 4.62, 4.75	1.62, 3.01, 4.62, 4.75
C-29	109.7	4.62, 4.75	1.70	1.70

TABLE 4. ^{13}C NMR (125 MHz) data of the isolated compounds from *D. suffruticosa* as compared to the previously reported data

Position	δ C	
	Active compounds of *D. suffruticosa*	Betulinic acid
	(3)	(a)
C-1	38.7	38.5
C-2	29.7	28.2
C-3	79.0	78.1
C-4	38.9	39.4
C-5	55.3	55.9
C-6	18.3	18.7
C-7	34.3	34.7
C-8	40.7	41.0
C-9	50.5	50.9
C-10	37.2	37.5
C-11	20.8	21.1
C-12	25.5	26.0
C-13	38.4	39.2
C-14	42.4	42.8
C-15	27.4	30.2
C-16	32.2	32.8
C-17	56.2	56.6
C-18	49.3	49.7
C-19	150.4	151.4
C-20	46.9	47.7
C-21	30.5	31.1
C-22	37.0	37.4
C-23	28.0	28.5
C-24	15.3	16.2
C-25	16.1	16.3
C-26	16.0	16.2
C-27	15.0	14.8
C-28	-	179.0
C-29	19.4	19.4
C-30	109.7	110.0

* These values may be interchanged.
Hess and Monache (1999)

CYTOTOXICITY OF THE ISOLATED COMPOUNDS

The three isolated compounds were subjected to MTT assay to evaluate the cytotoxic properties towards the selected cancer cell lines (MCF-7, MDA-MB-231 and HeLa). As illustrated in Table 5, compound **2** isolated from DCM extract of *D. suffruticosa* was potently cytotoxic towards MCF-7, MDA-MB-231 and HeLa as compared to the other two isolated compounds with IC_{50} values of 19.50±0.50, 4.37±0.15 and 5.17±0.29 µg/mL, respectively.

Interestingly, compound **2** exhibited significantly ($p<0.05$) higher cytotoxic properties towards MDA-MB-231 and HeLa compared to the selected chemotherapeutic drugs, tamoxifen and cisplatin.

Besides that, compound **2** was less cytotoxic ($p < 0.05$) towards the non-cancerous Swiss mouse embryo fibroblast cells (3T3 F442A) with an IC_{50} value of 15.00±1.00 µg/mL as compared to tamoxifen and cisplatin with IC_{50} values of 6.20±0.20 and 2.83±0.06 µg/mL, respectively. Other than compound **2**, compounds **1** and **3** also exhibit cytotoxicity towards the selected cancer cell lines.

TABLE 5. Cytotoxicity of the isolated compounds from *D. suffruticosa* on the selected cancer cell lines after 72 h of incubation

Compound /Cell line	IC_{50} (µg/mL)			
	MCF-7	MDA-MB-231	HeLa	3T3
Compound **(1)**	>30[a]	11.50±0.50[a]	19.17±1.04[a]	36.00±4.00[a]
Compound **(2)**	19.50±0.50[b]	**4.37±0.15**[b]	**5.17±0.29**[b]	15.00±1.00[b]
Compound **(3)**	14.00±1.00[c]	>30[c]	>30[c]	11.67±0.58[b]
Tamoxifen	**5.73±0.12**[d]	8.67±0.12[d]	*NA*	6.20±0.20[c]
Cisplatin	*NA*	*NA*	7.33±0.12[d]	**2.83±0.06**[c]

NA: Not applicable.
Data are expressed as mean ± SD of triplicates experiments. Data with different superscripts [(a, b, c, d, e)] in the same column are considered significant ($p < 0.05$)

CONCLUSIONS

In this study, a total of three triterpene compounds were isolated from roots of *D. suffruticosa* using various chromatographic techniques. All the isolated compounds demonstrated cytotoxic properties towards the selected cancer cell lines. Compound **2** isolated from DCM extract of *D. suffruticosa* was mostly cytotoxic towards MCF-7, MDA-MB-231, and HeLa compared to the other two compounds and selected chemotherapeutic drugs, tamoxifen, and cisplatin. The ability of the isolated compounds to inhibit the growth of cancer cells suggests that these compositions are the bioactive constituents in *D. suffruticosa* that is mainly responsible for the cytotoxicity of the plant. Thus, these isolated compounds could be used as a means for standardisation of herbal product from *D. suffruticosa*. Having said that, further studies are warranted to confirm the structure of compounds **1** and **2**, which are currently classified as unidentified ones. In the present study, the isolated compounds show higher cytotoxicity against breast cancer cells. Hence, further studies on the cytotoxicity of the isolated compounds against non-cancerous breast cells such as MCF-10A need to be performed to confirm the selectivity of these compounds on cancerous cells. Besides that, further studies to evaluate the anticancer activities of these compounds in *in vivo* model, especially in breast cancer, is also highly recommended to be carried out.

ACKNOWLEDGEMENTS

This study was funded by the Research University Grant Scheme (RUGS) of Universiti Putra Malaysia (04-01-09-0714RU) and the Fundamental Research Grant Scheme (04-04-10-884FR). The authors declare that there is no conflict of interest with respect to the data collected and procedures used within the study.

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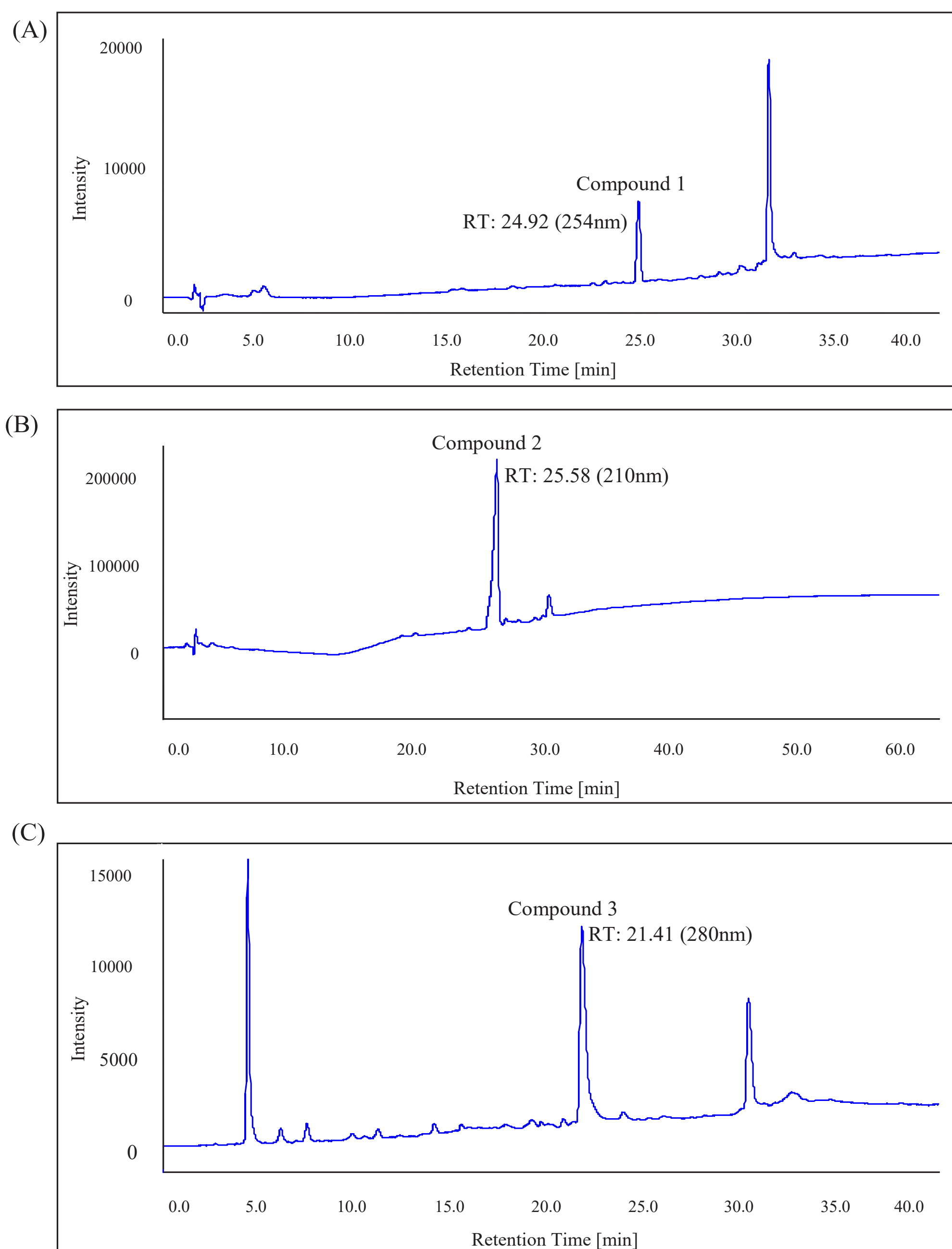

FIGURE S1. HPLC chromatogram of (A) compound **1** (isolated from D/F4/10), (B) compound **2** (isolated from D/F5/4) and (C) compound **3** (isolated from E/F4) of DCM and EtOAc extract of *D. suffruticosa*

TABLE S1. TLC profile and yield of D/F4 subfractions of DCM extract

Fraction	TLC Mobile Phase	TLC R*f* Values	Weight(mg)	Yields (%)
D/F4/1	$CHCL_3$, 100%	0.44, 0.50, 0.56, 0.86, 0.94	2.4	0.8
D/F4/2	$CHCL_3$-EtOAc, 9:1 v/v	0.54, 0.71, 0.76, 0.81, 0.87, 0.93	6.1	2.03
D/F4/3	$CHCL_3$-EtOAc, 9:1 v/v	0.60, 0.65, 0.72, 0.79, 0.96	55.0	18.33
D/F4/4	$CHCL_3$-EtOAc, 9:1 v/v	0.46, 0.54, 0.61, 0.96	34.4	11.47
D/F4/5	$CHCL_3$-EtOAc, 17:3 v/v	0.30, 0.34, 0.41, 0.53, 0.60, 0.67, 0.89	9.2	3.07
D/F4/6	$CHCL_3$-EtOAc, 17:3 v/v	0.24, 0.33, 0.41, 0.56, 0.66, 0.89	11.3	3.77
D/F4/7	$CHCL_3$-EtOAc, 4:1 v/v	0.36, 0.47, 0.54, 0.70, 0.75, 0.80, 0.90	15.6	5.20
D/F4/8	$CHCL_3$-EtOAc, 4:1 v/v	0.36, 0.45, 0.53, 0.70, 0.74, 0.90	15.3	5.10
D/F4/9	$CHCL_3$-EtOAc, 4:1 v/v	0.74, 0.79, 0.91, 0.94	20.0	6.67
D/F4/10	$CHCL_3$-EtOAc, 7:3 v/v	0.71, 0.91	46.0	15.33
D/F4/11	$CHCL_3$-EtOAc, 3:2 v/v	0.76, 0.79, 0.92	4.6	1.53
D/F4/12	$CHCL_3$-EtOAc, 1:1 v/v	0.50, 0.60, 0.77, 0.91	4.3	1.43
		Total	224.2	74.73

TABLE S2. TLC profile and yield of D/F4/10 subfractions of DCM extract

Fraction	TLC Mobile Phase	TLC R*f* Values	Weight(mg)	Yields (%)
D/F4/10/1	100% Toluene	0.70, 0.83, 0.90	2.2	1.69
D/F4/10/2	100% Toluene	0.60, 0.65, 0.73, 0.85	1.4	1.08
D/F4/10/3	100% Toluene	0.48, 0.55, 0.65, 0.80, 0.93	1.6	1.23
D/F4/10/4	100% Toluene	0.55, 0.58, 0.95	7.6	5.85
D/F4/10/5	Toluene-Acetone, 95:5 v/v	0.40, 0.90	2.8	2.15
D/F4/10/6	Toluene-Acetone, 95:5 v/v	0.40, 0.60, 0.85	8.9	6.85
D/F4/10/7	Toluene-Acetone, 95:5 v/v	0.28, 0.33, 0.45, 0.60, 0.93	2.8	2.15
D/F4/10/8	Toluene-Acetone, 95:5 v/v	0.13, 0.23, 0.35, 0.40, 0.48, 0.63, 0.80, 0.93	6.2	4.77
D/F4/10/9	Toluene-Acetone, 95:5 v/v	0.10, 0.25, 0.33, 0.45, 0.60	1.4	1.08
D/F4/10/10	Toluene-Acetone, 9:1 v/v	0.33, 0.43, 0.53, 0.63, 0.75	2.0	1.54
D/F4/10/11	CHCl3-MeOH, 97:3 v/v	0.08, 0.33, 0.50	1.7	1.31
D/F4/10/12	CHCl3-MeOH, 97:3 v/v	0.08, 0.13, 0.25, 0.48	2.8	2.15
D/F4/10/13	CHCl3-MeOH, 97:3 v/v	0.08, 0.13, 0.28, 0.50	3.4	2.62
D/F4/10/14	CHCl3-MeOH, 97:3 v/v	0.08, 0.15, 0.30, 0.50	5.9	4.54
D/F4/10/15	CHCl3-MeOH, 97:3 v/v	0.10, 0.18, 0.35, 0.55	13.0	10.00
D/F4/10/16	CHCl3-MeOH, 97:3 v/v	0.23, 0.40	4.2	3.23
D/F4/10/17	CHCl3-MeOH, 95:5 v/v	0.20, 0.33, 0.50	3.5	2.69
D/F4/10/18	CHCl3-MeOH, 95:5 v/v	0.23, 0.38, 0.58	2.1	1.62
D/F4/10/19	CHCl3-MeOH, 95:5 v/v	0.33, 0.50, 0.65	3.9	3.00
D/F4/10/20	CHCl3-MeOH, 95:5 v/v	0.30, 0.45, 0.58, 0.68	2.7	2.08
D/F4/10/21	CHCl3-MeOH, 95:5 v/v	0.13, 0.35, 0.48, 0.60, 0.70, 0.93	2.8	2.15
D/F4/10/22	CHCl3-MeOH, 95:5 v/v	0.13, 0.25, 0.35, 0.55, 0.68, 0.93	2.7	2.08
D/F4/10/23	CHCl3-MeOH, 95:5 v/v	0.18, 0.45, 0.60	3.3	2.54
D/F4/10/24	CHCl3-MeOH, 9:1 v/v	0.08, 0.18, 0.33, 0.48, 0.63	2.1	1.62
D/F4/10/25	CHCl3-MeOH, 9:1 v/v	0.20, 0.30, 0.68, 0.80	0.7	0.54

D/F4/10/26	CHCl3-MeOH, 9:1 v/v	0.10, 0.18, 0.28, 0.55, 0.68	0.7	0.54
D/F4/10/27	CHCl3-MeOH, 9:1 v/v	0.23, 0.38, 0.43, 0.73, 0.83	4.8	3.69
D/F4/10/28	100% MeOH, v/v	0.08, 0.50, 0.73, 0.80, 0.93, 0.98	10.8	8.31
D/F4/10/29	100% MeOH, v/v	0.40, 0.75, 0.03	8.9	6.85
		Total	116.9	89.92

TABLE S3. TLC profile and yield of D/F5 subfractions of DCM extract

Fraction	TLC Mobile Phase	TLC R*f* Values	Weight(mg)	Yields (%)
D/F5/1	$CHCL_3$, 100%	0.50, 0.56, 0.64, 0.70, 0.76, 0.83, 0.94	1.0	0.33
D/F5/2	$CHCL_3$-EtOAc, 9:1 v/v	0.06, 0.17, 0.49, 0.59, 0.94, 0.76, 0.84, 0.96	3.2	1.07
D/F5/3	$CHCL_3$-EtOAc, 9:1 v/v	0.23, 0.36, 0.41, 0.47, 0.53, 0.61, 0.66	40.1	13.37
D/F5/4	$CHCL_3$-EtOAc, 9:1 v/v	0.26, 0.39, 0.46	52.3	17.43
D/F5/5	$CHCL_3$-EtOAc, 9:1 v/v	0.19, 0.39, 0.44, 0.54, 0.59, 0.94	26.4	8.80
D/F5/6	$CHCL_3$-EtOAc, 9:1 v/v	0.19, 0.33, 0.47, 0.56, 0.93	25.9	8.63
D/F5/7	$CHCL_3$-EtOAc, 17:3 v/v	0.26, 0.33, 0.37, 0.51, 0.57, 0.94	16.5	5.50
D/F5/8	$CHCL_3$-EtOAc, 17:3 v/v	0.26, 0.33, 0.41, 0.54, 0.57, 0.94	7.1	2.37
D/F5/9	$CHCL_3$-EtOAc, 17:3 v/v	0.19, 0.24, 0.53, 0.91	10.0	3.33
D/F5/10	$CHCL_3$-EtOAc, 4:1 v/v	0.16, 0.23, 0.54, 0.56, 0.93	12.7	4.23
D/F5/11	$CHCL_3$-EtOAc, 4:1 v/v	0.5, 0.53, 0.57, 0.92	10.6	3.53
D/F5/12	$CHCL_3$-EtOAc, 7:3 v/v	0.59, 0.63, 0.9	13.3	4.43
D/F5/13	$CHCL_3$-EtOAc, 3:2 v/v	0.61, 0.70, 0.76, 0.91	16.4	5.47
D/F5/14	$CHCL_3$-EtOAc, 1:1 v/v	0.61, 0.73, 0.79, 0.91	10.0	3.33
D/F5/15	$CHCL_3$-EtOAc, 2:3 v/v	0.34, 0.46, 0.61, 0.64, 0.68, 0.75, 0.89	10.2	3.40
D/F5/16	$CHCL_3$-EtOAc, 3:7 v/v	0.44, 0.57, 0.64, 0.69, 0.79, 0.87	3.6	1.20
D/F5/17	$CHCL_3$-EtOAc, 1:4 v/v	0.56, 0.69, 0.89	16.0	5.33
		Total	275.3	91.77

TABLE S4. TLC profile and yield of subfractions of EtOAc extract

Fraction	TLC Mobile Phase	TLC R*f* Values	Weight(mg)	Yields (%)
E/F1	Hexane-Acetone, 9:1 v/v	0.13, 0.18, 0.25, 0.33, 0.38, 0.43, 0.58, 0.68, 0.83	14.7	0.29
E/F2	Toluene-Acetone, 9:1 v/v	0.08, 0.20, 0.35, 0.40, 0.50, 0.58, 0.65, 0.78, 0.88	15.6	0.31
E/F3	Toluene-Acetone, 9:1 v/v	0.18, 0.25, 0.33, 0.38, 0.50	95.2	1.9
E/F4	Toluene-Acetone, 9:1 v/v	0.10, 0.18, 0.23, 0.28, 0.31, 0.35, 0.40, 0.45, 0.48, 0.85	168.90	3.38
E/F5	Toluene-Acetone, 17:3 v/v	0.18, 0.21, 0.25, 0.30, 0.35, 0.40, 0.45, 0.54, 0.60, 0.65, 0.90	66.9	1.34
E/F6	Toluene-Acetone, 4:1 v/v	0.06, 0.15, 0.31, 0.39, 0.44, 0.48, 0.53, 0.61, 0.68, 0.86	136.8	2.74
E/F7	Toluene-Acetone, 4:1 v/v	0.13, 0.19, 0.28, 0.35, 0.40, 0.45, 0.53, 0.63, 0.75, 0.88	139.8	2.8
E/F8	Toluene-Acetone, 7:3 v/v	0.15, 0.30, 0.38, 0.43, 0.50, 0.63, 0.73, 0.90	82.2	1.64
E/F9	Toluene-Acetone, 7:3 v/v	0.13, 0.23, 0.35, 0.43, 0.48, 0.58, 0.63, 0.65, 0.70, 0.83, 0.90	50.0	1.0
E/F10	Toluene-Acetone, 7:3 v/v	0.13, 0.25, 0.30, 0.38, 0.43, 0.53, 0.63, 0.70, 0.75, 0.85, 0.93	67.8	1.36
E/F11	Toluene-Acetone, 3:2 v/v	0.28, 0.38, 0.45, 0.50, 0.58, 0.65, 0.76, 0.84, 0.93	139.7	2.79

E/F12	Toluene-Acetone, 3:2 v/v	0.13, 0.30, 0.40, 0.45, 0.50, 0.56, 0.65, 0.73, 0.79, 0.85, 0.93	70.6	1.41
E/F13	Toluene-Acetone, 1:1 v/v	0.23, 0.31, 0.38, 0.48, 0.56, 0.68, 0.78, 0.83, 0.88, 0.95	210.60	4.21
E/F14	Toluene-Acetone, 2:3 v/v	0.35, 0.45, 0.50, 0.55, 0.63, 0.70, 0.74, 0.80, 0.88, 0.93	77.6	1.55
E/F15	Toluene-Acetone, 3:7 v/v	0.28, 0.43, 0.50, 0.60, 0.81, 0.89, 0.95	24.6	0.49
E/F16	Toluene-Acetone, 3:7 v/v	0.30, 0.50, 0.58, 0.63, 0.80, 0.90, 0.95	10.3	0.21
E/F17	Toluene-Acetone, 3:7 v/v	0.30, 0.45, 0.63, 0.83, 0.90, 0.95	9.8	0.2
E/F18	Toluene-Acetone, 3:7 v/v	0.13, 0.25, 0.38, 0.43, 0.68, 0.79, 0.85, 0.95	25.9	0.52
E/F19	Toluene-Acetone, 3:7 v/v	0.10, 0.18, 0.23, 0.35, 0.43, 0.68, 0.75, 0.80, 0.85, 0.95	27.9	0.56
E/F20	Toluene-Acetone, 3:7 v/v	0.13, 0.18, 0.45, 0.63, 0.75, 0.85, 0.90, 0.95	11.7	0.23
E/F21	Toluene-Acetone, 3:7 v/v	0.13, 0.20, 0.49, 0.61, 0.65, 0.80, 0.90	42.2	0.84
E/F22	Toluene-Acetone, 3:7 v/v	0.10, 0.18, 0.45, 0.63, 0.68, 0.75, 0.80, 0.90, 0.96	10.6	0.21
E/F23	Toluene-Acetone, 3:7 v/v	0.18, 0.35, 0.55, 0.63, 0.73, 0.83	72.4	1.45
E/F24	Toluene-Acetone, 3:7 v/v	0.28, 0.38, 0.50. 0.78, 0.85, 0.95	9.2	0.18
E/F25	Toluene-Acetone, 3:7 v/v	0.35, 0.50, 0.73, 0.80, 0.85, 0.93	8.5	0.17
E/F26	Toluene-Acetone, 3:7 v/v	0.38, 0.48, 0.58, 0.75, 0.80, 0.95	6.6	0.13
E/F27	Toluene-Acetone, 3:7 v/v	0.38, 0.48, 0.60, 0.83, 0.95	37.5	0.75
E/F28	Toluene-Acetone, 3:7 v/v	0.13, 0.20, 0.33, 0.40, 0.75, 0.88	25.8	0.52
E/F29	Toluene-Acetone, 3:7 v/v	0.13, 0.19, 0.38, 0.43, 0.50, 0.75, 0.88, 0.95	52.0	1.04
E/F30	Toluene-Acetone, 3:7 v/v	0.11, 0.18, 0.35, 0.80, 0.88	14.8	0.3
E/F31	Acetonitrile-H_2O, 9:1 v/v	0.25, 0.50, 0.73, 0.93	231.2	4.62
E/F32	Acetonitrile-H_2O, 9:1 v/v	0.45, 0.53, 0.68, 0.88	116.2	2.32
E/F33	Acetonitrile-H_2O, 9:1 v/v	0.48, 0.65, 0.75	115.8	2.32
E/F34	Acetonitrile-H_2O, 9:1 v/v	0.45, 0.75	203.6	4.07
E/F35	Acetonitrile-H_2O, 9:1 v/v	0.48, 0.55, 0.68, 0.75	853.3	16.27
E/F36	Acetonitrile-H_2O, 9:1 v/v	0.35, 0.38, 0.43, 0.58, 0.65, 0.70, 0.85	458.2	9.16
E/F37	Acetonitrile-H_2O, 9:1 v/v	0.38, 0.40, 0.45, 0.53, 0.70, 0.85	36.9	0.74
E/F38	Acetonitrile-H_2O, 4:1 v/v	0.20, 0.50, 0.68, 0.70, 0.79, 0.93	12.0	0.24
E/F39	Acetonitrile-H_2O, 4:1 v/v	0.28, 0.55, 0.63, 0.73, 0.80, 0.90	17.2	0.34
E/F40	Acetonitrile-H_2O, 4:1 v/v	0.73, 0.80, 0.90	4.6	0.92
E/F41	Acetonitrile-H_2O, 4:1 v/v	0.59, 0.77, 0.88	9.7	0.19
E/F42	Acetonitrile-H_2O, 4:1 v/v	0.63, 0.70, 0.88, 0.94	25.8	0.52
E/F43	Acetonitrile-H_2O, 4:1 v/v	0.51, 0.70, 0.85, 0.90, 0.98	18.6	0.37
		Total	3829.3	76.6

TABLE S5. TLC profile and yield of E/F13 subfractions of EtOAc extract

Fraction	TLC Mobile Phase	TLC R*f* Values	Weight(mg)	Yields (%)
E/F13/1	Toluene-Acetone, 9:1 v/v	0.05, 0.15, 0.25, 0.33, 0.55, 0.70	5.3	2.79
E/F13/2	Toluene-Acetone, 7:3 v/v	0.20, 0.25, 0.30, 0.43, 0.55	1.8	0.95
E/F13/3	Toluene-Acetone, 7:3 v/v	0.23, 0.28, 0.35, 0.43, 0.48, 0.50	5.8	3.05
E/F13/4	Toluene-Acetone, 3:2 v/v	0.15, 0.23, 0.38, 0.55	3.3	1.74
E/F13/5	Toluene-Acetone, 3:2 v/v	0.18, 0.25, 0.33, 0.40	2.9	1.53
E/F13/6	Toluene-Acetone, 3:2 v/v	0.15, 0.25, 0.30, 0.35, 0.40, 0.68, 0.73	3.7	1.95
E/F13/7	Toluene-Acetone, 3:2 v/v	0.15, 0.25, 0.33, 0.38, 0.43, 0.50	2.6	1.37

E/F13/8	Toluene-Acetone, 1:1 v/v	0.33, 0.43, 0.53, 0.58, 0.63	9.9	5.21
E/F13/9	Toluene-Acetone, 9:11 v/v	0.28, 0.40, 0.50, 0.58, 0.63, 0.70	3.6	1.89
E/F13/10	Toluene-Acetone, 2:3 v/v	0.18, 0.33, 0.38, 0.48, 0.53, 0.60, 0.65, 0.73	4.7	2.47
E/F13/11	Toluene-Acetone, 2:3 v/v	0.10, 0.38, 0.48, 0.53, 0.58, 0.65, 0.75	4.8	2.53
E/F13/12	Toluene-Acetone, 3:7 v/v	0.53, 0.63, 0.70, 0.80	4.6	2.42
E/F13/13	Toluene-Acetone, 3:7 v/v	0.45, 0.53, 0.63, 0.70, 0.80	19.6	10.32
E/F13/14	Toluene-Acetone, 3:7 v/v	0.40, 0.45, 0.53, 0.60, 0.75	24.8	13.05
E/F13/15	Toluene-Acetone, 3:7 v/v	0.35, 0.40, 0.45, 0.53	25.4	13.37
E/F13/16	Toluene-Acetone, 3:7 v/v	0.40, 0.45, 0.68	14.2	7.47
E/F13/17	Toluene-Acetone, 3:7 v/v	0.28, 0.38, 0.48	1.6	0.84
E/F13/18	Acetone-MeOH, 3:7 v/v	0.68, 0.83, 0.88	11.3	5.95
		Total	149.9	78.9