UNIVERSITI SAINS MALAYSIA

UNIVERSITY RESEARCH GRANT FINAL REPORT
Geran Penyelidikan Universiti Laporan Akhir

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A.	**PARTICULARS OF RESEARCH** / *MAKLUMAT PENYELIDIKAN:*
(i)	**Title of Research:** Anticancer Bioactivities And Screening Of Essential Oils Of Selected Malaysian Plants For Development Of Anticancer Therapeutic Agents
(ii)	**Account Number:** 1001 / PFARMASI / 813021
B.	**PERSONAL PARTICULARS OF RESEARCHER** / *MAKLUMAT PENYELIDIK:*
(i)	**Name of Research Leader:** DR ROZIAHANIM BINTI MAHMUD
	Name of Co-Researcher: PROF MADYA AMIN MALIK SHAH BIN ABDUL MAJID PROF MADYA SABARIAH BINTI ISMAIL PUAN CHE NIN BINTI MAN
(ii)	**School/Institute/Centre/Unit :** PUSAT PENGAJIAN SAINS FARMASI
C.	**Research Platform (Please tick (/) the appropriate box):** *Pelantar Penyelidikan (Sila tanda (/) kotak berkenaan):*

☐	**A. Life Sciences** *Sains Hayat*
☐	**B. Fundamental** *Fundamental*
☐	**C. Engineering & Technology** *Kejuruteraan & Teknologi*
☐	**D. Social Transformation** *Transformasi Sosial*
☐	**E. Information & Communications Technology (ICT)** *Teknologi Maklumat & Komunikasi*
☐	**F. Clinical Sciences** *Sains Klinikal*
☑	**G. Biomedical & Health Sciences** *Bioperubatan Sains Kesihatan*

D. **Duration of this research :**
Tempoh masa penyelidikan ini :

***Duration :** 42 months
Tempoh :

From : 15 / 5 / 2009 **To** : 15 / 5 / 2012
Dari: *Ke :*

E. **ABSTRACT OF RESEARCH**

(An abstract of between 100 and 200 words must be prepared in **Bahasa Malaysia and in English**. This abstract will be included in the Annual Report of the Research and Innovation Section at a later date as a means of presenting the project findings of the researcher/s to the University and the community at large)

Abstrak Penyelidikan

(Perlu disediakan di antara 100 - 200 perkataan di dalam **Bahasa Malaysia dan juga Bahasa Inggeris**.
Abstrak ini akan dimuatkan dalam Laporan Tahunan Bahagian Penyelidikan & Inovasi sebagai satu cara untuk menyampaikan dapatan projek tuan/puan kepada pihak Universiti & masyarakat luar).

Sila lihat lampiran (ABSTRAK B. Malaysia &ABSTRACT B. Inggeris).

F. **SUMMARY OF RESEARCH FINDINGS**
Ringkasan dapatan Projek Penyelidikan

Sila lihat lampiran (SUMMARY).

G. **COMPREHENSIVE TECHNICAL REPORT**
Laporan Teknikal Lengkap
Applicants are required to prepare a comprehensive technical report explaining the project.
(This report must be attached separately)
Sila sediakan laporan teknikal lengkap yang menerangkan keseluruhan projek ini.
[Laporan ini mesti dikepilkan]

Sila Lihat Laporan yg dikepilkan.

List the key words that reflect our research:
Senaraikan kata kunci yang mencerminkan penyelidikan anda:

English	Bahasa Malaysia
Myristica fragrans essential oil	Minyak pati *Myristica fragrans*
Anti-cancer	Anti-kanser
Anti-angiogenesis	Anti-angiogenesis

H. **a) Results/Benefits of this research**
Hasil Penyelidikan

No. *Bil:*	Category/Number: *Kategori/ Bilangan:*	Promised	Achieved
1.	**Research Publications (Specify target journals)** *Penerbitan Penyelidikan (Nyatakan sasaran jurnal)*	2	6
2.	**Human Capital Development**		
	a. Ph. D Students		
	b. Masters Students	1	1
	c. Undergraduates (Final Year Project)	-	3
	d. Research Officers	-	-
	e. Research Assisstants	1	1
	f. Other: Please specify	-	-
3.	**Patents** *Paten*	-	-
4.	**Specific / Potential Applications** *Spesifik/Potensi aplikasin*	-	-
5.	**Networking & Linkages** *Jaringan & Jalinan*	Pusat Racun Negara & Pusat Penyelidikan Dadah & Ubat-Ubatan , USM	Pusat Racun Negara, & Pusat Penyelidikan Dadah & Ubat-Ubatan, USM
6.	**Possible External Research Grants to be Acquired** *Jangkaan Geran Penyelidikan Luar Diperoleh*	No	Yes (FRGS acquired 5/2011).

- Kindly provide copies/evidence for Category 1 to 6.

b) Equipment used for this research.
Peralatan yang telah digunakan dalam penyelidikan ini.

Items *Perkara*	Approved Equipment	Approved Requested Equipment	Location
Specialized Equipment Peralatan khusus	Incubator Multichannel micropipette Refridgerator	Refridgerator	J03/014
Facility Kemudahan	-	-	Pharmaceutical Chemistry Lab,PPSF Cell Culture Pharmacology Lab, PPSF Pusat Racun Negara,USM
Infrastructure Infrastruktur	-	-	-

- Please attach appendix if necessary.

I. **BUDGET** / *BAJET*

Total Approved Budget	: RM 98,000.00
Total Additional Budget	: RM 18,000.00
Grand Total of Approved Budget	: RM 116,000.00

Yearly Budget Distributed

Year 1	: RM 42,465.60
Year 2	: RM 55,534.40
Year 3	: RM -

Additional Budget Approved

Year 1	: RM -
Year 2	: RM 18,000.00
Year 3	: RM -

Total Expenditure	: RM 118,899.95
Balance	: - RM 2899.95

- **Please attach final account statement from Treasury**

Signature of Researcher
Tandatangan Penyelidik

Date
Tarikh

9/10/2012

Dr. Roziahanim Mahmud
Pensyarah
Pusat Pengajian Sains Farmasi
Universiti Sains Malaysia

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H.	**COMMENTS OF PTJ'S RESEARCH COMMITTEE** *KOMEN JAWATANKUASA PENYELIDIKAN PERINGKAT PTJ*

General Comments:
Ulasan Umum:

Telah melebihi dapatan yang dijangkakan dan mencapai matlamat kajian tersebut.

.....
Signature and Stamp of Chairperson of PTJ's Evaluation Committee
Tandatangan dan Cop Pengerusi Jawatankuasa Penilaian PTJ

Date : .....
Tarikh :.....

Signature and Stamp of Dean/ Director of PTJ
Tandatangan dan Cop Dekan/ Pengarah PTJ

Profesor Dr. Syed Azhar Syed Sulaiman
Dekan
Pusat Pengajian Sains Farmasi

Date : .....
Tarikh :.....

Year/ Period	Dr/Cr	Kod Akaun	Amaun	Keterangan	Rujukan 1	Rujukan 4
2009/7	Debit	1001.229.29412.PFARMASI.813021	700	HON: PRJ. ANTICANCER BIOACTIVITIES	MEMO 17.07.09	
2009/7	Debit	1001.111.11199.PFARMASI.813021	1,406.23	GAJI TAMBAHAN JULAI 2009- BK189036		
2009/8	Debit	1001.227.27102.PFARMASI.813021	60	NO. PO. USM:274620/D	INV 0907/112,22/7/2009	NO. DO. 0907/112
2009/8	Debit	1001.227.27799.PFARMASI.813021	220.4	NO. PO. USM:274753/D	INV 46699,27/7/2009	NO. DO.46699
2009/8	Debit	1001.227.27799.PFARMASI.813021	2,323.00	NO. PO. USM:274717/D	INV FB082504,7/8/2009	NO. DO. FB082504
2009/8	Debit	1001.227.27799.PFARMASI.813021	196	NO. PO. USM:274752/D	INV R3096/09,27/7/2009	NO. DO. R3096
2009/8	Debit	1001.227.27103.PFARMASI.813021	54	NO. PO. USM:274805/D	INV 29000663,6/8/2009	NO. DO. 29000663
2009/8	Debit	1001.111.11199.PFARMASI.813021	1,406.23	GAJI PROJEK OGOS 2009- BK189106		
2009/9	Debit	1001.227.27799.PFARMASI.813021	2,245.50	NO. PO. USM:274766/D	INV 00008451,12/8/2009	NO. DO. 00008451
2009/9	Debit	1001.227.27799.PFARMASI.813021	138	NO. PO. USM:274672/D	INV-182-07-09,13/8/2009	NO. DO. DO-182-07-09
2009/9	Debit	1001.111.11199.PFARMASI.813021	1,406.23	GAJI PROJEK SEPT 2009 - BK 189201		
2009/10	Debit	1001.227.27799.PFARMASI.813021	152	NO PO USM:274959/D	INV AT01GP/1001,3/8/2009	NO. DO. AT01GP/1001
2009/10	Debit	1001.227.27799.PFARMASI.813021	105.5	NO PO USM:274739/D	INV 09-3135,17/9/2009	NO. DO. DO09-3135

2009/10	Debit	1001.227.27799.PFARMASI.813021	702	NO PO USM:274941/D	INV 00008671,29/9/2009	NO. DO. 00008671
2009/10	Debit	1001.111.11199.PFARMASI.813021	1,406.23	GAJI PROJEK OKT 2009 - BK189290		
2009/11	Debit	1001.227.27702.PFARMASI.813021	105	NO PO USM:274948/D	INV HBS270709-1, 27/7/09	NO DO HSBDO270709-1
2009/11	Debit	1001.111.11199.PFARMASI.813021	1,406.23	GAJI PROJEK OKT 2009 - BK189349		
2009/12	Debit	1001.227.27299.PFARMASI.813021	695	NO PO USM:275277/D	INV 3108, 17.11.09	NO DO 2287
2009/12	Debit	1001.227.27799.PFARMASI.813021	130	NO PO USM:275233/D	INV SINV008825, 27/10/09	NO DO SDO008825
2009/12	Debit	1001.227.27799.PFARMASI.813021	678.6	NO PO USM:275218/D	INV 47620, 29/10/09	NO DO 47620
2009/12	Debit	1001.227.27799.PFARMASI.813021	866	NO PO USM:275167/D	INV 3444/AH, 23/10/09	NO DO 3444/AH
2009/12	Debit	1001.227.27799.PFARMASI.813021	752.3	NO PO USM:275219/D	INV 47618, 28/10/09	NO DO 47618
2009/12	Debit	1001.227.27799.PFARMASI.813021	179	NO PO USM:274992/D	INV AT01RL/1001,AT01BP,AT01MD	NO DO AT01RL,AT01GP,AT01MD
2009/12	Debit	1001.227.27799.PFARMASI.813021	731		INV 47614, 28/10/09	NO DO 47614
2009/12	Debit	1001.111.11199.PFARMASI.813021	1,406.23	GAJI PROJEK DISEMBER 2009 - BK189413	GAJI PROJEK DISEMBER 2009	
2009/12	Debit	1001.227.27103.PFARMASI.813021	750	NO PO USM:275278/D	INV 79697, 19/11/09	NO DO 79697
2009/12	Debit	1001.227.27799.PFARMASI.813021	821.2	NO PO USM:275234/D	INV BG09-026, 20/11/09	NO DO BG09-026
2009/12	Debit	1001.227.27799.PFARMASI.813021	55	NO. Q 137762	NO.RUJ. 760/11/2009	
2009/12	Debit	1001.335.35999.PFARMASI.813021	4,650.00	NO PO USM:274745/D	INV -192-08-09, 16/10/09	NO DO -192-08-09
2009/12	Debit	1001.S150.35999.PFARMASI.813021	4,650.00	NO PO USM:274745/D	INV -192-08-09, 16/10/09	NO DO -192-08-09

2009/12	Credit	1001.S150.35999.PFARMASI.813021	-4,650.00	AK00036042		
2009/12	Debit	1001.227.27799.PFARMASI.813021	1,520.01	NO PO USM:274675/D	INV 11.1030, 23/11/09	NO DO 11.1027
2009/12	Debit	1001.227.27799.PFARMASI.813021	202	NO. Q 137721	NO. RUJ. BK:232/09	
2010/1	Debit	1001.111.11199.PFARMASI.813021	1,406.23	GAJI PROJEK JANUARI 2010 - BK189551	GAJI PROJEK JANUARI 2010	
2010/2	Debit	1001.111.11199.PFARMASI.813021	1,406.23	GAJI PROJEK FEB 2010-BK 189568	GAJI PROJEK FEB 2010	
2010/3	Debit	1001.227.27103.PFARMASI.813021	90	NO PO USM:318960/D	INV PG/128/10, 3/3/10	NO DO PG/064/10
2010/3	Debit	1001.227.27799.PFARMASI.813021	265.01	NO PO USM:318787/D	INV 8516053240, 1/3/10	NO DO 421743289
2010/3	Debit	1001.111.11199.PFARMASI.813021	1,406.23	GAJI PROJEK MAC 2010 - BK189583	GAJI PROJEK MAC 2010	
2010/4	Debit	1001.227.27799.PFARMASI.813021	209	NO. Q 137883	NO. RUJ. 211/3/2010	
2010/4	Debit	1001.227.27799.PFARMASI.813021	891.79	Bayaran: NO PO USM:274676/D	INV 11.1029	NO DO 11.1028
2010/4	Credit	1001.227.27799.PFARMASI.813021	-178.83	Bayaran: NO PO USM:274676/D	No:CN 01.2010, 19/1/10	
2010/4	Debit	1001.227.27799.PFARMASI.813021	46	NO. Q 137896	NO. RUJ. 260/4/2010	
2010/4	Debit	1001.227.27799.PFARMASI.813021	220	NO PO USM:318980/D	INV AT01GL/1001/02-03-10,AT01MC	DO AT01GL/1001/02-03-10,AT01MC
2010/4	Debit	1001.111.11199.PFARMASI.813021	1,406.23	GAJI PROJEK APRIL 2010	BK 189598	
2010/5	Debit	1001.227.27799.PFARMASI.813021	180	Bayaran:PFPFARMASI1000000000215	INVM48706	NO.DO N37838
2010/5	Debit	1001.227.27799.PFARMASI.813021	381	Bayaran:PFPFARMASI1000000000254	INV1144/RK	NO.DO 1144/RK
2010/5	Debit	1001.227.27799.PFARMASI.813021	215	NO. Q 137956	NO. RUJ. 318/4/2010	
2010/5	Debit	1001.227.27799.PFARMASI.813021	500	BAY.TUNAI PROJEK-ROZIAHANIM	MEMO 24/5/2010	
2010/5	Debit	1001.111.11199.PFARMASI.813021	1,406.23	GAJI PROJEK MEI 2010- BK 189533	GAJI PROJEK MEI 2010	
2010/6	Debit	1001.227.27799.PFARMASI.813021	1,065.00	Bayaran:PFPFARMASI1000000000438	INV:1210/RK	NO.DO 1210/RK

2010/6	Debit	1001.227.27799.PFARMASI.813021	1,020.49	Bayaran:PFPFARMASI1000000000350	INV:: 8516056863	NO.DO 422541351
2010/7	Debit	1001.227.27799.PFARMASI.813021	30	NO. Q 137962	NO. Q 137962	
2010/7	Debit	1001.221.21101.PFARMASI.813021	60	TP 6/2010-ROZIAHANIM MAHMUD	T208774,02.07.2010	
2010/7	Debit	1001.221.21104.PFARMASI.813021	50	TP 6/2010-ROZIAHANIM MAHMUD	T208774,02.07.2010	
2010/7	Debit	1001.221.21199.PFARMASI.813021	30	TP 6/2010-ROZIAHANIM MAHMUD	T208774,02.07.2010	
2010/7	Debit	1001.227.27799.PFARMASI.813021	55	NO. Q 137986	NO. RUJ. 435/6/2010	
2010/7	Debit	1001.227.27799.PFARMASI.813021	456	NO. Q 137973	NO. RUJ. BK:126/10	
2009/9999	Credit	1001.111.11199.PFARMASI.813021	-8,437.38	Year 2009 t/f to retained earnings		
2009/9999	Credit	1001.227.27102.PFARMASI.813021	-60	Year 2009 t/f to retained earnings		
2009/9999	Credit	1001.227.27103.PFARMASI.813021	-804	Year 2009 t/f to retained earnings		
2009/9999	Credit	1001.227.27299.PFARMASI.813021	-695	Year 2009 t/f to retained earnings		
2009/9999	Credit	1001.227.27702.PFARMASI.813021	-105	Year 2009 t/f to retained earnings		
2009/9999	Credit	1001.227.27799.PFARMASI.813021	-12,017.51	Year 2009 t/f to retained earnings		
2009/9999	Credit	1001.229.29412.PFARMASI.813021	-700	Year 2009 t/f to retained earnings		
2009/9999	Credit	1001.335.35999.PFARMASI.813021	-4,650.00	Year 2009 t/f to retained earnings		
2010/7	Debit	1001.221.21102.PFARMASI.813021	207	BTP USM : 48730	INV 6670,30.06.2010	
2010/7	Debit	1001.229.29399.PFARMASI.813021	182.4	TP 2/2010-SUTHAGAR PILLAI	T209321,02.07.2010	

2010/9	Debit	1001.229.29107.PFARMASI.813021	400	NO PO USM:322054/D	INV:ICNP 2010/USM/06,03/09/2010	
2010/9	Debit	1001.111.11199.PFARMASI.813021	4,890.49	GAJI TAMB 9/2010 - BK189654	GAJI TAMB 9/2010	
2010/10	Debit	1001.227.27799.PFARMASI.813021	697	NO PO USM:319801/D	INV:10-2829,21/09/2010	NO DO:DO10-2829
2010/10	Debit	1001.221.21106.PFARMASI.813021	1,418.00	BAYARAN:BIL 2010/6	INV:63950PB10P1030	WPM:A475323
2010/10	Debit	1001.111.11199.PFARMASI.813021	1,300.00	GAJI PROJEK OKTOBER 2010- BK189662	GAJI PROJEK 10/2010	
2010/11	Debit	1001.111.11199.PFARMASI.813021	1,300.00	GAJI PROJEK NOV 2010 - BK 189673	GAJI PROJEK 11/2010	
2010/12	Debit	1001.227.27799.PFARMASI.813021	370	NO. Q 149316	NO. Q 149316	
2010/12	Debit	1001.227.27799.PFARMASI.813021	398	Bayaran:PSPFARMASI1000000000138	INV:1426/RK	NO DO:1426/RK
2010/12	Debit	1001.221.21106.PFARMASI.813021	300	Bayaran:PFPFARMASI1000000000955	INV:PGINV0001945	
2010/12	Debit	1001.111.11199.PFARMASI.813021	1,300.00	GAJI PROJEK DEC 2010 - BK 189686	GAJI PROJEK 12/2010	
2011/1	Debit	1001.229.29199.PFARMASI.813021	150	Bayaran:PSPFARMASI1000000000204	INV:PG/006/11	NO DO:PG/002/11
2011/1	Debit	1001.111.11199.PFARMASI.813021	1,300.00	GAJI PROJEK JAN 2011-BK189731	GAJI PROJEK 1/2011	
2011/2	Debit	1001.227.27799.PFARMASI.813021	585	Bayaran:PSPFARMASI1000000000341	INV:AZT/INV/11/020	NO DO:AZT/DO/11/020
2011/2	Debit	1001.227.27799.PFARMASI.813021	131.8	Bayaran:PDPFARMASI1000000000093	INV:PDPFARMASI1000000000093	
2011/2	Debit	1001.111.11199.PFARMASI.813021	1,300.00	GAJI PROJEK FEBRUARI 2011 - BK189759	GAJI PROJEK 2/2011	
2011/3	Debit	1001.227.27799.PFARMASI.813021	1,953.00	Bayaran:PSPFARMASI1000000000311	INV:00011626	NO DO:00011626
2011/3	Debit	1001.227.27799.PFARMASI.813021	114.5	Bayaran:PSPFARMASI1000000000340	INV:1025/RK	NO DO:1025/RK
2011/3	Debit	1001.227.27799.PFARMASI.813021	2,050.00	Bayaran:PSPFARMASI1000000000304	INV:1018/RK	NO DO:1018/RK
2011/3	Debit	1001.227.27799.PFARMASI.813021	90	Bayaran:PDPFARMASI1000000000106	INV:PDPFARMASI/106	
2011/3	Debit	1001.111.11199.PFARMASI.813021	1,300.00	GAJI PROJEK 3/2011 - BK 189766		

2011/3	Debit	1001.227.27799.PFARMASI.813021	49.96	Bayaran:PDPFARMASI1000000000128	INV:PDPFARMASI/128	
2011/3	Debit	1001.227.27799.PFARMASI.813021	661	Bayaran:PSPFARMASI1000000000464	INV:TMS/10-6503	DOTMS/10-6503
2011/3	Debit	1001.227.27799.PFARMASI.813021	125	Bayaran:PSPFARMASI1000000000463	INV:I024852	NO DO:DO-028834
2011/4	Debit	1001.227.27103.PFARMASI.813021	1,188.00	Bayaran:PSPFARMASI1000000000499	INV:21030596	NO DO:21030596
2011/4	Debit	1001.227.27199.PFARMASI.813021	142.5	Bayaran:PSPFARMASI1000000000499	INV:21030596	NO DO:21030596
2011/4	Debit	1001.227.27799.PFARMASI.813021	141	Bayaran:PDPFARMASI1000000000032	INV:PDPFARMASI1000000000032 (1)	
2011/4	Debit	1001.227.27799.PFARMASI.813021	367.5	Bayaran:PSPFARMASI1000000000462	INV:: 8516066586	NO DO:425196145
2011/4	Debit	1001.227.27799.PFARMASI.813021	322	Bayaran:PSPFARMASI1000000000534	INV:11-1033	DO11-1033
2011/4	Debit	1001.227.27799.PFARMASI.813021	375	Bayaran:PSPFARMASI1000000000533	INV:FB103494	NO DO:FB103494
2011/4	Debit	1001.111.11199.PFARMASI.813021	1,300.00	PEL GAJI PROJEK APRIL 2011-BK 189776	BK 189776	
2011/5	Debit	1001.227.27799.PFARMASI.813021	80	Bayaran:PSPFARMASI1000000000531	INV:N52060	NO DO:041556
2011/5	Debit	1001.227.27799.PFARMASI.813021	375.8	Bayaran:PSPFARMASI1000000000565	INV:11-1158	NO DO:DO11-1158
2011/5	Debit	1001.227.27799.PFARMASI.813021	509	Bayaran:PSPFARMASI1000000000627	INV:MDG11-1239	NO DO:MDG11-1239
2011/5	Debit	1001.227.27799.PFARMASI.813021	1,048.00	Bayaran:PSPFARMASI1000000000532	INV:491723	
2011/5	Debit	1001.227.27799.PFARMASI.813021	1,460.00	Bayaran:PSPFARMASI1000000000626	INV:1084/11/RK	NO DO:1084/11/RK
2011/5	Debit	1001.227.27799.PFARMASI.813021	84.5	Bayaran:PSPFARMASI1000000000625	INV:8516067691	NO DO:425495928
2011/5	Debit	1001.227.27799.PFARMASI.813021	315.99	Bayaran:PDPFARMASI1000000000142	INV:PDPFARMASI/142	
2011/5	Debit	1001.227.27799.PFARMASI.813021	2,362.00	Bayaran:PSPFARMASI1000000000564	INV:FB104698	NO DO:FB104698
2011/5	Debit	1001.111.11199.PFARMASI.813021	1,300.00	Bayaran:GAJI BULAN MEI 2011	GP/05/2011/100001	
2011/6	Debit	1001.227.27799.PFARMASI.813021	1,294.00	Bayaran:PSPFARMASI1000000000664	INV:11-1459	NO DO:DO11-1459
2011/6	Debit	1001.221.21101.PFARMASI.813021	90	Bayaran:PFPFARMASI1000000001314	INV:TP/6/2011(1)	
2011/6	Debit	1001.221.21102.PFARMASI.813021	60	Bayaran:PFPFARMASI1000000001314	INV:TP/6/2011(1)	
2011/6	Debit	1001.221.21104.PFARMASI.813021	111.4	Bayaran:PFPFARMASI1000000001314	INV:TP/6/2011(1)	
2011/6	Debit	1001.227.27799.PFARMASI.813021	6,180.00	Bayaran:PSPFARMASI1000000000661	INV:1125/11/RK	DO:1113/11/RK

2011/6	Debit	1001.227.27799.PFARMASI.813021	1,700.00	Bayaran:PSPFARMASI1000000000663	INV:1126/11/RK	DO:1114/11/RK
2010/9999	Credit	1001.111.11199.PFARMASI.813021	-15,821.64	Year 2010 t/f to retained earnings		
2010/9999	Credit	1001.221.21101.PFARMASI.813021	-60	Year 2010 t/f to retained earnings		
2010/9999	Credit	1001.221.21102.PFARMASI.813021	-207	Year 2010 t/f to retained earnings		
2010/9999	Credit	1001.221.21104.PFARMASI.813021	-50	Year 2010 t/f to retained earnings		
2010/9999	Credit	1001.221.21106.PFARMASI.813021	-1,718.00	Year 2010 t/f to retained earnings		
2010/9999	Credit	1001.221.21199.PFARMASI.813021	-30	Year 2010 t/f to retained earnings		
2010/9999	Credit	1001.227.27103.PFARMASI.813021	-90	Year 2010 t/f to retained earnings		
2010/9999	Credit	1001.227.27799.PFARMASI.813021	-6,820.46	Year 2010 t/f to retained earnings		
2010/9999	Credit	1001.229.29107.PFARMASI.813021	-400	Year 2010 t/f to retained earnings		
2010/9999	Credit	1001.229.29399.PFARMASI.813021	-182.4	Year 2010 t/f to retained earnings		
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2011/8	Debit	1001.227.27799.PFARMASI.813021	218.99	Bayaran:PSPFARMASI1000000000926	INV:8516071072	DO:426246445
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2012/3	Debit	1001.335.35901.PFARMASI.813021	3,388.00	Bayaran:PBPFARMASI1000000000032	INV:FB114658	1001/KPP/PFARMASI/12/813021/15
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2012/8	Debit	1001.111.11199.PFARMASI.813021	1,500.00	PEL BV692936-GAJI PROJEK JULAI 2012	PEL BV692936	

FINAL REPORT FOR RESEARCH UNIVERSITY (RU) GRANT

Title: Anticancer Bioactivities and Screening of Essential Oils of Selected Malaysian Plants for Development of Anticancer Therapeutic Agents

Research leader: Dr.Roziahanim Mahmud

Co-researchers: Assoc. Prof. Dr. Amin Malik Shah Abdul Majid

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Project Account No : 1001/PFARMASI/813021

Duration: 15 Jun 2009-15 May 2012

ABSTRACT

The essential oil *Myristica fragrans* and its fractions were screened for their anti-cancer activities on cancer cell lines of colorectal cancer (HCT-116) and breast cancer (MCF-7) and anti-angiogenic activities in *ex vivo* rat aortic ring assay. Chemical composition and standardization of the crude essential oil of *M. fragrans* were determined by various spectroscopic and chromatographic analyses (TLC, FT-IR and UV-Vis). The chemical composition of the essential oil was determined quantitatively using GCMS. Pure standards of myristicin, limonene, terpinen-4-ol, and β-pinene were used to validate the GCMS analysis. The studies showed that both the crude extract and its fractions showed cytotoxic activities in a dose dependent manner. Both the extract and it fractions were more sensitive on HCT-116 compared to MCF-7. Of all fractions, fraction F3 showed most bioactive activity against HCT-116. The mechanism of action of the crude extract and the potent anti-cancer fraction F3 were studied by DNA fragmentation assay. Fraction F3 showed distinct bands (fragments) on the gel forming DNA ladder. Crude essential oil of *M. fragrans* and fraction F3 showed significant anti-angiogenic activity compared to positive control (suramin) in a dose dependant manner. Both the crude extract and fraction F3 also showed cytotoxic effect on human umbilical vein endothelial cells (HUVEC), a reference cell line. In the antioxidant activities, fraction F3 showed higher activity than the crude extract. Fraction F3 was found to exhibit significant pharmacological activities in anti-cancer, anti-angiogenic and antioxidant activities. This is due to the presence of higher content of bioactive chemical constituents such as myristicin, limonene, eugenol, and terpinen-4-ol in fraction F3.

ABSTRAK

Minyak pati *Myristica fragrans* mentah dan fraksi-fraksinya telah dikaji dalam sel-sel kanser kolorektal (HCT-116) dan kanser payudara (MCF-7) dan anti-angiogeniknya ke atas cerakin gelang aortik tikus *ex vivo*. Komposisi kimia dan pemiawaian minyak pati mentah *M. fragrans* ditentukan menggunakan pelbagai jenis analisis spektroskopik dan kromatografik. Komposis kimia minyak pati tersebut telah ditentukan secara kuantitatif menggunakan GCMS. Standard-standard tulen miristisin, limonin, terpinin-4-ol, and β-pinin digunakan untuk mengesahkan analisis GCMS. Kajian menunjukkan kedua-dua ekstrak mentah dan pecahan-pecahannya menunjukkan aktiviti sitotoksik secara dos bergantung. Kesemua pecahan, pecahan F3 menunjukkan aktiviti paling bioaktif dalam HCT-116. Mekanisma tindakan ekstrak mentah dan pecahan mujarab anti-kanser telah dikaji menngunakan cerakin fragmentasi DNA. Pecahan F3 menunjukkan jalur-jalur ketara (fragmen) ke atas gel yang membentuk tangga DNA menandakan aktiviti sitotoksik berasaskan apoptotik pecahan F3. Seterusnya, kajian minyak pati *Myristica fragrans* mentah dan pecahan F3 ke atas cerakin gelang aortik tikus *ex vivo* menunjukkan aktiviti anti-angiogenik secara ketara berbanding kawalan positif (suramin) secara dos bergantung. Tambahan pula, kedua-dua ekstrak mentah dan pecahan F3 menunjukkan kesan sitotoksik ke atas sel-sel endothelium pembuluh umbilical (HUVEC), sel rujukan. Dalam aktiviti anti-oksidan, pecahan F3 menunjukkan aktiviti yang lebih tinggi berbanding ekstrak mentah Pecahan F3 didapati menunjukkan aktiviti farmakologi yang ketara dalam aktiviti-aktiviti anti-kanser, anti-angiogenik dan antioksidan. Ini adalah kerana kandungan kimia seperti miristisin, limonin, eugenol dan terpinen-4-ol adalah tinggi dalam pecahan F3.

INTRODUCTION

Angiogenesis is characterised as the development of new blood vessels by proliferation of new capillaries from previously existing microvessels. Most normal angiogenesis occurs in the embryo. It creates the primary vascular tree as well as a sufficient vasculature for the growth and development of organs (Folkman, 1971). The angiogenesis process is triggered by environmental and genetic changes. The examples include metabolic stress, cytokines from inflammatory, hypoxia, pH variation response as well as genetic mutations initiated by up-regulation of oncogenes such and down-regulation of tumour suppressor gene (Pang & Poon, 2006; Carmeliet & Jain 2000). Angiogenesis also happens during physiological repair processes such as wound healing and during the menstrual cycle (Klagsbrun and D'Amore, 1991). In addition to that, some other pathological conditions such as inflammatory diseases such as rheumatoid arthritis, atherosclerosis, proliferative retinopathies, diabetes, obesity, psoriasis as well as cancers take place via angiogenesis (Mojzis *et al.,* 2008). Apart from that, angiogenesis is also stimulated via carcinogenesis and tumour progression due to the stimulation of hormones such as androgen, progesterone and oestrogens. It has been shown increased tumor extracellular levels of VEGF in *in vitro* and *in vivo* hormone-dependent cancers such as prostate and breast cancers (Jain *et al.,* 1998; Wu *et al.,* 2004; Debrosin *et al.,* 2003; Pang & Poon, 2006).

The angiogenesis proceeds following an initial process called vasculogenesis. Vasculogenesis is a process of formation of a network of blood vessels where the vessels are formed *de novo* from endothelial cell precursors which is termed angioblasts. During vasculogenesis, angioblasts multiply and develop into a primitive network of vessels known as the primary capillary plexus. These endothelial cell grids created by vasculogenesis subsequently serves as a platform for angiogenesis (Risau, 1997). The new blood vessels will then start to proliferate and branch from the existing one via angiogenesis upon formation of primary capillary plexus.

Cancer is the effect of a number of genetic mutations that causes the functional alterations in oncogenes, tumor-suppressor genes and microRNA genes. It affects various parts of the body by means of proliferation characterized by fast and uncontrolled abnormal cell development (Vanhoecke *et al.*, 2005).

In 2008, it was estimated that there were 12.7 million cancer cases and 7.6 million deaths (within 5 years of diagnosis), globally. The cancers fatality rate is more common among men than women with sex ratio of male to female for cancer deaths is 1.3.

Disease development starts from a genetic modification known as mutation of a cell within a tissue. A mutation permits the cell to multiply at a very high rate and forms a group of fast reproducing cells with normal appearance known as hyperplasia. Seldom, some hyperplastic cells will re-mutate and produce abnormally looking cells called dysplasia. Further frequent mutations of dysplastic cells will eventually lead to the formation of a tumor, which may either remain localized at its place of origin (malignant tumor), or enter the neighboring tissues via invasion and create new tumors (metastases).

Nutmeg (*Myristica fragrans* Houtt.) is a medium-sized topical evergreen dioecious tree cultivated in tropical regions. *M. fragrans* is known as pala in Indonesia and Malaysia, luk jan in Thailand and nikuzuku in Japan.

Myristica fragrans is widely used in medicines development technology and as spices in dishes. It also has been reported to show aphrodisiac and carminative effects (Burkill, 1966), as stomachic (Khory & Katrak, 1985), used as a tonic (Chopra *et al.,* 1958). In addition to that, nutmeg is also used as an astringent and nervous stimulant (Ainslie, 1979; Nadkarni, 2002). The powdered seed of nutmeg had been used to treat sores caused by guinea worm (Burkill, 1985). Nutmeg is also used in traditional medicine perform for prenatal and postnatal treatment during pregnancy and lactation (Lavy, 1987). The aril part of the nutmeg has been used as the main ingredient to prepare medicated ointment to heal headache, insect bites and rheumatism. The infusion of the leaves of nutmeg is used to treat hypertension and gastrointestinal problems such as flatulence and intestinal spasm (Flach &Willink, 1999). The fresh juice extracted from the fleshy pericarp the fruits was drunk to induce sedation and helps in curing nausea (Burkill, 1966; Chooi, 2004).

The nutmeg (endosperm) and mace (fleshy orange-red aril around the seeds of nutmeg) were reported to have high medicinal values with enormous phytochemical diversity (Latha *et al.,* 2005). Studies showed that the plant possesses biological activities such as anti-bacterial, anti-viral, anti-diabetic, carminative, hypolipidaemic, antithrombotic, antiplatelet aggregating, antifungal, aphrodisiac, anxiogenic, anti-ulcerogenic, antitumor and anti-inflammatory activities (Latha *et al.,* 2005; Yang *et al.,* 2006; Chirathaworn *et al.,* 2007; Morita *et al.,* 2003). Nutmeg is also one of the most widely used spices in the preparation of Ayurvedic drugs (Nisamony, 2001).

Myristica fragrans Houtt has been reported to contain 25–30% fixed oils and 5–15% volatile oils such as camphene, elemicin, eugenol, isoelemicin, isoeugenol, methoxyeugenol, pinene, sabinene, safrol, etc., and other chemical substances such as dihydroguaiaretic acid, elemicin, myristic acid, myristicin and lignan compounds (Janssen *et al.,* 1990).

The constituent myristicin have been reported to induce cytotoxicity in human neuroblastoma SK-N-SH cells by means of apoptotic mechanism in A/J mice with an accumulation of cytochrome and activation of caspase 3 in the cytosol (Henderson *et al.,* 1998). D-limonene has been reported to inhibit tumor growth and metastasis by inhibiting the angiogenesis and lymphangiogenesis of gastric cancer *in vivo* in nude mice and *in vitro* study using cell strains.

PHYTOCHEMICAL STUDIES OF ESSENTIAL OIL OF *MYRISTICA FRAGRANS*

Plant material

The fresh fruits of *M. fragrans* were obtained from Balik Pulau, Penang, Malaysia. Voucher specimens (11253) *M. fragrans* was authenticated by botanist, Mr. V. Shunmugam and deposited at the Herbarium Unit of the School of Biological Sciences, Universiti Sains Malaysia. The commercially purchased *M. fragrans* essential oil from Botanicessence Essential Oils was stored at -20°C prior to analysis.

Extraction of essential oil

The fresh fruits of the *M. fragrans* were submitted to water distillation for 3 h using a modified Clevenger's apparatus. The ground samples (200 g) were boiled with water (200 mL) for 3 h in a L liter round bottom flask fitted with a condenser. The extracted essential oils were dried over anhydrous sodium sulphate and after filtration, stored in amber glass vial at -20°C until tested and analyzed.

Fractionation and profiling of *Myristica fragrans* crude essential oil

Column chromatography was used to fractionate of the essential oil. 50 g of silica gel 60 (0.063-0.200 mm, Merck) was premixed with toluene and packed into a flash glass column measuring 60 cm long and 3 cm in diameter and left overnight. 10 g of essential oil of nutmeg was then loaded and fractionated, equilibrated with toluene and eluted

sequentially with various solvents (100 ml each), toluene, toluene-ethyl acetate mixture (95:5, 90:10, 85:15, 80:20, 70:30 and 50:50 v/v) and ethyl acetate, respectively. Hundred-eight fractions (8 mL) were obtained. The eluted fractions were evaluated and pooled into ten fractions (F1-F10) by thin-layer chromatography (TLC) analysis. The TLC analysis was achieved using aluminium silica gel plates (0.063-0.200 mm, Merck) with toluene–ethyl acetate (85:15) as the mobile phase. The chromatogram was revealed by spraying with anisaldehyde reagent and subsequent heating. The organic solvents were removed from the pooled fractions by evaporation under reduced pressure. The concentrated fractions were stored at -20 ^{0}C prior to analysis.

Qualitative analysis of *Myristica fragrans* crude essential oil

Development and validation of GCMS method

Chromatographic condition

The GC-MS and MS analysis was carried out using HP6890 GC (Agilent Gas Chromatography) coupled with HP5973 (Agilent Mass Spectrometer) mass spectrometer detector. The GC/MS was operated under the following conditions. Separation was performed on HP-5MS capillary column (30 m × 0.25 mm i.d × 0.25 μm film thickness). The injector was set at 250 °C using splitless mode. The initial oven temperature was set at 50 °C (hold for 2 min) and increased at a rate of 10 °C/min to 300 °C and held for 3 min. The flow rate of the carrier gas (helium) was maintained at 1.1 mL/min. The total run time was 30 min. Selective ion monitoring (SIM) mode was used in the analysis.

Qualitative GCMS analysis of *M. fragrans* crude essential oil and its fractions

Qualitative analysis of volatile compounds in *M. fragrans* crude essential oil and its fractions was carried out according to the method described above. An amount of 2 mg of sample was weighed and dissolved in 2 ml of hexane (HPLC grade) to give a solution of concentration 1 mg/ml. The components were identified based on the comparison of their retention indices and mass spectra with those of standards, commercially available spectral, (National Institute of Standards and Technology (NIST) mass spectral search program for NIST/EPA/NIH Mass Spectral Library V2.0) and literature data (Adams, 2001). Retention index (RI) value of each component was determined relative to the retention times (RT) of a series of C_8–C_{40}, *n*-alkanes using the following expression:

$$RI_{(x)} = 100(z) + 100\left[\frac{RT_{(x)} - RT_{(z)}}{RT_{(z+1)} - RT_{(z)}}\right]$$

Where:

$RI_{(x)}$ is the retention index of the unknown compound *x*,

z is the number of carbon atoms of the *n*-alkane eluted before the unknown compound *x*,

$z + 1$ is the number of carbon atoms of the *n*-alkane eluted after the unknown compound *x*,

$RT_{(x)}$ is the retention time of the unknown compound *x*,

$RT_{(z)}$ is the retention time of the *n*-alkane eluted before the unknown compound *x*,

$RT_{(z+1)}$ is the *n*-alkane eluted after the unknown compound *x*.

Assay for total phenolic contents of *Myristica fragrans* crude essential oil

Total phenolics constituent of the *M. fragrans* crude essential oil was determined using Folin–Ciocalteu reagent according to the method of Slinkard & Singleton, 1997. 0.1 ml of the sample solution containing 1000µg/ml of the sample was taken in a volumetric flask; 46 ml of distiled water and 1 ml Folin–Ciocalteu reagent were added. The flask was shaken thoroughly. After 3 min, 3 ml of 2% sodium bicarbonate (Na_2CO_3) solution was added and the mixture was allowed to stand for 2 hrs in the dark at room temperature with intermittent shaking. Absorbance was measured using Perkin-elmer 45 UV-Vis spectrometer at 760 nm. The gallic acid solution (6.25-100 µg/ml) was used as the standard. The concentration of total phenolic contents in sample was determined as gallic acid equivalent (GAE), in µg/mg of extract by using an equation that was obtained from standard gallic acid graph.

The experiment was carried out in triplicate and gallic acid equivalent values were determined as mean ± SD (Oktay *et al.,* 2003; Tepe *et al.,* 2005).

Total flavanoid contents of *Myristica fragrans* crude essential oil

Natural flavonoids are able to scavenge the free radicals *in vivo* and *in vitro*. Flavonoid contents were determined according to the method described by Zhishen et al. (1999). 250 µl with concentration of 1 mg/ml of *M. fragrans* crude essential oil was mixed with 1.25 ml of doubly-distilled water (dd H_2O) and 75 µl of 5% sodium nitrate ($NaNO_2$) solution. 150 µl of 10% anhydrous aluminium chloride ($AlCl_3.H_2O$) solution were added after 6 min incubation. 0.5 ml of 1 M sodium hydroxide (NaOH) solution was added after 5 min and then the total volume was made up to 2.5 ml with dd H_2O. The solution was

mixed thoroughly. The absorbance was read against blank at 510 nm. Catechin was used as a standard to construct the standard curve (0.0625 -1 mg/ml; $y = 2.7553x + 0.0579$; $r^2 = 0.999$; y is the absorbance; x is the solution concentration). The results were expressed as mg catechin equivalents (CAT)/mg of oil extract. The experiment was analyzed and values were determined as mean ± SD of triplicates.

Results

Qualitative GCMS analysis of *Myristica fragrans* essential oil extract and its fractions

Thirty seven compounds were indentified representing 98.4% of the *M. fragrans* crude essential oil. Qualitative analysis results are shown in Table 1. The *M. fragrans* crude essential oil is made up of monoterpenes (67.1%), oxygenated monoterpenes (22.2%), sesquiterpenes(1.4%), phenolic ether(6.8%) and phenylpropanoids (0.9%). Oxygenated monoterpenes accounted for 22.2% with terpineol-4 (21.3%) as the main component followed by linalool (0.7%) and d-citronellol (0.2%). The sesquiterpene hydrocarbon only accounted for 1.4% of the essential oil with geranyl acetate (0.4%) as the main component. The phenolic ether constitutes 6.8% of the oil with myristicin (6.8%) as a major component. The trans β-caryophyllene (0.3%), trans α-bergamotene (0.1%), trans β-farnesene (0.1%) and trans methyl iso-eugenol(0.1%) are the sesquiterpenes isomers (0.5%) of the *M. fragrans* oil. The phenylpropanoids constitute 0.9% with eugenol (0.2%), methyl eugenol (0.1%) and elemicin(0.6%). The aliphatic hydrocarbons accounted for 1.7% of the oil.

The *M. fragrans* crude essential oil in the study had a composition similar to previously analyzed samples and the main differences were identified in the relative quantities of individual compounds. The differences might be the result of variability associated with origins of herb, collection seasons or environmental growing conditions. The chemical composition of each fraction is shown in Table 1. Major compound in each fraction is as following:

F1; sabinene, 21.72%, F2; myristicin, 85.27%, F3; myristicin, 82.64%, F4; terpinene-4-ol,64.24%, F5; methoxyeugenol, 44.67%, F6; β-fenchyl alcohol, 57.87%, F7; β-fenchyl alcohol, 61.65%, F8; cis-sabinene hydrate, 64.10%, F9; myristic acid, 55.86%, F10; benzaldehyde:100%.

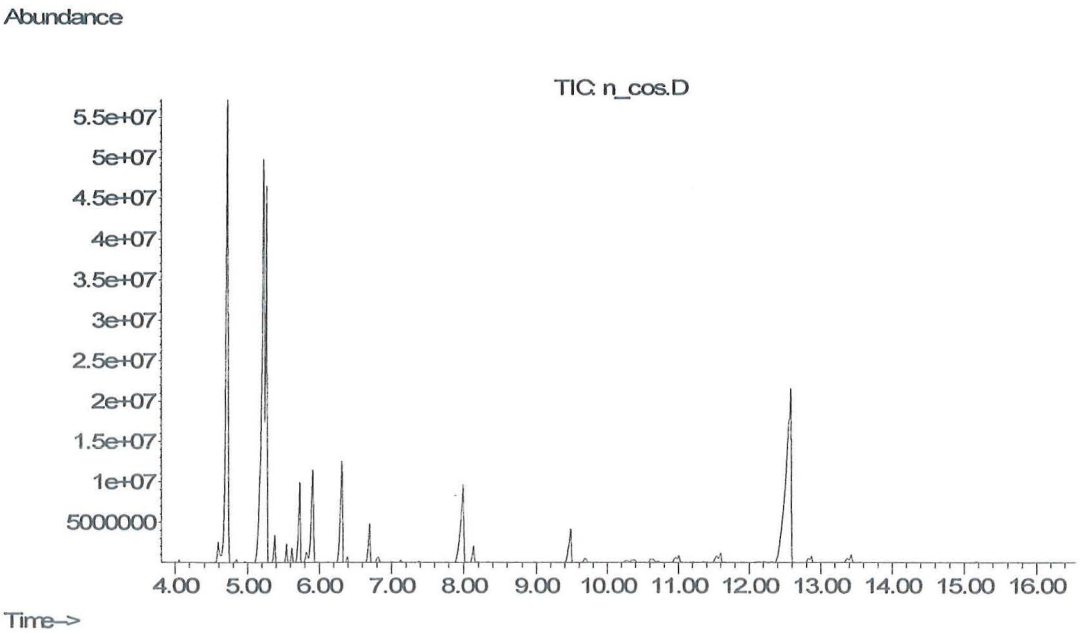

Figure 1: Total ion chromatogram of essential oil of *Myristica fragrans.*

Table 1: GCMS analysis of crude essential oil extract of *Myristica fragrans* and its 10 fractions (F1-F10).

No	Compound	Rentention time (min)	Retention index (RI)	Percent composition (%)									
				Crude extract	F1	F2	F3	F4	F5	F6	F7	F8	F9	F10
1	β-phellandrene	4.59	884	0.2	0.64	–	0.15	0.15	0.35	–	–	0.7	–	–
2	α-pinene	4.69	896	8.5	8.97	1.05	0.19	–	–	–	–	–	–	–
3	Camphene	4.84	907	0.1	0.15	–	–	–	–	–	–	–	–	–
4	Benzaldehyde	4.97	915	0.4	0.36	0.18	0.35	0.32	0.72	0.45	0.57	0.82	5.58	100
5	Sabinene	5.19	928	0.6	21.72	2.4	–	–	–	–	–	0.97	–	–
6	β-pinene	5.23	931	3.5	10.92	1.63	–	–	–	–	–	–	–	–
7	Mycrene	5.37	939	2.5	2.14	0.38	–	–	–	–	–	–	–	–
8	α-phellandrane	5.53	949	0.3	0.14	0.18	–	–	–	0.21	0.54	0.58	–	–
9	delta-3-carene	5.61	954	0.1	0.87	–	–	–	–	–	–	–	–	–
10	α-terpinene	5.7	959	9.8	0.21	0.83	–	0.41	0.45	–	0.31	0.87	–	–
11	benzene,1-methyl-2-(1-methylethyl)	5.79	964	0.1	–	0.28	–	–	–	–	–	–	–	–
12	Cymene	5.84	967	0.4	18	–	–	0.14	–	–	–	–	–	–
13	1,8 cineole eucalyptol	5.86	968	0.1	–	–	–	0.33	–	–	–	–	–	–
14	Limonene	5.87	969	7.8	11.07	–	0.49	–	–	–	–	–	–	–
15	benzyl alcohol	5.91	972	1.2	–	–	–	–	–	0.31	0.51	–	–	–

Table 1: Continued

16	γ-terpinene	6.28	994	9.9	2.34	1.82	–	0.31	–	0.22	0.28	1.2	–	–
17	sabinene hydrate	6.37	999	0.3	–	–	–	0.13	0.89	9.34	–	–	–	–
18	Terpinolene	6.67	1018	5.1	1.56	–	–	0.14	–	–	–	0.52	–	–
19	benzene(2-methyl-1-propenyl)	6.68	1019	0.1	–	–	–	–	–	0.25	–	–	–	–
20	α-terpinolene	6.8	1026	1	–	0.69	–	–	1.23	–	–	–	–	–
21	cis-sabinene hydrate	6.82	1027	0.8	–	–	–	–	–	–	6.09	64.1	29.56	–
22	Linalool	6.89	1032	0.7	–	–	–	1.09	–	–	–	–	–	–
23	Adamantanol	6.91	-	0.1	0.31	–	–	–	–	–	–	–	–	–
24	D-fenchyl alcohol	7.01	1039	0.1	–	–	–	0.17	–	–	–	–	–	–
25	2-cyclohexen-1-ol,1-methyl-4-(-1-methylethyl)	7.13	1047	0.1	–	–	–	–	2.5	4.37	–	–	–	–
26	Terpineol	7.38	1062	0.7	–	–	–	–	0.45	1	–	2.16	–	–
27	terpinene-1-ol	7.42	1065	0.1	–	–	–	–	–	–	11.25	–	–	–
28	4,5-nonadiene,2-methyl	7.64	1078	0.1	0.13	–	–	–	–	–	–	–	–	–
29	Borneol	7.75	1085	1.3	–	–	–	–	0.96	0.83	–	–	–	–
30	terpinene-4-ol	7.77	1086	21.3	–	–	–	64.24	43.19	1.01	0.53	0.49	–	–
31	benzenemethanol,α-trimethyl	7.92	1096	–	–	–	–	–	0.71	–	–	–	–	–
32	β-fenchyl alcohol	8.03	1103	8.1	–	–	–	–	–	57.87	61.65	1.37	–	–
33	citronellol	8.22	1115	0.2	–	–	–	–	–	–	0.76	–	–	–

34	Geraniol	8.62	1141	0.3	–	–	–	–	–	0.89	1.5	–	–	–

Table 1: Continued

35	bornyl acetate	8.97	1164	0.2	–	–	2.71	0.61	–	–	–	–	–	–
36	Safrole	9.41	1193	0.4	7.89	3.05	–	–	–	–	–	–	–	–
37	4-methyl 1,4-heptadiene	9.47	1197	0.1	–	–	–	–	1.29	–	–	–	–	–
38	3,4 octadiene,7-methyl	9.57	1204	0.1	–	–	–	–	–	1.18	–	–	–	–
39	2-carene	9.6	1206	0.1	–	–	0.76	–	–	–	–	–	–	–
40	benzene-1-methoxy-4-penthyl	9.68	1212	0.1	0.76	0.23	–	–	–	–	–	–	–	–
41	α-cubebene	10.27	1253	0.1	0.3	–	–	–	–	–	–	–	–	–
42	2,6-octadiene,2,6-dimethyl	10.33	1257	0.1	–	–	0.56	–	–	–	–	–	–	–
43	Eugenol	10.37	1260	0.2	–	–	2.33	2.73	–	–	–	–	–	–
44	α-copaene	10.62	1278	0.2	1.71	0.25	–	–	–	–	–	–	–	–
45	2,6-octadiene,2,6-dimethyl acetate	10.69	1283	0.4	–	–	0.89	0.98	–	–	–	–	–	–
46	geranyl acetate	10.72	1285	0.4	–	–	0.57	–	–	–	–	–	–	–
47	Vanilline	10.9	1297	0.3	–	–	0.2	0.31	–	–	0.49	–	–	–
48	Methyleugenol	10.99	1304	0.1	–	–	–	8.88	–	–	–	–	–	–
49	α-bergamotene	11.37	1332	0.1	0.16	–	–	–	–	–	–	–	–	–
50	α-farnesene	11.4	1334	0.2	0.15	–	–	–	–	–	–	–	–	–
51	isoeugenol	11.54	1344	0.1	–	–	2.12	4.67	–	–	–	–	–	–
52	methyl isoeugenol	12.11	1387	0.1	–	–	–	0.84	–	–	–	–	–	–
53	anisyl acetone	12.16	1390	0.1	–	–	–	0.58	–	–	–	–	–	–
54	Myristicin	12.44	1412	6.8	2.43	85.2	82.6	1.83	–	–	–	–	–	–

						7	4							

Table 1: Continued

55	Elemicin	12.83	1443	0.6	–	–	–	4.32	0.56	–	–	–	–	–
56	Methoxyeugenol	13.43	1490	0.2	–	–	–	–	44.67	15.04	1.32	1.1	–	–
57	myristic acid	15.28	1647	1.5	–	–	–	–	–	–	3.49	15.47	55.86	–
Total				98.4	92.93	98.24	93.96	93.18	97.97	92.97	89.29	90.35	91	100

Total phenolic content of *Myristica fragrans* essential oil extract

Based on the absorbance values of both essential oils which have reacted with Folin–Ciocalteu reagent and compared with the standard solutions of GAE, as described above, results of the colorimetric analysis of total phenolic content values were calculated. Total phenolic content (TPC) was determined in comparison with standard gallic acid and the results were expressed in terms of μg GAE/mg of extract. The TPC value of *M. fragrans* crude essential oil was determined at 43.4±2.0 μg of GAE/mg of extract.

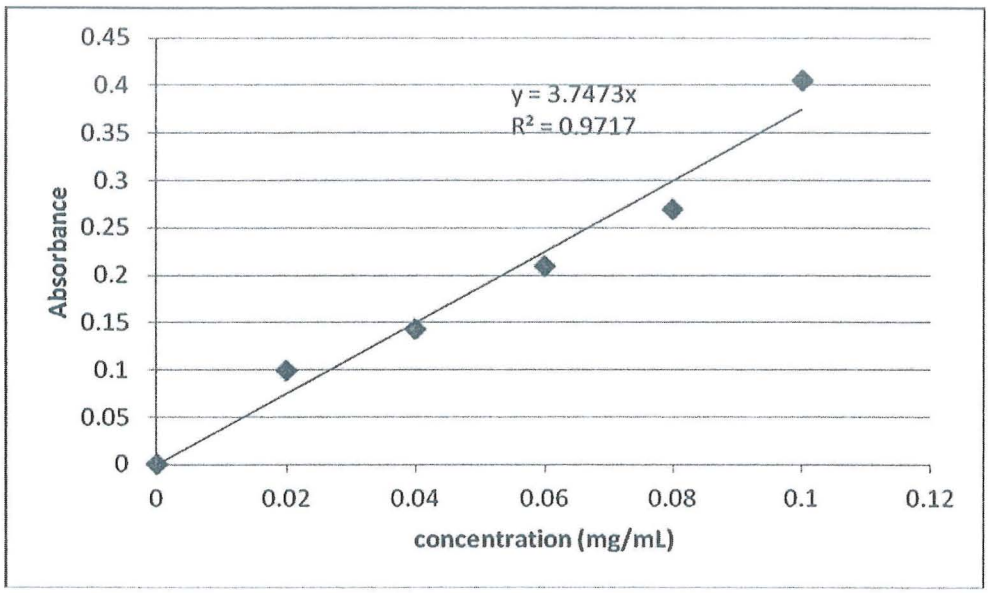

Figure 1: Calibration curve of gallic acid for total phenolics study.

Total flavonoid content of *Myristica fragrans* essential oil extract

Flavonoids are likely to be the most vital phenolics since the presence of the compounds is broad with various types. These compounds possess a broad spectrum of chemical and biological activities including radical scavenging properties. The total flavonoids content of the *M. fragrans* crude essential oil was determined to be 11.00±1.6 µg of CAT/mg of extract. The high potential of polyphenols to scavenge free radicals may be due to the presence of many phenolic hydroxyl groups. Thus, the presence of hydroxyl groups in the phenolic compounds makes the compound to be oxidized very easily.

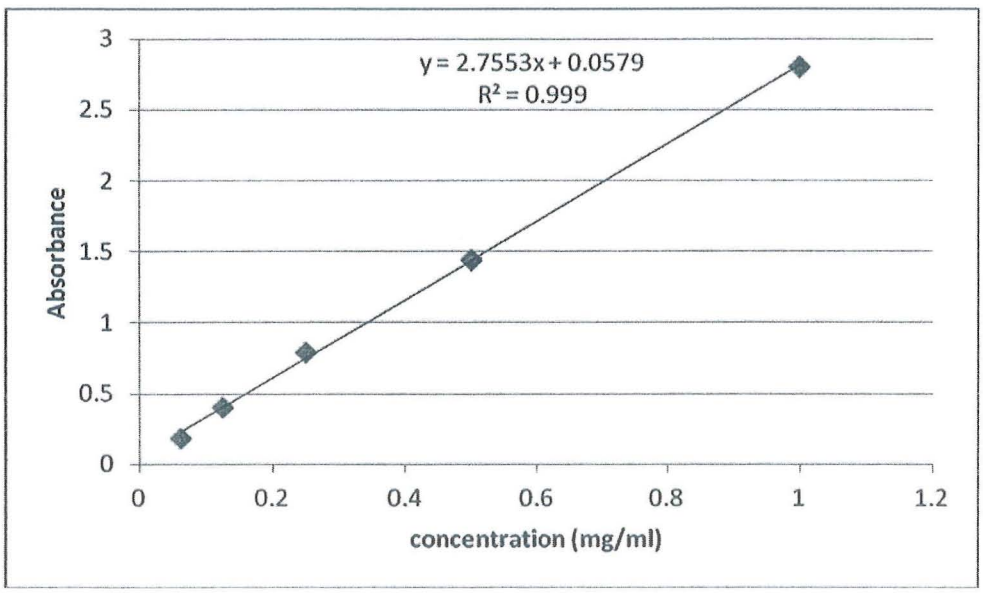

Figure 2: Calibration curve of catechin for total flavonoid study.

IN VITRO CYTOTOXICITY STUDIES OF ESSENTIAL OIL OF *MYRISTICA FRAGRANS* AND ITS FRACTIONS

Cytotoxicity activity

Anti-proliferative activity of *Myristica fragrans* crude essential oil

The cytotoxicity was measured using MTT assay developed by Mosmann (1983) with slight modifications. The cytotoxicity of *M.fragrans* essential oil and its fractions were studied against human tumour cell lines (HCT 116) derived from human colorectal cancer and MCF 7 derived from oestrogen dependent breast cancer cells. Briefly, after being harvested from culture flasks, the cells were seeded at $1.5x10^4$ cells/mL in each well of 96-well plate containing 100 µL of fresh growth medium per well and cells were permitted to adhere for 24h. The cells were then treated with 100 µL of culture medium containing the test extracts with various concentrations into each well, with 1% dimethyl sulfoxide (DMSO) served as a negative control whereas the medium only well functioned as a blank. After 48 h of treatment the media in the wells were aspirated and replaced by 100 µl of 1:9 solution of MTT reagent in PBS to culture media. The MTT reagent was prepared as 5 mg/ml in PBS and mixed well with the appropriate amount of the culture medium. Then, the plates were incubated for another at 37°C for 3 h. After incubation, the media was aspirated and 100 µl of DMSO were added into each well in order to dissolve the formazan crystals formed. The optical density of each well was then read using a microplate reader (Tecan, Switzerland) at a test wavelength of 570 nm and 620 nm as the reference filter. Standard anti-cancer clinical drugs 5-fluorouracil and tamoxifen were used as positive controls for HCT-116 while and MCF-7, respectively.

All test concentrations were run in triplicate and the experiments were repeated thrice. The percentage of inhibition towards various concentrations of each test sample was then calculated using the following equation:

$$\text{Percentage of inhibition} = [(A_{sample}-A_{blank})/(A_{control}-A_{blank})] \times 100,$$

Where, A_{sample} = absorbance of the treated cells at a certain concentration of the extract,

A_{blank} = absorbance of the blank,

$A_{control}$ = absorbance of the cells treated with 1% DMSO.

The half maximal inhibitory concentrations (IC_{50}) of each test sample were then determined by plotting the values of percentage of inhibition against their respective concentrations. Consequently, the logarithmic regression equations obtained were used to calculate the IC_{50}. The results were expressed as mean ± SEM (n=3). The fraction with lowest IC_{50} will be further selected for angiogenesis and antioxidant activities.

DNA fragmentation assay

The assay was carried out following the method previously described by Zhivotosky & Orrenius, 2001. HCT 116 cells were seeded at 5 x 10^6 cells per well before treating with various concentrations of the samples (25 and 50 µg/ml for F3 sample and *M.fragrans* crude oil, respectively) for 24 h. Betulinic acid (20 µg/ml) and DMSO (1%) served as positive and negative control, respectively. Then, DNA of the cells was extracted using the Wizard® SV Genomic DNA Purification Kit (Promega, USA) following the

manufacturer's instructions. The extracted DNA was then subjected to electrophoresis for 2 h at 100 V on 1.2% agarose gel (Dumont *et al.,* 2000) and the gel was stained with ethidium bromide to ease visualisation under ultraviolet light. The image of the gel was captured using gel documentation system (BioRad, USA). The experiment was repeated twice.

Statistical analysis

Statistical analysis was carried out by utilising SPSS software version 16.0 (SPSS, Chicago, IL). All experimental values shown are of mean ± SEM. In analysing the results, comparisons of the samples to controls were done via analysis of variance (ANOVA) followed by post-hoc test treatment of Dunnett test, where $P < 0.001$ or $P< 0.05$ was considered significant.

Results and discussion

Anti-cancer activity of *Myristica fragrans* crude essential oil

The antiproliferative effects of the nutmeg essential oil on human cancer cell lines were examined by MTT assay. A series of concentrations of *M. fragrans* crude essential oil were tested on colorectal carcinoma cell line (HCT-116) and breast carcinoma cell line (MCF-7). The *M. fragrans* crude essential oil showed anticancer activity in both carcinoma cell lines in a dose-dependent manner. The IC_{50} values of *M. fragrans* crude essential oil were determined at 66.45± 10.09µg/mL and 78.61±11.42µg/mL in HCT-116 and MCF-7, respectively. The sample was found to be more selectively cytotoxic on HCT-116 compared to MCF-7. The sample also showed significant inhibitory activity at

all concentrations ($p<0.05$ and $p<0.001$) except at 100 µg/mL ($p<0.001$) compared to its positive control, 5-fluorouracil for HCT-116 (Figure 5) and significant activity ($p<0.05$ and $p<0.001$) was observed for all concentrations compared to tamoxifen for MCF-7 (Figure 6).

Anti-cancer activity of fractions of *Myristica fragrans* crude essential oil

The fractionation of the essential oil yielded 10 fractions (F1-F10). The fractions were tested for their cytotoxic activity in HCT-116 and MCF-7 cell lines. The IC_{50} values for all fractions on both cell lines were calculated and shown in Table 2. All fractions showed dose dependent cytotoxic effects in HCT-116 (Figure 3) and MCF-7 (Figure 4), respectively. All the fractions showed no significant inhibitory activity at higher concentrations ($p<0.05$ and $p<0.001$) compared to 5-fluorouracil in HCT-116. The IC_{50} value of fraction 3 in HCT-116 was found to be the lowest compared to other fractions and determined at 20.5±0.8 µg/mL while 5-fluorouracil (positive control) showed strong cytotoxic response with IC_{50} value of 6.4±0.8 µg/mL. Fraction 3 also showed higher inhibitory activity in comparison to the anticancer activity of crude extract in HCT-116. In addition to that, fraction 3 was also studied to be cytotoxic in MCF-7 with IC_{50} value of 29.7±1.0 µg/ml. The standard of cytotoxic or non-cytotoxic was adapted from the guidelines set by the National Cancer Institute (NCI) which indicated that plant extracts or compounds with IC_{50} values less than 20 µg/ml are considered to be cytotoxic (active) and non-cytotoxic if otherwise (Bezivin *et al.*, 2003).

All the fractions tested on MCF-7 showed no significant inhibitory activity at highest concentrations ($p<0.001$ and $p<0.05$). Fraction 1 showed the lowest IC_{50} value on MCF-7 compared to other fractions. The IC_{50} values of Fraction 1 and tamoxifen were determined at 21.6 ± 0.9 µg/mL and 10.1 ± 0.4 µg/mL, respectively. Fraction 3 was selected for further study on DNA fragmentation since it showed potent bioactivity with lowest IC_{50} value in HCT-116 compared to other fractions.

DNA fragmentation

In this study, F3 (most bioactive fraction) showed significant cytotoxic activity on HCT-116 with IC_{50} 20.5µg/mL. The agarose gel electrophoresis was shown in Figure 5. The fraction showed distinct bands (fragments) on the gel forming DNA ladder at both 50µg/mL (Lane 2) and 25µg/mL (Lane 3). It is believed that the chemical components in F3 may contribute to the cytotoxic activity in which lead to DNA fragmentation.

Table 2: Anti-cancer activity of essential oil of *Myristica fragrans*, its fractions and 5-FU. Values are shown as IC_{50} ± SD (n=3).

Sample fractions	IC_{50}(μg/ml)	
	HCT-116	MCF-7
F1	27.7±1.2	21.6±0.9
F2	24.7±0.9	37.4±1.9
F3	20.5±0.8	29.7±1.0
F4	23.1±0.8	38.6±2.1
F5	27.8±0.9	33.5±2.2
F6	23.0±1.0	27.9±1.2
F7	27.6±1.2	36.7±2.7
F8	27.3±0.5	28.5±0.7
F9	25.7±0.5	27.8±0.8
F10	28.4±1.1	48.6±2.9
5-FU	6.4 ± 0.8	-
Tamoxifen	-	10.1 ± 0.4

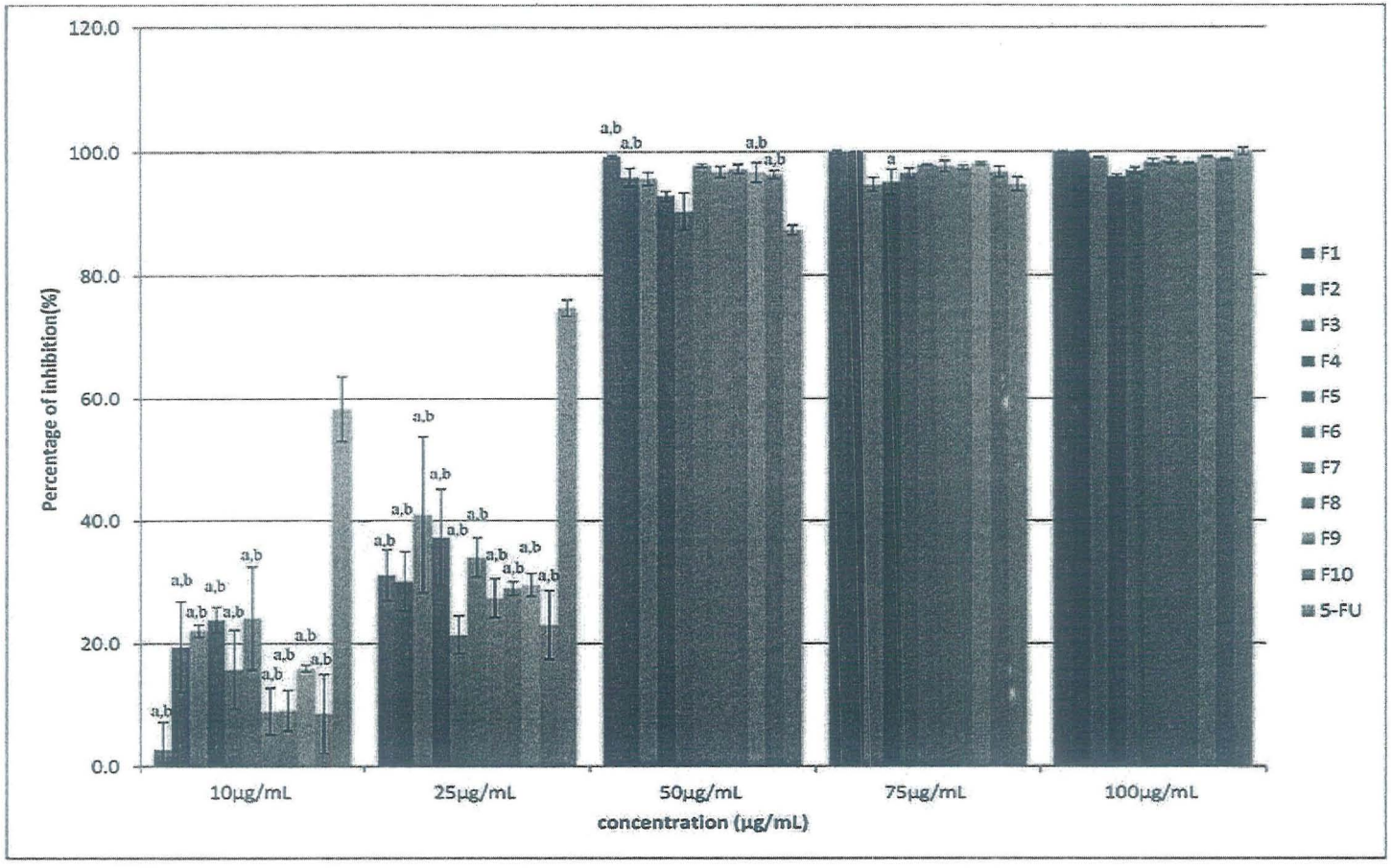

Figure 3: Anti-cancer activity of essential oil of *Myristica fragrans* and its fractions on colorectal cancer cell line (HCT-116) (n=3). Values are presented as mean±SEM (µg/mL).

* a= $p<0.05$ and b=$p<0.001$ compared to positive control (5-flurouracil)

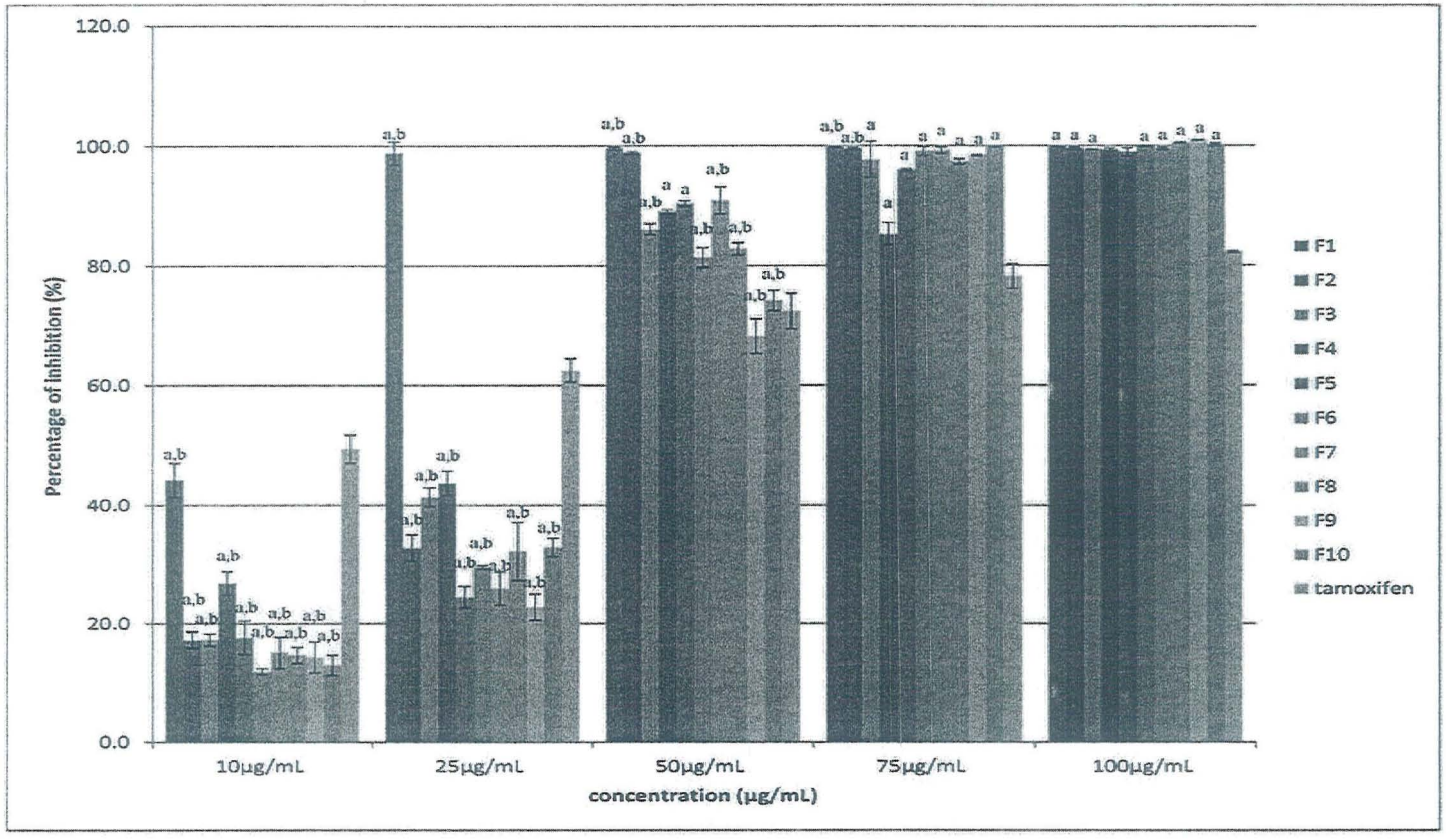

Figure 4: Anti-cancer activity of essential oil of *Myristica fragrans* and its fractions on breast cancer cell line (MCF-7) (n=3). Values are presented as mean±SEM (µg/mL).

* a= $p<0.05$ and b=$p<0.001$ compared to positive control (tamoxifen)

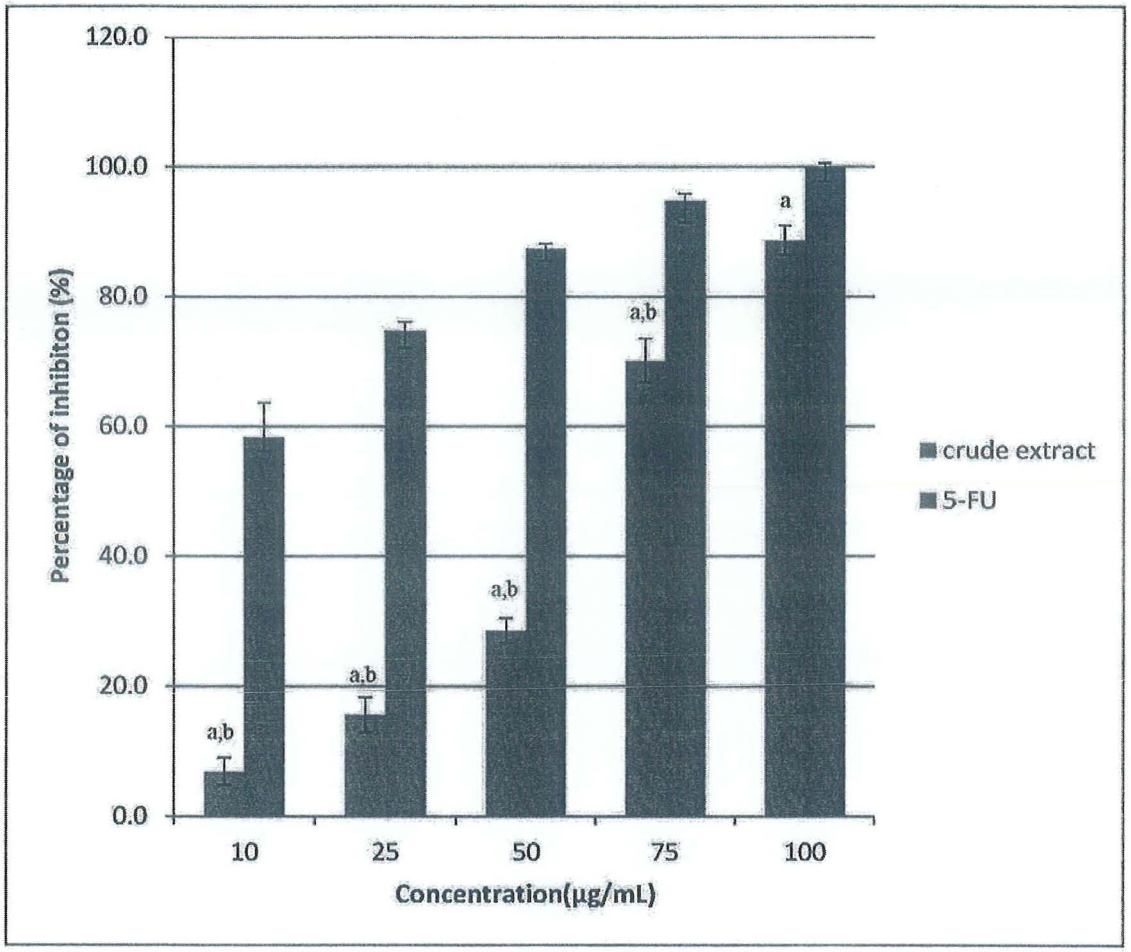

Figure 5: Anti-cancer activity of essential oil of *Myristica fragrans* and 5-flurouracil on colorectal (HCT-116) cancer cell line (n=3). Values are presented as mean±SEM (µg/mL).

* a= $p<0.05$ and b=$p<0.001$ compared to positive control (5-flurouracil)

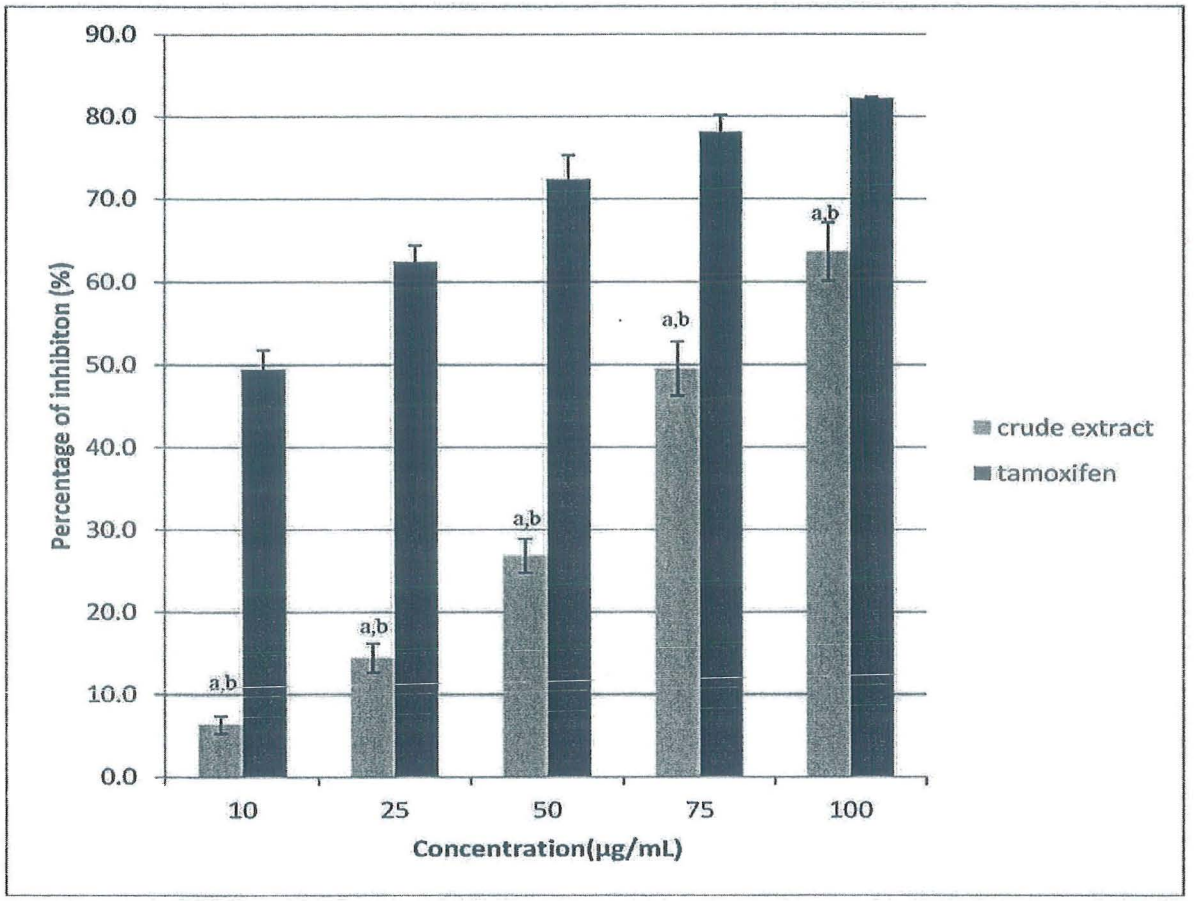

Figure 6: Anti-cancer activity of essential oil of *Myristica fragrans* and tamoxifen on breast (MCF-7) cancer cell line (n=3). Values are presented as mean±SEM (µg/mL).
* a= $p<0.05$ and b=$p<0.001$ compared to positive control (tamoxifen)

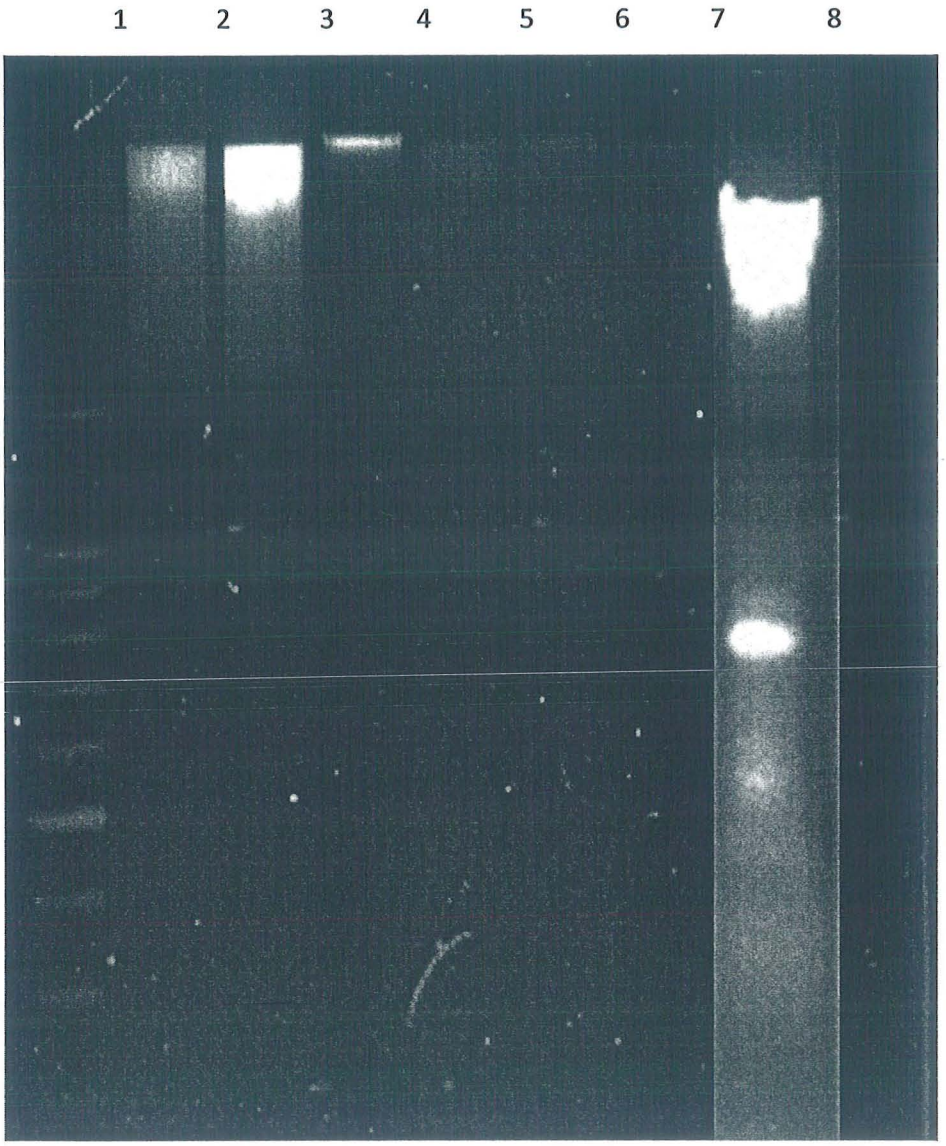

Figure 5. Effect of essential oil of *Myristica fragrans* and its fraction, F3 on DNA fragmentation of HCT 116 cells. Lane 2 and 3 indicated the effects of F3 (25 and 50 µg/ml) while Lane 4 and 5 showed effects of essential oil of *Myristica fragrans* (25 and 50 µg/ml), Lane 1 showed fragmentation of 100 bp DNA marker, Lane 6 and 7 showed fragmentation of negative control (1% DMSO) and Lane 8 showed fragmentation of positive control (betulinic acid, 20 µg/ml). The experiment was repeated twice.

ANGIOGENESIS AND ANTI-PROLIFERATIVE ACTIVITIES OF CRUDE ESSENTIAL OIL OF *MYRISTICA FRAGRANS* AND ITS FRACTION, F3.

Antiangiogenic assay

Antiangiogenic effects on rat aortic rings

Antiangiogenic effect of the essential oil and fractions was investigated *ex vivo* on rat aortic rings according to the protocol reported by Brown *et al.* (1996). Briefly, the cleansed thoracic aortas were cross sectioned into thin rings of about one millimeter thickness. One ring was placed in the center of each well of 48-well plate containing 500 µl of M199 basal medium supplied with fibrinogen, aprotinin and L-glutamine at 3mg/ml, 5µg/ml and 1% w/v, respectively. Then, 10µl thrombin was added to each well. After 90 min incubation at 37°C, another 300 µl M199 medium supplied with; fetal bovine serum (FBS) at 20% v/v, L-glutamine at 1%, 0.1% 6-aminocaproic acid, fungizone at 1% and gentamicin at 0.6% were added on top of the solidified bottom layer. The *M. fragrans* crude essential oil and its fractions (6.25-100µg/ml range) were also included in the top layer medium. The top layer medium was replaced with fresh one containing the samples after 4 days incubation at 37°C in 5% CO_2. After five days of incubation, the explants were visualised in order to study the growth of microvessels which were expected to occur spontaneously at a basal rate from the cut surfaces of the aortic rings. The magnitude of blood vessel outgrowth were quantified as described by Nicosia et al., 1997 using inverted microscope (Olympus, Japan) supplied with a digital camera (Lieca CCD, Japan) and Leica QWin computerized imaging software. Three independent experiments were conducted and the results were calculated as mean percentage of inhibition ± SD

(n=3). Suramin was used as a positive control while 1% DMSO served as a negative control. The inhibition of blood vessels was calculated using the following formula:

Percentage of blood vessels inhibition = $(1-(A_s/A_o)) \times 100\%$:

Where, A_s= distance of blood vessels growth of aorta rings treated with extracts (in μm).

A_o= distance of blood vessels growth of aorta rings treated with DMSO (in μm).

Cytotoxicity activity

***In vitro* assay for cytotoxic activity on HUVEC of *Myristica fragrans* crude essential oil and F3 (MTT assay)**

Cellular viability of HUVEC was monitored by the MTT method. F3 showed the most potent anti-cancer activity against HCT-116 cell line among the fractions tested. Briefly, cells were seeded into a 96-well microtitre plate, and grown to confluence. The cells were treated with *M. fragrans* crude essential oil and F3 (bioactive fraction) which were serially diluted with growth medium to obtain various concentration (100, 50, 25, 12.5, 6.25, 3.125 μg/mL). 100 μL of each concentration was added to each well. After 48 h of treatment the medium was aspirated and the cells were washed once with sterile phosphate buffered saline (PBS). 5 mg/ml of MTT in PBS was added to each well at 10% v/v and the plate was incubated at 37^0C in 5% CO_2 for 3 h. The medium was discarded and 200 μL of dimethylsulfoxide (DMSO) was added to each well to dissolve the dark blue crystals of formazan salt. After incubation at 37^0C for 10 min, the absorbance was measured at a primary wavelength of 570 nm and a reference wavelength of 650 nm,

using a microplate reader. Each plate contained the essential oil, fraction, negative control (0.1% DMSO) and blank. Cytotoxicity is expressed as IC_{50} denoting the concentration resulting in 50% loss of cell viability. All tests and analyses were run in triplicate.

Statistical analysis

Statistical analysis was carried out by utilising SPSS software version 16.0 (SPSS, Chicago, IL). All experimental values shown are of mean ± SEM. In analysing the results, comparisons of the samples to controls were done via analysis of variance (ANOVA) followed by post-hoc test treatment of Dunnett test, where $P < 0.001$ or $P< 0.05$ was considered significant.

Results and discussion

Antiangiogenic effects of *Myristica fragrans* crude essential oil and F3 on rat aortic rings

The rat aortic assay is regarded to mimic the *in vivo* environment because it involves not only endothelial cells but also nonendothelial cells besides the endothelial cells. The endothelial cells are not preselected by sub-culturing or in the proliferation state as in a typical *in vitro* model. So the employed *ex vivo* method is more representative of a real life endothelial model (Robert *et al.*, 2003). The assay is based on the capability of the aortic wall to produce neo-vessels in bio-matrix gels after mechanically inflicted wound on the aortic ring or upon addition in the assay as factors that can stimulate angiogenesis.

The angiogenic activity of *M. fragrans* crude essential oil was investigated by rat aortic ring assay using various concentrations (6.25-100μg/mL). Figure 7 showed the graph of percentage of inhibition of blood vessel formation versus concentration for *M. fragrans* crude essential oil, F3 and the positive control (suramin), respectively. Based on this graph, the IC_{50} of the crude extract and suramin were determined at 56.07±8.9 μg/mL and 4.15±0.7 μg/mL, respectively. The percentage inhibition of the crude extract has been seen to be increased from 2.08±0.5% at 6.25μg/mL to 82.61±8.2% at 100μg/mL. The anti-angiogenic activity of the crude extract and suramin were observed to be significant ($p<0.001$ and $p<0.05$) in a dose-dependent manner (Figure 7). Suramin, a well recognized anti-angiogenic agent, showed almost 100% of inhibition at 100μg/mL (Figure 9(c)). In Figure 9 the inhibition of blood vessel growth from the rat aortic ring by *M. fragrans* crude essential oil can be visualized as the concentration increases. This meant a complete inhibition at the highest concentration (100 μg/mL) of the crude extract.

The anti-angiogenic potential was also investigated using anti-cancer bioactive fraction of *M.fragrans* crude essential oil, fraction F3. The angiogenic activity of the fraction was studied at various concentrations (6.25-100μg/mL). The IC_{50} of F3 was calculated at 11.10±1.3 μg/mL and the fraction also showed significant inhibitory activity ($p<0.05$ and $p<0.001$) compared to positive control (Figure 7). Similarly, Figure 7 showed dose dependent effect of fraction F3 in rat aortic ring assay. Fraction F3 showed complete inhibition of the growth of new blood vessel from aortic ring from concentration as low as 25μg/mL (Figure 10).

In vitro assay for cytotoxic activity on HUVEC of _Myristica fragrans_ crude essential oil and F3 (MTT assay)

The anti-proliferative effect of both *M. fragrans* crude essential oil and fraction F3 respectively in HUVEC were tested in a series of concentration in the MTT assay. The HUVEC was used as reference cell line to investigate the relationship between cytotoxicity of the studied plant samples with angiogenesis. The IC_{50} values of *M. fragrans* crude essential oil and F3 in HUVEC were determined at 23.59±7.4 μg/mL and 16.19±3.6 μg/mL, respectively. Betulinic acid was used as a positive control and the IC_{50} value of the positive control was determined at 3.19 ± 0.23 μg/mL. Both samples showed anti-proliferative response in dose-dependent manner significantly (Figure 8). The crude extract showed low cytotoxic effect on HUVEC based on the guidelines set by National Cancer Institute (NCI) that IC_{50} value of more than 20 μg/ml is considered to be less or non cytotoxic (inactive) (Bezivin *et al.,* 2003). The crude extract was found to be significantly cytotoxic ($p<0.05$ and $p<0.001$) in HUVEC at the lower concentration (25 μg/mL). On the other hand, F3 showed potent cytotoxic response in HUVEC with its IC_{50} value less than 20 μg/ml. Thus, strong anti-angiogenic activity in fraction F3 was observed in rat aortic ring assay.

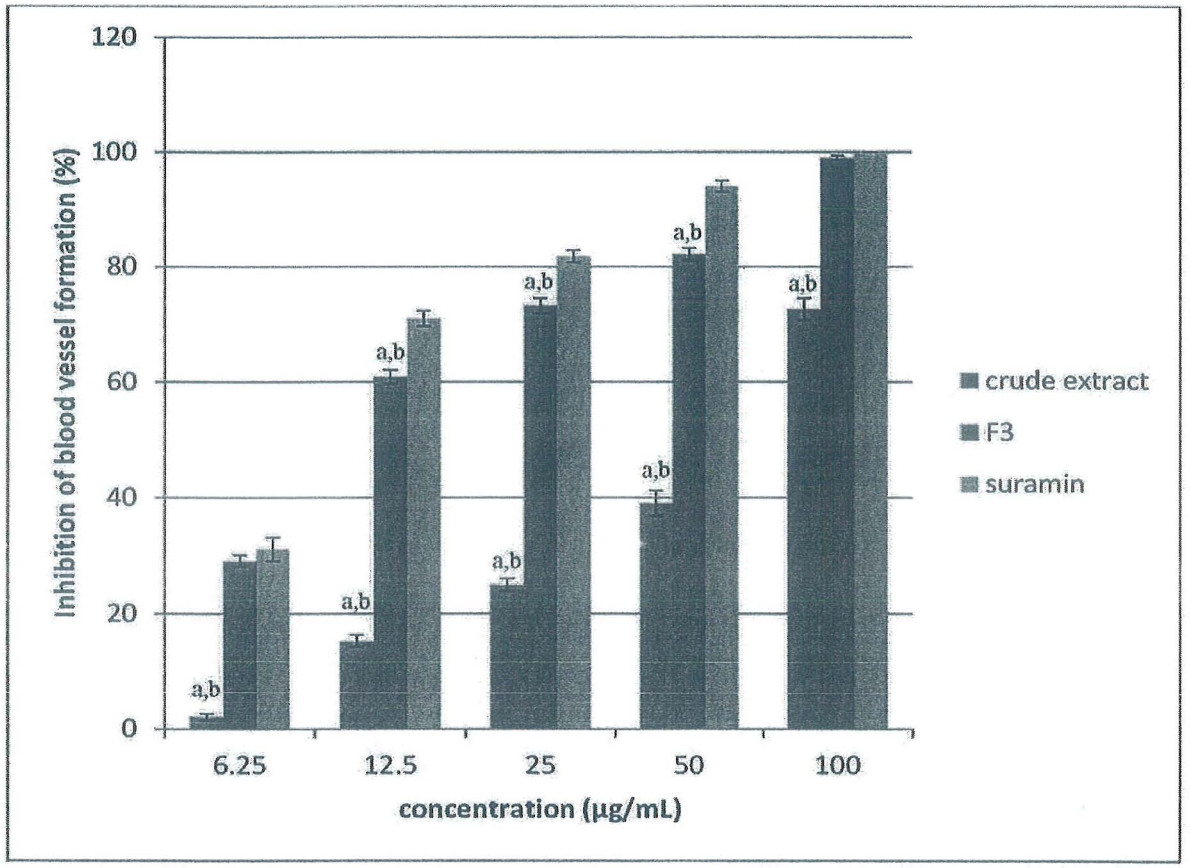

Figure 7: Dose dependent relationship of crude essential oil of *Myristica fragrans*, fraction F3 and suramin in rat aortic ring assay. Values are presented as mean±SEM (µg/mL).

* a= $p<0.05$ and b=$p<0.001$ compared to positive control (suramin)

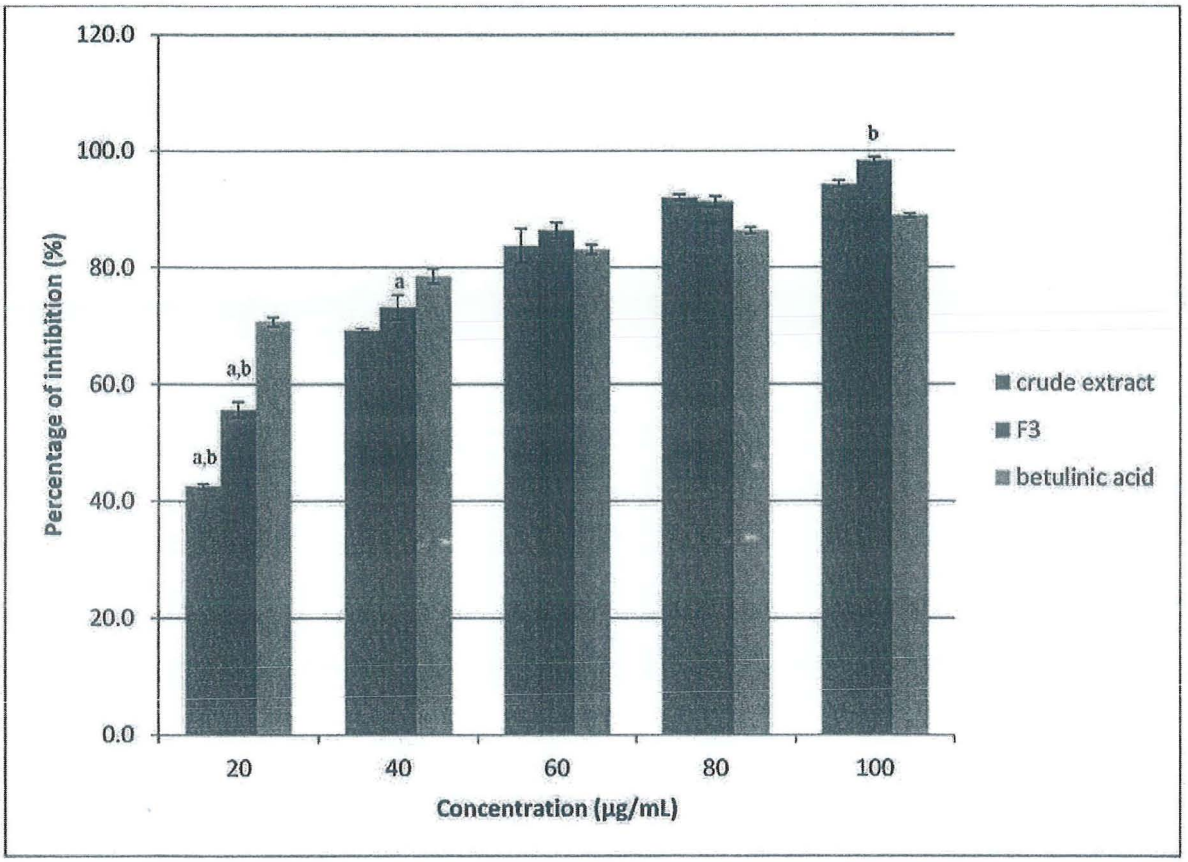

Figure 8: Proliferative activity of (a) crude ssential oil of *Myristica fragrans* and (b) its fraction F3 on HUVEC cell line (n=3). Values are presented as mean±SEM (µg/mL).
* a= $p<0.05$ and b=$p<0.001$ compared to positive control (betulinic acid)

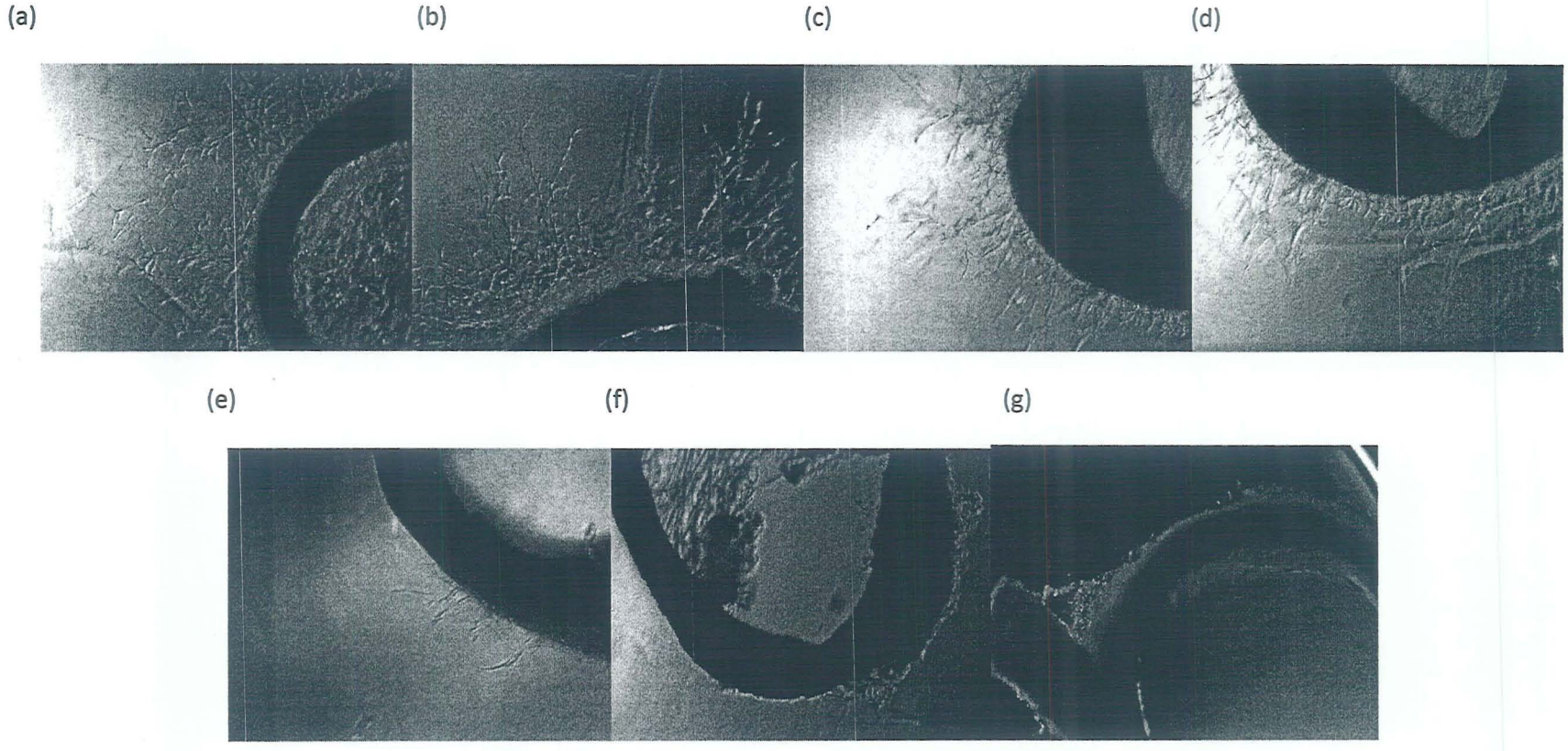

Figure 9: The effect of essential oil of *Myristica fragrans* on rat aortic ring assay. The rings were treated with (a) 1% DMSO, (b) 6.25 µg/mL, (c) 12.5 µg/mL, (d) 25 µg/mL, (e) 50 µg/mL, (f) 100 µg/mL and (g) Suramin 100 µg/mL as a positive control.

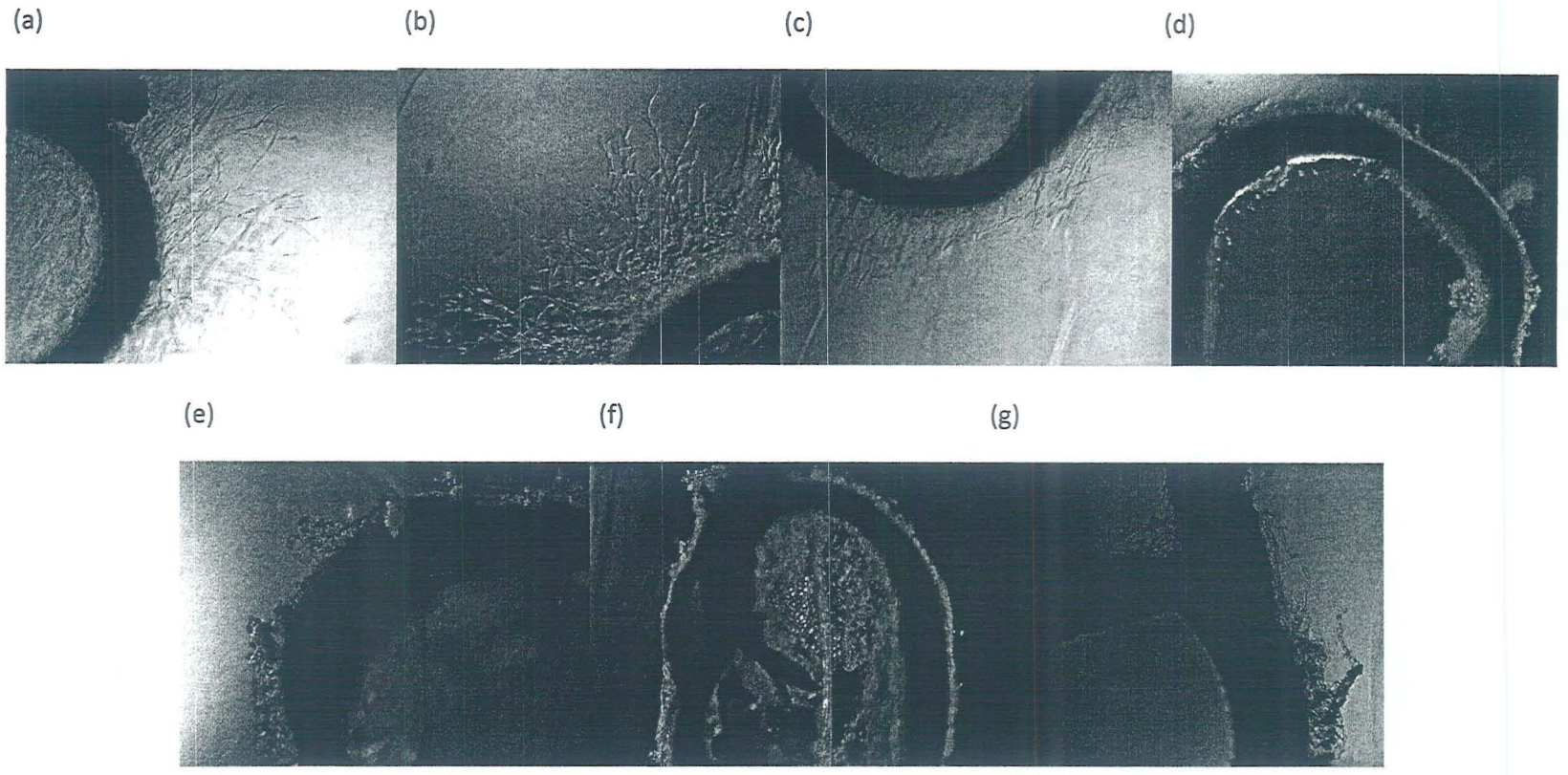

Figure 10: The effect of fraction F3 on rat aortic ring assay. The rings were treated with (a) 1% DMSO, (b) 6.25 µg/mL, (c) 12.5 µg/mL, (d) 25 µg/mL, (e) 50 µg/mL, (f) 100 µg/mL and (g) Suramin 100 µg/mL as a positive control.

ANTIOXIDANT STUDIES OF ESSENTIAL OIL OF *MYRISTICA FRAGRANS* AND ITS BIOACTIVE FRACTION, F3

Free radical scavenging activity

The hydrogen atoms or electrons donation ability of the corresponding samples were measured from the bleaching of purple coloured methanol solution of DPPH. The DPPH-radical scavenging activity was determined as per method of Shimada *et al.*, 1992. A volume of 50 µl of 1 mM DPPH (Sigma-Aldrich) in methanol was added to 200 µL of various concentrations of *M. fragrans* oil and bioactive fraction (F3) in methanol solution (6.25-100 µg/mL range), respectively. The solutions were incubated at room temperature for 30 min in the dark. The absorbance of the solutions, including a blank (containing all reagents except the sample) and a positive control (BHT, tert-butylated hydroxytoluene) was measured at 517 nm. The percentage of DPPH radical scavenged compared to blank was determined using the following formula:

$$I\% = (A_{blank} - A_{sample}/A_{blank}) \times 100$$

A_{blank} = Absorbance of blank

A_{sample} = Absorbance of sample

Extract concentration giving 50% inhibition (IC_{50}) was calculated from interpolation of the graph of inhibition percentage versus concentration of extract. Each sample assay was carried out in triplicate and data presented as a mean of the three values.

β-Carotene/linoleic acid bleaching assay

In this assay, antioxidant activity was determined by measuring the inhibition of volatile organic compounds and conjugated diene hydroperoxides arising from linoleic acid oxidation. The method described by Miraliakbari & Shahidi (2008) was adapted with slight modifications. A stock solution of β-carotene and linoleic acid was prepared with 0.5 mg of β-carotene in 1 mL chloroform, 25 µl of linoleic acid and 200 mg Tween 40. The chloroform was evaporated under vacuum and 100 mL of aerated distilled water was then added to the residue. The samples (2 g/l) were dissolved in DMSO and 350 µl of *M.fragrans* oil and F3 were added to 2.5 mL of the above mixture in different test tubes. The test tubes were incubated in a hot water bath at 50° C for 1 h, together with two blanks, one contained the antioxidant BHT as a positive control and the other contained the same volume of DMSO instead of the samples. The test tube with BHT maintained its yellow colour during the incubation period. The absorbance was measured at 470 nm on an ultraviolet spectrophotometer. Antioxidant activities (inhibition percentage, I%) of the samples were calculated using the following equation:

$$I\% = (A_{\beta\text{-carotene after 1h assay}}/A_{\text{initial }\beta\text{-carotene}}) \times 100$$

Where $A_{\beta\text{-carotene after 1 h assay}}$ is the absorbance of β-carotene after 1 h assay remaining in the samples and $A_{\text{initial }\beta\text{-carotene}}$ is the absorbance of β-carotene at the beginning of the experiments. All tests were carried out in triplicate and inhibition percentages were reported as means± SEM of triplicates.

Estimation of reducing power

The reducing power was determined according to the method of Oyaizu (1986). The *M. fragrans* crude oil and its fraction F3 (31.25–500 µg/mL in methanol), respectively were mixed with 2.5 mL of 200 mM sodium phosphate buffer (pH 6.6) and 2.5 mL of 1% potassium, and the mixture was incubated at 50° C for 20 min. Then, 2.5 mL of 10% trichloroacetic acid was added, the mixture was centrifuged at 200 g for 10 min. The upper layer (2.5 mL) was mixed with 2.5 mL of de-ionized water and 0.5 mL of 0.1% ferric chloride. The absorbance of the mixture was measured at 700 nm against blank. A higher absorbance indicates a higher reducing power. EC_{50} value (µg/mL) is the effective concentration at which the absorbance was 0.5 for reducing power and was obtained by interpolation from linear regression analysis of graph absorbance at 700nm versus concentration. Similar procedure was repeated for ascorbic acid which served as a positive control.

Statistical analysis

Statistical analysis was carried out by utilising SPSS software version 16.0 (SPSS, Chicago, IL). All experimental values shown are of mean ± SEM. In analysing the results, comparisons of the samples to controls were done via analysis of variance (ANOVA) followed by post-hoc test treatment of Dunnett test, where $P < 0.001$ or $P< 0.05$ was considered significant.

Results and discussion

Antioxidant assay

The antioxidant potential of the essential oil can be mainly attributed to its reducing power, radical scavenging ability and singlet oxygen quenching ability. The effect of antioxidants of *M.*

fragrans oil and its fraction F3 on the DPPH radical scavenging was determined by their hydrogen donating ability. DPPH• is a stable free radical and accepts an electron or hydrogen radical to become a stable diamagnetic molecule (Gulcin *et al.*, 2002). It is visualised as a discoloration of the reaction mixture. The scavenging effects of *M. fragrans* crude oil and F3 on DPPH were examined at different concentrations (6.25 µg/mL-100 µg/mL) and shown in Figure 11. A higher DPPH radical-scavenging activity is associated with a lower IC_{50} values. As shown in Figure 11, both samples exhibited significant activity compared to positive control ($p<0.05$ and $p<0.001$). The antioxidant activity of the *M. fragrans* crude oil was determined at IC_{50} of 136.00µg/mL while IC_{50} for fraction F3 was determined at 30.67µg/mL (Table 3). The antioxidant activities were compared with the standard commercial synthetic antioxidant, butylated hydroxytoluene (BHT). The IC_{50} of BHT was evaluated at 18.46µg/mL (Table 3). Antioxidants have been reported to show potent antiangiogenic properties by regulating angiogenesis modulators such as vascular endothelial growth factor (VEGF).

β-Carotene/linoleic acid bleaching assay

At 1mg/mL concentration, the oxidation of linoleic acid was effectively inhibited by *M. fragrans* crude essential oil (88.68±0.1%), while fraction F3 exhibited 91.66±0.4% of inhibition. The positive control BHT showed the highest activity at 93.20±0.1% (Table 4). Both *M. fragrans* crude essential oil and fraction F3 showed no significant inhibiting activity ($p<0.05$ and $p<0.001$) against positive control (Figure 12). In β-carotene/linoleic acid bleaching assay, β-carotene undergoes fast discoloration in the absence of an antioxidant. This is due to the generation of free radicals by coupled oxidation of β-carotene and linoleic acid. The free radicals of linoleic acid

formed upon the extraction of a hydrogen atom from one of its diallylic methylene group attacks the highly unsaturated β-carotene molecules. As a result, β-carotene is oxidized and broken down.

Reducing power

Reducing power of the *M. fragrans* essential oil and F3 is presented in Figure 13. As can be seen from the table *M. fragrans* oil and fraction F3 showed antioxidant activity with an EC_{50} value of 181.4µg/mL and 65.5µg/mL, respectively. Ascorbic acid as a positive control showed EC_{50} value of 36.1µg/mL. The *M. fragrans* essential oil and fraction F3 showed significant antioxidant activity ($p<0.05$ and $p<0.001$) against positive control. The other way of defense mechanism in preventing body against the harmful effects of free radicals is by reducing these radicals by the antioxidant substances. The reducing capacity of a compound may serve as a significant indicator of its potential antioxidant activity. Reducing powers of nutmeg essential oil was strong and increased with increasing concentration, and it showed good reducing ability in comparison with ascorbic acid. The reducing powers of *M. fragrans* essential oil and fraction F3 might be due to their hydrogen-donating ability. The components present in the studied samples have already shown to contain high phenolic content which act as good reductants leading to stabilization and termination of free radical chain reactions. Chemically, phenolic structures are good reducing agents and play vital roles as antioxidants.

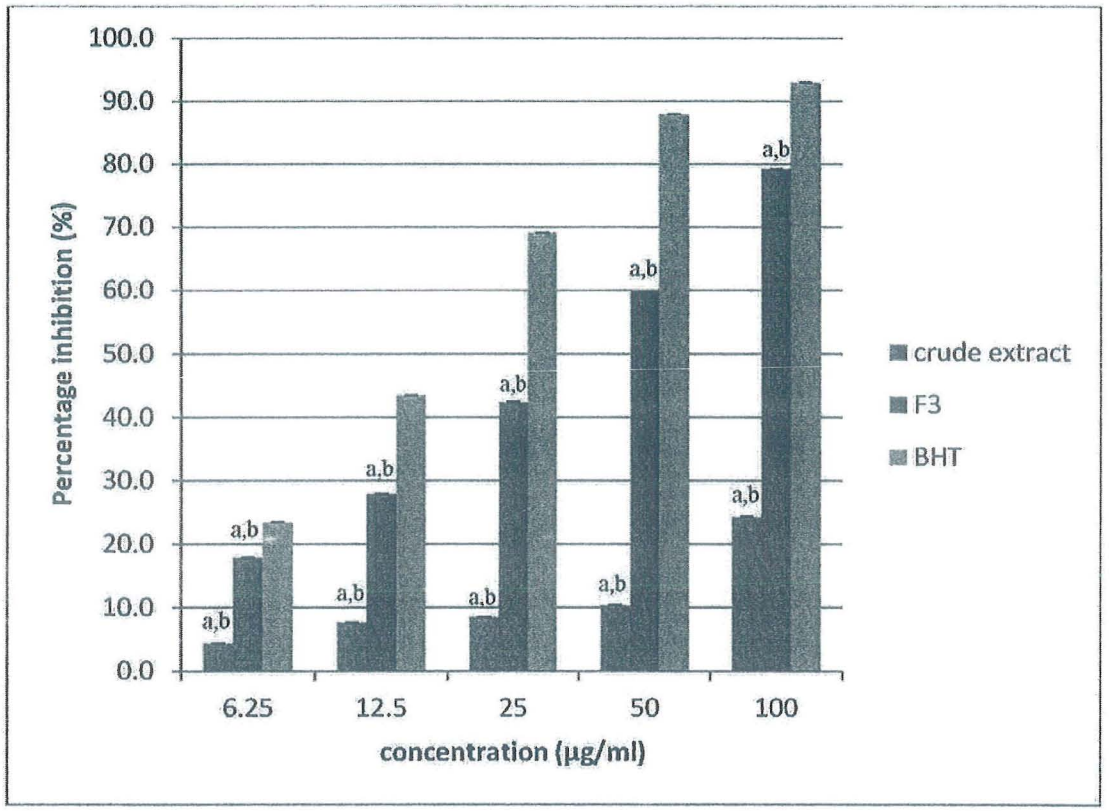

Figure 11: DPPH free radical scavenging activity of essential oil of *Myristica fragrans*, fraction F3 and BHT (n=3).
* a= $p<0.05$ and b=$p<0.001$ compared to positive control (BHT)

Table 3: IC_{50} value of free radical scavenging activity of essential oil of *M.fragrans*, fraction F3 and BHT.

Sample	IC_{50} (ug/mL)
M.fragrans oil	236.09
Fraction F3	30.67
BHT	18.43

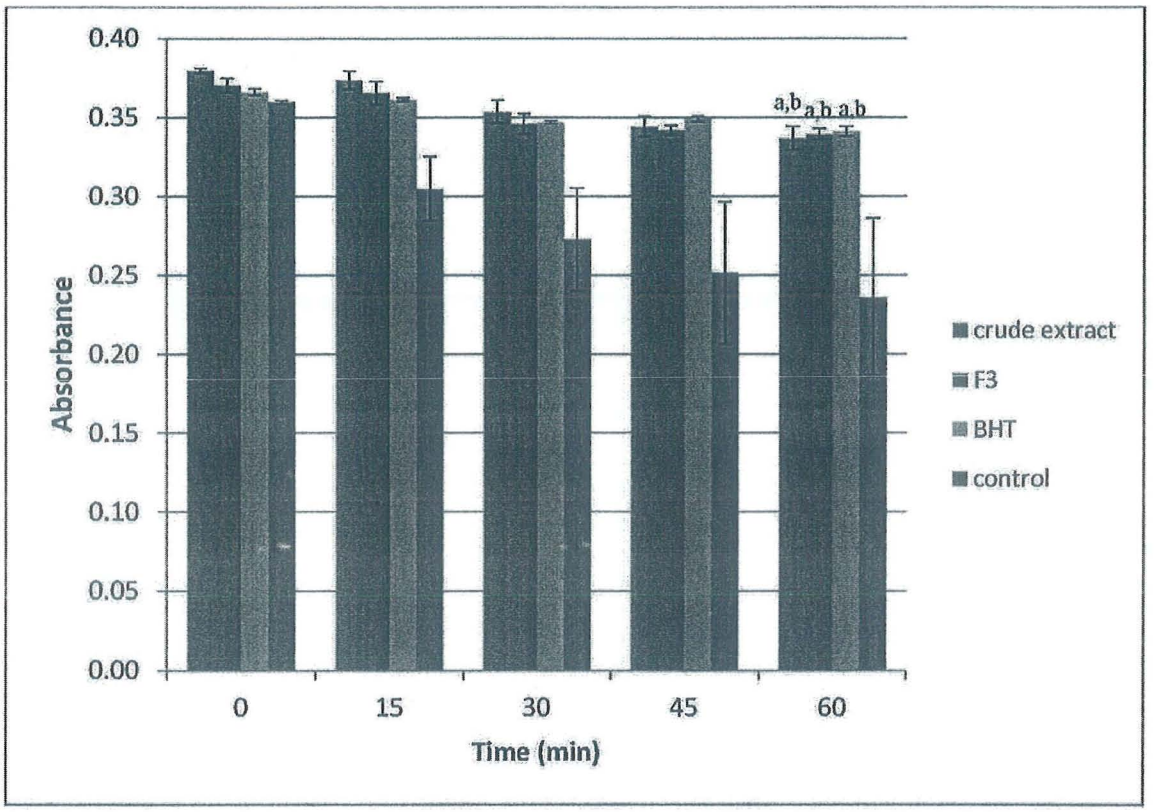

Figure 12: Beta carotene-linoleic acid bleaching activity of essential oil of *M. fragrans*, fraction F3 and BHT (n=3). Values are presented as mean±SEM (µg/mL).
* a= $p<0.05$ and b=$p<0.001$ compared to negative control.

Table 4: Beta carotene-linoleic acid bleaching activity of essential oil of *M. fragrans*, fraction F3 and BHT (n=3). Percentage inhibition values are presented as mean±SEM (µg/mL).

Sample	Absorbance (nm)					Percentage of inhibition (%)
	0min	15min	30min	45min	60min	
Essential oil of *Myristica fragrans*	0.3799	0.3737	0.3536	0.3445	0.3369	88.68±0.01
F3	0.3705	0.3657	0.3460	0.3421	0.3396	91.66±0.01
BHT	0.3663	0.3615	0.3470	0.3493	0.3414	93.2±0.00
Control	0.3600	0.3049	0.2726	0.2515	0.2360	65.56±0.02

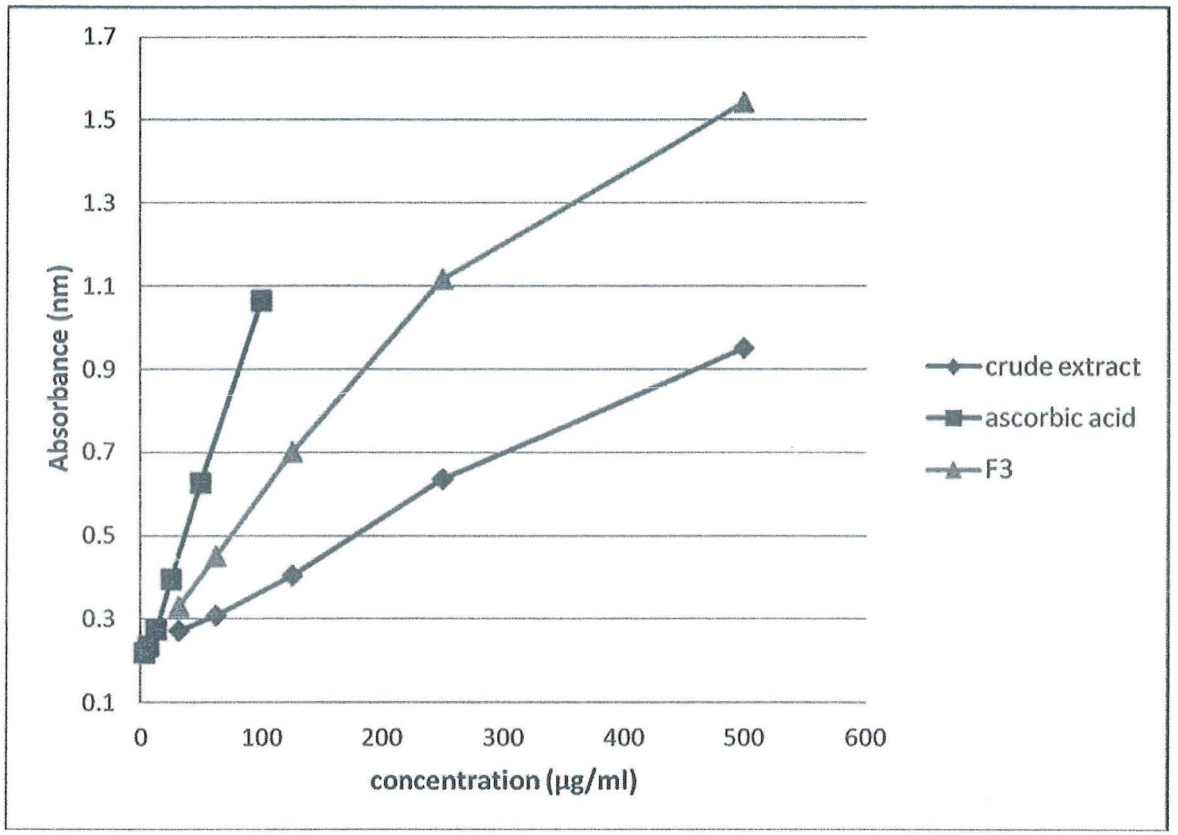

Figure 13: Reducing power activity of essential oil of *M.fragrans*, fraction F3 and BHT (n=3).

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SUMMARY

The present work investigated the phytochemical properties, anti-cancer and anti-angiogenesis activities of essential oil of *M. fragrans* and its fractions. Following the phytochemical studies further identification and characterization of essential oil of *M. fragrans* were performed by various chromatographic and spectroscopic methods which serve as a fingerprinting method to identify the plant sample containing *M. fragrans*. The GCMS method have been developed to identify the different chemical constituents and to determine the percent composition of the constituents in essential oil of *M. fragrans* and its fractions. Terpinen-4-ol and myristicin were found to be the major compounds present in the crude extract and fraction F3, respectively. Standards markers of myristicin, limonene, terpinen-4-ol and β-pinene were used to validate the GCMS method and the quantitative method of analysis developed was found to be accurate and precise. The anticancer activities of the essential oil of *M. fragrans* and its fractions were investigated in the colorectal cancer (HCT-116) and the breast cancer (MCF-7) cell lines using *in vitro* MTT assay. The extract and its fractions were found to show significant cytotoxic activity against HCT-116 compared to MCF-7. Fraction F3 was found to exhibit significantly the cytotoxic activity against HCT-116 in a dose-dependent manner. DNA fragmentation assay was conducted on both crude essential oil of *M. fragrans* and fraction F3 showed laddering of DNA. This suggests that both samples contain constituents that can induce cytotoxic effects via apoptotic mechanism selectively. Angiogenesis studies in both crude and its fraction F3 respectively using *ex vivo* rat aortic ring assay model showed significant anti-angiogenic activity in a dose-dependent manner. This indicates that both samples significantly inhibit the branching of new blood vessels from the pre-existing vessels. The crude extract and potent bioactive fraction F3 showed potent proliferation inhibition effect on HUVECs, indicating potent cytotoxic

response and strong anti-angiogenic activity of both samples. The antioxidant studies showed that crude and potent bioactive fraction F3 possess strong antioxidant properties which were contributed by the present composition of potent antioxidant chemical constituents in the crude extract and fraction F3.

The investigation reveals that higher content of bioactive chemical constituents such as myristicin, limonene, eugenol, and terpinen-4-ol in fraction F3 as determined by GCMS analysis have exhibited significant anti-cancer and anti-angiogenic activities compared to crude essential oil of *M. fragrans*. The significant anti-cancer activity of fraction F3 in colorectal cancer cell lines (HCT-116) can be due to the apoptotic induction which happened to be one of the selective mechanisms involved in cytotoxic effects. The cytotoxic effect of fraction F3 in HUVECs justifies the significant anti-angiogenesis response.

In addition to that, presence of higher antioxidant properties in fraction F3 has been strongly correlated to its significant pharmacological activities.

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Determination of Antibacterial Activity of Essential Oil of *Myristica fragrans* Houtt. using Tetrazolium Microplate Assay and its Cytotoxic Activity Against Vero Cell Line

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Abstract: Essential oils and volatile constituents of *Myristica fragrans* (nutmeg) are widely used as antioxidants, antidiabetic agents and for the prevention and treatment of different human diseases such as cancer, cardiovascular diseases and bacterial and viral infections. The aim of the study is to determine the antimicrobial activity and *in vitro* cytotoxicity of essential oil of nutmeg on normal cell line (Vero cell line). The Minimum Inhibitory Concentration (MIC) of the antimicrobial activity was determined using tetrazolium microplate assay. Eight gram negative and 2 g positive species of test microorganism were selected to determine the MIC exhibited by the essential oil. Cytotoxicity assay of the essential oil against normal cell line was conducted using Vero cell line. The percentage of inhibition was calculated and expressed as IC_{50}. Essential oil exhibited variant antimicrobial activity against the tested strains. The MIC values of nutmeg oil ranged from 0.031 to 1 mg mL^{-1} and showed higher activity against *Shigella dysenteriae* with MIC value of 0.031 mg mL^{-1}. The nutmeg oil possessed low cytotoxicity against Vero cell line with IC_{50} at 24.83 μg mL^{-1}. Nutmeg essential oil exhibited strong antibacterial activity against the *Shigella dysenteriae* with low cytotoxic effects. Thus, the essential oil can be further studied on its action of mechanism and fractionation will be carried out to determine the strength of its antibacterial activity.

Key words: Nutmeg, antibacterial, cytotoxicity, Vero cell line, essential oil

INTRODUCTION

Since long time back, aromatic herbs and spices have been added to various foods as ingredients to improve the taste, flavor and organoleptic properties. Essential oils are secondary metabolites which derived from plants, herbs and spices are made up of volatile compounds comprising volatile constituents like lipids, terpenoids, ketones, phenols and oxygenated derivatives. They are characterized by a strong odor and are formed by aromatic plants (Bakkali *et al*., 2008).

The antimicrobial activities of plant oils and extracts have formed the foundation of many applications such as in raw and processed food preservation, pharmaceuticals, alternative medicine and natural therapies (Lis-Balchin and Deans, 1997).

Nutmeg (*Myristica fragrans* Houttuyn) is the seed kernel of inside the fruit and mace is the lacy covering (aril) on the kernel (Nadkarni, 1988) and cultivated in tropical regions and indigenous to the Maluku Province of Indonesia. It has been studied to having anthelmintic, hepatoprotective anti-inflammatory, aphrodisiac, insecticide properties and a treatment for rheumatism, diarrhea, asthma, atherosclerosis and flatulence (Burkill, 1966; Ozaki *et al*., 1989; Pooja *et al*., 2012). In addition to that nutmeg mace was found to exhibit strong antifungal and antibacterial activities (Singh *et al*., 2005). Eugenol, a component of nutmeg, is widely used in dentistry as root canal sealers reported to show antibacterial activity against oral bacteria (Lai *et al*., 2001).

To our knowledge, though extensive studies on nutmeg have been conducted but less research has been studied on the volatile mixtures of the plant species. Therefore, the aim of this study is to investigate the antibacterial activity and *in vitro* cytotoxicity of essential oil of nutmeg on normal cell line. The cytotoxic study is important to determine the safety of the essential oil for therapeutic application since few compounds especially myristicin, one of chemical compounds of nutmeg is reported to be toxic (Pooja *et al*., 2012).

MATERIALS AND METHODS

Plant material: The fresh fruits of nutmeg (*Myristica fragrans*) were obtained from Balik Pulau, Penang, Malaysia. Voucher specimens (11253) *Myristica fragrans*

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Studies on the Phytochemical Properties and Brine Shrimp Toxicity of Essential Oil Extracted from *Myristica fragrans* Houtt. (Nutmeg)

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Abstract: The essential oil of *Myristica fragrans* (nutmeg) was extracted by steam distillation. The obtained essential oil was analysed for phytochemical properties and cytotoxic activity. Flavonoids, terpenoids, anthraquinones, cardiac glycosides and steroids were identified to be present in the oil by phytochemical studies. LC_{50} of the essential oil was determined in the brine shrimp toxicity test to be 5434.78 ± 23.2 µg/ml, which is not cytotoxic (LC_{50} > 1000 µg/mL).

Key words: Essential oil, *Myristica fragrans*, phytochemical properties, brine shrimp toxicity test.

Introduction

Many natural compounds isolated from plants have shown a broad spectrum of biological activities. Among these various kinds of natural compounds, essential oils possess promising biological activities and have been of potential sources of natural products [1]. They have been studied for their benefits as alternative remedies for the treatment as well as preventive agents of many infectious diseases and are confirmed to have various pharmacological effects, such as spasmolytic, carminative, hepatoprotective, antiviral and anticarcinogenic effects [2,3].

The essential oils are extracted from aromatic and medicinal plants using several methods such as water or steam distillation, solvent extraction, expression under pressure, supercritical fluid and subcritical water extractions.

The increased findings in herbs and spices as sources of natural antioxidants have initiated researchers to look for natural antioxidants with low cytotoxicity [4].

Nutmeg (*Myristica fragrans* Houtt.), a medium sized tree cultivated in tropical regions [5] is the source of the high value medicinal spices, nutmeg (endosperm) and mace (the reddish aril) with enormous phytochemical diversity [6]. Recent reports on its antibacterial, antiviral, antidiabetic, antileukaemic effects and other biological activities [6,7,8] suggest its enormous therapeutic potential. The nutmeg also showed a potent hepatoprotective activity against liver damage caused by certain chemicals [9]. The protective activity was correlated with myristicin, a major constituent of nutmeg and recently it was found that myristicin induces cytotoxicity in human neuroblastoma SK-N-SH cells by an apoptotic mechanism [10].

To the best of the author's knowledge, although a detailed investigation of nutmeg species was

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Chemical composition, anti-angiogenic and cytotoxicity activities of the essential oils of *Cymbopogan citratus* (lemon grass) against colorectal and breast carcinoma cell lines

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The essential oil of *Cymbopogan citratus* (lemon grass) was isolated by steam distillation method and subjected to cytotoxicity activity using two different cell lines, human colon carcinoma (HCT-116), breast carcinoma cell lines (MCF-7) and anti-angiogenic activity. The cytotoxicity activity study was determined using the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2,4-tetrazolium bromide] assay and anti-angiogenic activity using rat aortic ring model. The chemical composition of the essential oil was determined by gas chromatography–mass spectrometry (GC–MS) and GC–flame ionization detection (FID). Forty-one compounds, representing 88.5% of the lemon grass oil was identified and the main components were neral (29.8%) and geranial (44.6%). The free radical scavenging activity of the essential oil showed an IC_{50} value of 156.1 μg/mL. The results showed that essential oil of lemon grass possessed potential cytotoxic effect on HCT-116 and with the IC_{50} value of 27.41 ± 4.3 μg/mL comparing to MCF-7 with IC_{50} value of 41.90 ± 1.2 μg/mL. Significant anti-angiogenic activity of the sample was observed with 99 ± 0.8% of inhibition at 100 μg/mL.

Keywords: angiogenesis; *Cymbopogan citratus*; cytotoxicity; essential oil; essential oil composition

Introduction

Recently, essential oils have gained popularity and scientific interest. Essential oils are natural, complex, multi-component systems composed mostly of terpenes with some other non-terpene components (1). The essential oils are demonstrated to show various pharmacological effects, such as spasmolytic, carminative, hepatoprotective, antiviral and anticarcinogenic effects (2, 3). *Cymbopogon citratus* (lemon grass) is a widely used herb, particularly in southeast Asia and Brazil, where it is used as a food flavoring, as a perfume, and has been found to possess medicinal properties (4) and contain 1 to 2% essential oil on a dry basis (5). Studies have shown that lemon grass has antidepressant, antioxidant, antiseptic, astringent, bactericidal, fungicidal, nervine and sedative properties (6) and the lemon grass oil is reported to have significant anticancer activity and leads to loss of tumor cell viability (7, 8). The anticancer activity of lemon grass oil was demonstrated by induction of Glutathione S-Transferase (GST). GST has been suggested to increase the host's ability to detoxify xenobiotics, including carcinogens.

Angiogenesis is a process of sprouting of new blood vessels from pre-existing vessels which is an essential feature of tissue remodeling associated with wound healing, solid tumor development, proliferative retinopathies, atherosclerosis and rheumatoid arthritis (9). Furthermore, angiogenesis is important for growth and metastasis of most solid malignancies and vascular endothelial growth factor-A (VEGF-A) which is assumed to be essential for tumor angiogenesis (10). Nicosia and Ottinetti (11) demonstrated that rat aorta rings reproducibly make micro vessel outgrowths in fibrin or collagen gels and supply a sensitive assay for the study of angiogenic agonists and antagonists in a chemically defined environment. In addition to that, tumor angiogenesis is considered to be a vital pharmacological target for cancer prevention and treatment (12–14).

Early reports also have indicated that essential oil components in lemon grass, especially monoterpenes, have multiple pharmacological effects on mevalonate metabolism which could account for the terpene–tumor suppressive activity (15). Monoterpenes have been shown to exert chemopreventive as well as chemotherapeutic activities in mammary tumor models and thus may represent a new class of therapeutic agents (16).

The aim of this study was to determine the cytotoxicity affect of essential oil extracted from locally grown lemon grass (*Cymbopogan citratus*) on colorectal (HCT-116) and breast (MCF-7) carcinoma cell lines using MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2,4-tetrazolium bromide] assay and angiogenic activity on rat aorta ring.

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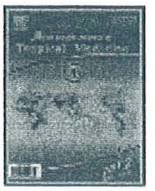

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Antioxidant and antiangiogenic activities of the essential oils of *Myristica fragrans* and *Morinda citrifolia*

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ABSTRACT

Objective: Toinvestigate the anti-angiogenic activity and antioxidant properties of *Myristica fragrans (M. fragrans)* (nutmeg) and *Morinda citrifolia* (*M. citrifolia*)(mengkudu) oils. **Methods:** The nutmeg and megkudu essential oils were obtained by steam distillation. The antioxidant activities of both essential oils were determined by beta-carotene/linoleic acid bleaching assay and reducing power while the anti-angiogenic activity was investigated using rat aortic ring assay using various concentrations. **Results:** The results showed that nutmeg oil has higher antioxidant activity than mengkudu oil. The nutmeg oil effectively inhibited the oxidation of linoleic acid with (88.68±0.1)% while the inhibition percentage of oxidation of linoleic acid of the mengkudu oil is (69.44±0.4)%. The nutmeg oil and mengkudu oil showed reducing power with an EC_{50} value of 181.4 μg/mL and 3 043.0 μg/mL, respectively. The antiangiogenic activity of nutmeg oil showed significant antiangiogenic activity with IC_{50} of 77.64 μg/mL comparing to mengkudu oil which exhibits IC_{50} of 109.30 μg/mL. **Conclusion:** Bioactive compound(s) will be isolated from the nutmeg essential oil to be developed as antiangiogenic drugs.

1. Introduction

Essential oils are volatile aromatic oily liquids which are identified by a strong smell and are produced by aromatic plants as secondary metabolites. Essential oil can be isolated using a number of isolation methods. The aromatic plants and spices are commonly used in phytotherapy and mostly related to various activities of their essential oils, such as antimicrobial, antioxidant, antifungal spasmolytic, carminative, hepatoprotective, antiviral and anticarcinogenic activities[1].

The increased discoveries in herbs and spices as sources of natural antioxidants have initiated researchers to look for natural antioxidants with low cytotoxicity[2]. The free radicals which cause oxidative stress are believed to play an essential role in human health. They are produced in body system in the form of reactive oxygen species (ROS) which can be obtained from the environment. These free radicals cause harm to the components of the cell resulting in cellular and metabolic disruption such as cardiovascular diseases, inflammation and cancer[3].

Nutmeg is the seed of *Myristica fragrans (M. fragrans)* Houttuyn, a medium-sized tree cultivated in tropical regions and native to the Maluku Province of Indonesia. The essential oil of nutmeg was able to block lipid peroxidation in chicken tissue homogenates and egg yolk fat and showed hepatoprotective activity against liver damage[4]. Pepper and nutmeg are one of the most widely used spices in preparation of ayurvedic drugs[5]. Nutmeg is a widely used spice and flavouring ingredient in food products, with possible health beneficial effects, such as anti-inflammatory and antimicrobial activities[6,7].

Morinda citrifolia (*M. citrifolia*) L. (Rubiaceae), known as "Noni" or Indian mulberry, "mengkudu" in Malaysia,

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Abstract

BACKGROUND: In this study the chemical composition, antioxidant activities and cytotoxic effect of the essential oils of *Myristica fragrans* (nutmeg) and *Morinda citrifolia* (mengkudu) were determined.

RESULTS: Thirty-eight compounds in nutmeg oil and six compounds in mengkudu oil were identified by gas chromatography–mass spectrometry. The free radical scavenging activity of nutmeg oil was superior of that mengkudu oil. The MTT assay of nutmeg oil on human colorectal carcinoma (HCT-116) and human breast carcinoma (MCF-7) cell lines showed IC_{50} values of 78.61 and 66.45 μg mL^{-1}, respectively. The mengkudu oil exhibited IC_{50} values of 91.46 and 78.15 μg mL^{-1} for HCT-116 and MCF-7, respectively.

CONCLUSION: The results showed that nutmeg oil can be developed as potent anti-cancer and antioxidant drugs.
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Keywords: essential oil; *Myristica fragrans*; *Morinda citrifolia*; antioxidant activity; cytotoxic activity

INTRODUCTION

For centuries, herbal and natural products have been used for various purposes.[1] In recent decades, plants yielding essential oils and other extracts have gained attention and scientific interest as sources of natural products. Essential oils are claimed to have antibacterial, antifungal and antioxidant properties and have potential for food preservation.[2,3]

The ever increasing findings concerning herbs and spices as sources of natural antioxidants have stimulated researchers to look for natural antioxidants with low cytotoxicity.[4] Free radicals, which cause oxidative stress, are believed to play a crucial role in human health. These free radicals cause damage to the components of the cell, resulting in cellular and metabolic injury such as cardiovascular diseases, inflammation and cancer.

Nutmeg is the seed of *Myristica fragrans* Houttuyn, a medium-sized tree cultivated in tropical regions and indigenous to the Maluku Province of Indonesia.[5] The essential oil of nutmeg was found to inhibit lipid peroxidation in chicken tissue homogenates and egg yolk fat and showed hepatoprotective activity against liver damage.[6]

Morinda citrifolia L. (Rubiaceae), known as 'Noni' or Indian mulberry, 'mengkudu' in Malaysia, 'painkiller bush' in the Caribbean or 'cheese fruit' in Australia, is a shrub that grows in tropical Asia and Polynesia.[7] *Morinda citrifolia* has been used traditionally for a broad range of therapeutic uses such as antimicrobial, anti-cancer, anti-inflammatory and antioxidant agents for decades.[8]

To the best of the authors' knowledge, studies on the volatile oil mengkudu species have been little reported elsewhere. Therefore the present work aimed to determine the chemical profile of mengkudu, as well as nutmeg oils, using gas chromatography–mass spectrometry (GC–MS), to evaluate their antioxidant properties by 2,2-diphenyl-2-picrylhydrazyl (DPPH). A cytotoxicity assay was carried out. This assay was a novel biological screening method for both oils.

MATERIALS AND METHODS

Plant material

Fresh fruits of *Myristica fragrans* and *Morinda citrifolia* were obtained from Balik Pulau, Penang, Malaysia. Voucher specimens (11 032) *Myristica fragrans* and (11 049) *Morinda citrifolia* were authenticated and deposited at the Herbarium Unit of the School of Biological Sciences, Universiti Sains Malaysia.

Extraction of essential oil

The collected fresh fruits of nutmeg and mengkudu were submitted to water distillation for 3 h using a modified Clevenger's

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