BHARATHIDASAN UNIVERSITY, TIRUCHIRAPPALLI – 620 024
Phone No.: 0431-2407092, Fax : 0431-2407045, Email: reg@bdu.ac.in
Website : www.bdu.ac.in
(Accredited with "A" Grade by NAAC in the Third Cycle)

Dr. G.GOPINATH
REGISTRAR i/c

Ref.No. 20566/P4/2021 dated 05.04.2021

To

The Secretary
University Grants Commission
Bahadurshah Zafar Marg
New Delhi – 110 002.

Sir,

Sub: Dr. G. Mathan, Associate Professor, Department of Bio-Medical Science, Bharathidasan University, Trichy-24 – Project Completion Report & Unspent amount refunded – reg.

Ref: 1. UGC Sanction Lr.No.F.41-1289/2012/(SR), dt.26.07.2012.
2. Your Mail dated: 25.03.2021

I would like to inform you that the unspent balance amount of Rs.6,382/- from the project entitled "Studies...Cells" was refunded to the funding agency through NEFT, vide UTR No.SBIN717321998408 on 17.11.2017. Now, I am forwarding herewith the Project Completion Report for the period from 01.07.2012 to 30.11.2015 submitted by Dr. G. Mathan, PI of the UGC Project "Studies...Cells", Department of Bio-Medical Science, Bharathidasan University, Trichy-24 for your kind consideration.

Yours faithfully,

REGISTRAR (i/c)

Encl.: As above

Copy to:

Dr. G. Mathan, Associate Professor, Department of Bio-Medical Science,
Bharathidasan University, Trichy-24.

BHARATHIDASAN UNIVERSITY, TIRUCHIRAPPALLI – 620 024

Phone No.: 0431-2407092, Fax : 0431-2407045, Email: office@bdu.ac.in
Website : www.bdu.ac.in,**(Re-Accredited with "A" Grade by NAAC)**

Dr. R. Babu Rajendran,
REGISTRAR i/c

Ref.No. 20566/E4/2011 dt. 23.01.2017.

To

The Secretary
University Grants Commission
Bahadurshah Zafar Marg
New Delhi – 110 002.

Sir

Sub: UGC Project "Studies ..... Cells" – Forwarding of Statement of Expenditure, Utilisation Certificate and Audit Certificate – Reg.

Ref: UGC Sanction Lr.No.F.41-1289/2012(SR), dt. 26.07.2012.

****

With reference to the above, I am forwarding herewith Statement of Expenditure, Utilisation Certificate and Audit Certificate for the period from 01.07.2012 to 30.11.2015, in respect of UGC Project "Studies ..... Cells" as received from Dr. G. Mathan, Assistant Professor, Department of Bio-Medical Science , Bharathidasan University, Tiruchirappalli – 620 024, for your kind consideration.

Yours faithfully,

REGISTRAR

Copy to:

Dr. G. Mathan, Assistant Professor, Department of Bio-Medical Science, Bharathidasan University, Tiruchirappalli – 620 024.

UNIVERSITY GRANTS COMMISSION

University : Bharathidasan University
Sanction Letter No : **41-1289/2012 (SR)**
Date : **26.07.2012**
Statement of Actual Expenditure during 01.07.2012 to 30.11.2015.
And Estimated Expenditure for 01.07.2012 to 30.11.2015.

Consolidated Statement of Expenditure incurred during 01.07.2012 to 30.11.2015 in the Department of Biomedical Science, Bharathidasan University under **UGC Major Research Project**

Item(s) of Expenditure	Total Grant Approved by the UGC in Rs.	Grant Released		Total expenditure Incurred in Rs	Unspent balance amount in Rs
		1st Installment	2nd installment		
A. Non- recurring 1. Books & Journals	-Nil-	-Nil-	-Nil-	-Nil-	-Nil-
2. Equipments	4,82,000	4,82,000	-Nil-	4,82,000	-Nil-
B. Recurring 1. Contingency	30,000	15,000	12,000	27,000	-Nil-
2.Chemicals/ Consumables/ Glassware's	2,00,000	1,00,000	80,000	1,79,618	382
3. Project Fellow	-Nil-	-Nil-	-Nil-	-Nil-	-Nil-
4. Overhead charges	23,000	23,000	-Nil-	23,000	-Nil-
5.Travel/Facilities	15,000	7,500	6,000	7,500	6,000
6. Honorarium to Principal Investigator	-Nil-	-Nil-	-Nil-	-Nil-	-Nil-
7.Special need	Nil-	-Nil-	-Nil-	-Nil-	-Nil-
8. Hiring Services	-Nil-	-Nil-	-Nil-	-Nil-	-Nil-
Total (A+B)	**7,50,000**	**6,27,500**	**98,000**	**7,19,118**	**6,382**

1. Total Amount Received as on 07th October, 2014 : Rs.7,25,500/-
2. Total Expenditure on 30th November, 2015 : Rs.7,19,118/-
3. Unspent Amount Balance : Rs.6,382/-

CERTIFICATE

1. Certified that the grant has been utilized for the purpose for which it was sanctioned and in accordance with the terms and conditions attached to the grant.
2. If as a result of check or audit objection, some irregularity is noticed at a later stage, action will be taken to refund, adjust or regularize the objected amount.

Principal Investigator **Finance Officer** **Registrar** **Statutory Auditor**

Dr.G.MATHAN
PRINCIPAL INVESTIGATOR
UGC MAJOR RESEARCH PROJECT
DEPARTMENT OF BIOMEDICAL SCIENCE
BHARATHIDASAN UNIVERSITY
TIRUCHIRAPPALLI-620 024

Finance Officer,
BHARATHIDASAN UNIVERSITY,
TIRUCHIRAPPALLI - 620 024

REGISTRAR
BHARATHIDASAN UNIVERSITY
TIRUCHIRAPPALLI 620 024

Assistant Director of
Local Fund Audit
Audit Unit
Bharathidasan University
Trichirappalli-620 024

Office of Assistant Director,
Local Fund Audit,
Bharathidasan University,
Tiruchirappalli-24

AUDIT CERTIFICATE

As per the statements of expenditure enclosed and an examination of records and registers, it is ascertained in audit that a sum of Rs.7,50,000/- (Rupees Seven Lakh Fifty Thousand only) has been sanctioned as grant in aid by the UGC for conducting the project entitled "STUDIES ON INTERVENE EFFECTS OF ANTICANCER, BUTRIN/ISOBUTRIN, ON CELL CYCLE REGULATION IN HT-29 HUMAN COLON CANCER CELLS." implemented by Dr.G.Mathan, Assistant Professor, Department of Bio-Medical Science, Bharathidasan University, Trichy-24. Out of the sanctioned grant of Rs.7,50,000/- (Rupees Seven Lakh Fifty Thousand only) a sum of Rs.7,25,500 /- (Rupees Seven Lakh Twenty Five Thousand and Five Hundred only) received as grant vide UGC Reference F.No. 41-1289/2012(SR), Dated:26.07.2012 and 25.08.2014.

It is further certified that on an examination of relevant records it is ascertained in audit that out of the total grant of Rs.7,50,000/- (Rupees Seven Lakh Fifty Thousand only) received a total sum of Rs.7,25,500/- (Rupees Seven Lakh Twenty Five Thousand and Five Hundred only) have been incurred as expenditure for conducting the project during the period from 01.07.2012 to 30.11.2015 as detailed in the final statements of expenditure enclosed.

The above Audit certificate is issued subject to the conditions that

On a cross verification of the expenditure incurred, an amount of Rs.6,382/- (Rupees Six Thousand Three Hundred and Eighty Two only) is kept as unspent balance which has to be refunded to the Funding Agency and the same may be pointed out to audit.

6/1/17

Assistant Director(i/c),
Local Fund Audit,
Bharathidasan University,
Trichy-24

6/1/17

UNIVERSITY GRANTS COMMISSION
BAHADUR SHAH ZAFAR MARG
NEW DELHI – 110 002

Utilization certificate

It is certified that the amount of **Rs.7,19,118/-** (Seven Lakhs Nineteen Thousand One Hundred Eighteen rupees only) has been utilized as on **30.11.2015** from the first and second installment of **Rs. 7,25,500/-** (Seven Lakhs Twenty Five Thousand and Five Hundred only) received from UGC out of the total grant of **Rs.7,50,000/-** sanctioned to the Department of Biomedical Science, Bharathidasan University, Tiruchirappalli - 24 under the scheme of support for Major Research Project entitled **"Studies on intervene effects of anticancer, butrin/Isobutrin, on cell cycle regulation in HT-29 human colon cancer cells."** vide UGC letter No. **F. 41-1289/2012(SR)** dated: **26.07.2012.** It has been fully utilized for the purpose for which it was sanctioned in accordance with the terms and conditions laid down by the University Grants Commission and the unspent balance amount is **Rs. 6,382/-**.

If as a result of check or audit objection some irregularities are noticed at a later stage, action will be taken to refund, adjust or regularize the objected amount.

SIGNATURE OF THE PRINCIPAL INVESTIGATAR
(Seal)
Dr. S. MATHAN
PRINCIPAL INVESTIGATOR
UGC MAJOR RESEARCH PROJECT
DEPARTMENT OF BIOMEDICAL SCIENCE
BHARATHIDASAN UNIVERSITY
TIRUCHIRAPPALLI - 620 024.

6/1/17

STATUTORY AUDITOR
(Seal)
Assistant Director of
Local Fund Audit
Audit Unit
Bharathidasan University
Trichirappalli-620 024

SIGNATURE OF THE FINANCE OFFICER
(Seal)
Finance Officer,
BHARATHIDASAN UNIVERSITY,
TIRUCHIRAPPALLI - 620 024.

SIGNATURE OF THE REGISTRAR
(Seal)
6/6/16
REGISTRAR
BHARATHIDASAN UNIVERSITY
TIRUCHIRAPPALLI 620 024

Annexure-VIII

UNIVERSITY GRANTS COMMISSION
BAHADUR SHAH ZAFAR MARG
NEW DELHI – 110 002

Final report (01.07.2012 to 30.11.2015) of the work done on the Major Research Project entitled"Studies on intervene effects of anti-cancer compounds, Butrin/Isobutrin isolated from Butea monosperma on cell cycle regulation in HT-29, human colon cancer cells"

1. Project report No. 1st /2nd /3rd/Final : **Final**
2. UGC Reference No : **F.No. 41-1289/2012 (SR) Dated. 26.07.2012**
F.No.41-1289/2012 (SR) Dated. 07/10/2014

3. Period of report : **1st July 2012 to 30th November 2015**

4. Title of research project : **Studies on intervene effects of anti-cancer compounds, Butrin/Isobutrin isolated from Butea monosperma on cell cycle regulation in HT-29, human colon cancer cells**

5. (a) Name of the PrincipalInvestigator : **Dr.G.Mathan**
(b) Department : **Biomedical Science**
(c) University/College where work has progressed : **Bharathidasan University Tiruchirappalli, TamilNadu**

6. Effective date of starting of the project: **01.07.2012**

7 Grant approved and expenditure incurred during the period of the report
a. Total amount approved Rs : **7,50,000/-**
b. Total Grant Released Rs : **7,25,500/-**
(1st Installment: Rs. 6,27,500 + 2nd Installment Rs. 98,000)
c. Total expenditure Incurred Rs : **7,19,118/-**
d. Unspent Balance Rs. : **6,382/-**
e. Report of the work done : **See Annexure-I**

i. Brief objective of the project

- To assess the biological activity of *Butea monosperma* floral isolates, Butrin and Isobutrin, effect in cell growth and death in HT-29 human colon cancer cells in time and dose dependent manner

- To investigate, the possible inhibitory effect of Butrin / Isobutrin on cell cycle regulation in HT-29 human colon cancer cells by flow cytometry analysis

- To measure the active compound, Butrin/Isobutrin, impact on Cyclins A,B,D and E), Cyclin dependent kinases (CDK1, 2, 4 and 6) and CDK inhibitory proteins (p16 Ink4, p21 Waf1 and p27 Kip1) expression in cell cycle regulatory mechanism of HT-29 human colon cancer cells based on the flow cytometry analysis

.

ii. Work done so far and results achieved and publications

1.Boopathi Subramaniyan, Navaneethakrishnan Polachi and **Ganeshan Mathan**. Isocoreopsin: An active constituent of n-butanol extract of Butea monosperma flowers against colorectal cancer (CRC). *Journal of Pharmaceutical Analysis,* **2016**, Vol 6 (5), 318-325. **(Impact Factor: 2.673)**

2. Navaneethakrishnan polachi, Boopathi Subramaniyan,Prashantha Nagaraja, Kannan Rangiah and **Mathan Ganeshan**, Extract from Butea monosperma inhibits β-catenin/Tcf signaling inSW480 human colon cancer cells. *Gene Reports,* 10, 79-89 **(2018)**

iii. Has the progress been according to original plan of work and towards achieving the objective. if not, state reasons

The progress of the project has been achieved towards the objectives and additional Colon cancer cell lines, SW480 cells, used for validation of biomarker, β-catenin

iv. Has the progress been according to original plan of work and towards achievingthe objective. if not, state reasons

The progress of the project has been achieved towards objectives

v. Please indicate the difficulties, if any, experienced in implementing the project

-Nil-

V. If project has not been completed, please indicate the approximate time by whichit is likely to be completed. A summary of the work done for the period (Annual basis)may please be sent to the Commission on a separate sheet.

Project work is complete

Vi. If the project has been completed, please enclose a summary of the findings ofthe study. One bound copy of the final report of work done may also be sent toUniversity Grants Commission.

See Annexure I & II

vii Any other information which would help in evaluation of work done on the project.

At the completion of the project, the first report should indicate the output, such as (a)Manpower trained (b) Ph. D. awarded (c) Publication of results (d) other impact, if any

(a) **Manpower trained : 1**

(b) **Ph. D. awarded : 1 (Mr.S.Boopathi)**

(c) **Publication of results : 2**

(d) **Other impact, if any : Nil**

Signature of the Principal Investigator

Dr.G.MATHAN
PRINCIPAL INVESTIGATOR
UGC MAJOR RESEARCH PROJECT
DEPARTMENT OF BIOMEDICAL SCIENCE
BHARATHIDASAN UNIVERSITY
TIRUCHIRAPPALLI - 620 024.

Registrar/Principal

(Seal)

REGISTRAR
BHARATHIDASAN UNIVERSITY
Tiruchirappalli - 620 024

Annexure-IX

UNIVERSITY GRANTS COMMISSION
BAHADUR SHAH ZAFAR MARG
NEW DELHI – 110 002

PROFORMA FOR SUBMISSION OF INFORMATION AT THE TIME OF SENDING THE FINAL REPORT OF THE WORK DONE ON THE PROJECT

1. Title of the Project	: **Studies on intervene effects of anti-cancer Compounds, Butrin/Isobutrin isolated from Butea monosperma on cell cycle regulation in HT-29, human colon cancer cells**
2. Name and address of the Principal Investigator	: **Dr. G.Mathan** **Assistant Professor** **Department of Biomedical Science**
3. Name and address of the Institution	: **Bharathidasan University** **Tiruchirappalli-620024** **TamilNadu**
4. UGC Reference No	: F.No. 41-1289/2012 (SR) Dated.26.07.2012 F.No.41-1289/2012 (SR) Dated. 07/10/2014
5.Date of Implementation	: **01.07.2012**
6.Tenure of the project	: 3 **years (01.07.2012 to 30.11.2015)**
7.Total grant allocated	: **Rs. 7,50,000/-**
8.Total grant received	: **Rs. 7,25,500/-**
9.Final expenditure	: **Rs. 7,19,118/-**
10. Title of the project	: **Studies on intervene effects of anti-cancer Compounds, Butrin/Isobutrin isolated from Butea monosperma on cell cycle regulation in HT-29, human colon cancer cells**

11. **Objectives of the project** :

- To assess the biological activity of Butea monosperma floral isolates, Butrin and Isobutrin, effect in cell growth and death in HT-29 human colon cancer cells in time and dose dependent manner

- To investigate, the possible inhibitory effect of Butrin / Isobutrin on cell cycle regulation in HT-29 human colon cancer cells by flow cytometry analysis

- To measure the active compound, Butrin/Isobutrin, impact on Cyclins A,B,D and E), Cyclin dependent kinases (CDK1, 2, 4 and 6) and CDK inhibitory proteins (p16 Ink4, p21 Waf1 and p27 Kip1) expression in cell cycle regulatory mechanism of HT-29 human colon cancer cells based on the flow cytometry analysis

12. Whether objectives were achieved: **Yes**

13. **Achievements from the project :**

1.Boopathi Subramaniyan, Navaneethakrishnan Polachi and **Ganeshan Mathan**. Isocoreopsin: An active constituent of n-butanol extract of Butea monosperma flowers against colorectal cancer (CRC). *Journal of Pharmaceutical Analysis,* **2016**, Vol 6 (5), 318-325. **(Impact Factor: 2.673)**

2. Navaneethakrishnan polachi, Boopathi Subramaniyan,Prashantha Nagaraja, Kannan Rangiah and **Mathan Ganeshan**, Extract from Butea monosperma inhibits β-catenin/Tcf signaling inSW480 human colon cancer cells. *Gene Reports,* 10, 79-89 **(2018)**

14. Summary of the findings : **See annexure –II**

15. Contribution to the society : **See annexure –III**

16. Whether any Ph.D. enrolled/
Produced out of the project : 1 **(Mr.S.Boopathi)**

17. No. of publications out of the Project 2

Signature of the Principal Investigator

Dr.G.MATHAN
PRINCIPAL INVESTIGATOR
UGC MAJOR RESEARCH PROJECT
DEPARTMENT OF BIOMEDICAL SCIENCE
BHARATHIDASAN UNIVERSITY
TIRUCHIRAPPALLI - 620 024.

Registrar/Principal

REGISTRAR i/c
BHARATHIDASAN UNIVERSITY
Tiruchirappalli - 620 024

Introduction:

The colorectal cancer (CRC) is diagnosed as third most common form of cancer in men (6,63,000 cases, 10% of the total cancers) and second in women (5,70,000 cases, 9.4% of the total cancers) (Jemal et al., 2011). Aberrant activation of Wnt/β-catenin signaling pathway is a hallmark of the majority of CRCs, results in increased stability of protein β-catenin (Barker and Clevers, 2006). Continuous activation of Wnt/β-catenin signaling is believing to be an initiating event in colorectal carcinogenesis (Fodde et al., 2001; Giles et al., 2003).

Recently, Herbal medicines and their derived phytocompounds are being raised as a complementary treatment for cancer. In this series, Butea monosperma Lam. Kuntze (Fabaceae), commonly known as flame of the forest, have been illustrated as a valuable traditional medicinal plant with> 45 medicinal attributes. Among its different parts, flower is the one which is associated with several pharmaceutical effects (Burlia and Khadeb, 2007; Choedon et al., 2010). Phytochemical analysis of B. monosperma flowers constituents showed the presence of flavonoid glucosides such as butrin, isobutrin, butein, butin, sulfurein, coreopsin, isocoreopsin, monospermoside and isomonospermoside (Wagner et al., 1986; Gupta et al., 1970). Ethanol extract of B. monosperma flowers have been shown as anti-hyperglycemic, anti-diabetic and anti-fertility activity (Somani et al., 2006; Razdan et al., 1970). The Same extract has also possess hepatoprotective and anti-tumorigenic effect (Sehrawat and Sultana, 2006; Mathan et al., 2011). Whereas, the petroleum ether extract of B. monosperma flowers has been showed to exhibit anticonvulsive activity, due to the presence of triterpenes (Kasture et al., 2002). These constructive effects were attributed to several class of compounds identified in B. monosperma flowers extracts. The preliminary reports of B. monosperma flower extract had showed the cytotoxic effect against human colon cancer cells. So far there are no reports of this extract against wnt/β-catenin signaling mediated CRC on SW480 cells. Based on these reports, this present study was aimed to assess the n-butanol fraction of B. monosperma floral extracts (NBF-BMFE) inhibitory effects on Wnt/β-catenin signaling proteins against CRC in SW480 cells.

Research Findings:

The Butea monosperma flowers were collected from the Bharathidasan University campus (Fig.1). The collected flowers were dried at room temperature and powdered. The dried flowers were subjected for soxhlet extraction with methanol and then the compound was

concentrated by rotator evaporator. Methanol extract further partitioned between water and ethyl acetate for 3 times. After removal of the ethyl acetate fraction, the remaining water phase was treated with n-butanol. The ethyl acetate fraction concentrated into powder by rotator evaporator.

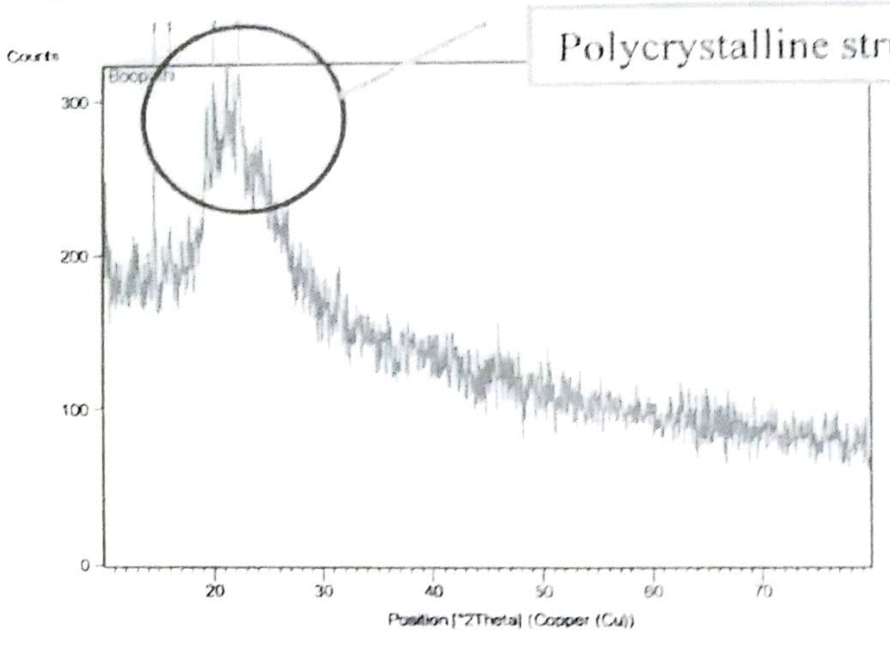
Fig. 1. *Butea monosperma*

The powered compound was characterized by solubility of different solvent (Table: 1). besides, the complete solubility achieved in ethyl acetate, methanol composition and its solubility confirmed by TLC with suitable solvents. The TLC result shows 3 distinguished spot; moreover, the second spot has polycrystalline structure, which was confirmed by powder XRD result (Fig: 2). Based on the spot, I have worked on isolation of single compound from ethyl acetate powder through the column chromatography by using ethyl acetate and methanol at different ratio. Further the collected single fraction was confirmed by TLC.

Table: 1, Solubility test results:

S.No	Solvent system I	Solvent system II	TLC result
1	Ethyl acetate	Hexane	No spot
2	Chloroform	Methanol	UV active spot only
3	Acetone	Hexane	No Spot
4	Dichloromethane	Methanol	2 spot
5	Dichloromethane	Ethanol	3 spot (2 yellow spot and 1 UV spot)
6	Ethyl acetate	Methanol	3 spot (2 yellow spot and 1 UV spot)

Fig: 2 Powder XRD results of second fraction



2Theta:0.001; Minimum step size Omega: 0.001
Sample stage=PW3071/xx Bracket
Diffractometer system=XPERT-PRO
Measurement program=Thin Film Samples - Gonio scan - 2 mi

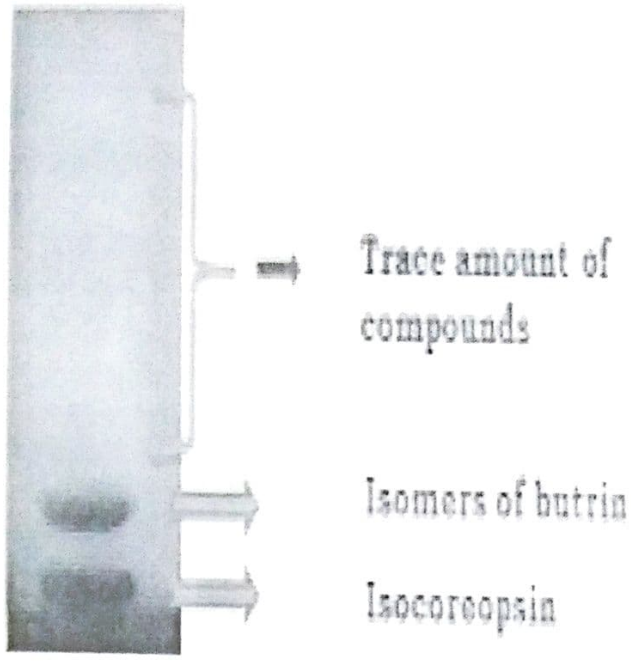
Start Position [°2Th.]	10.0381
End Position [°2Th.]	79.9381
Scan Type	Continuous
Divergence Slit Size [°]	0.4785
Specimen Length [mm]	10.00
Measurement Temperature [°C]	25.00
Anode Material	Cu

Peak List: (Bookmark3)

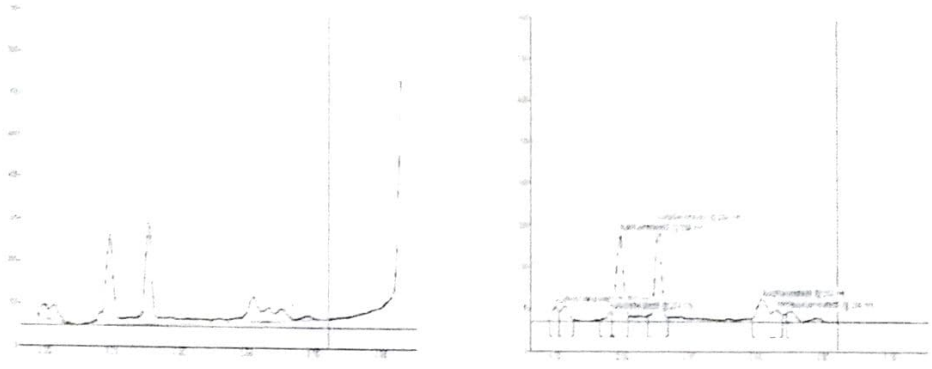
Pos. [°2Th.]	Height [cts]	FWHM Left [°2Th.]		
14.65(2)	48(25)	0.21(5)	6.04189	100.00
16.1(1)	30(183)	0.4(4)	5.51233	62.98
20.10(3)	35(31)	0.22(8)	4.41347	73.77
22.4(1)	43(297)	1(4)	3.96771	88.83

Preparative TLC

The preparation of methanolic extract from *B. monosperma* flowers were done by using Soxhlet apparatus. From this extract, the three individual fractions were collected namely water, ethyl acetate and n-butanol. Among these fractions, the n-butanol fraction showed high solubility, consequently this fraction is used for further studies. Further, the NBF-BMFE was screened by HPTLC to identify the active compounds. As a result, the results showed the presence of isocoreopsin (R_f : 0.20) and isomers of butrin and isobutrin (R_f : 0.32) were majorly found and other compounds were existed in small amounts (Fig. 3.a). The chromatogram results showed the maximum Rf values of 0.19, 0.31, 0.62, 0.68 and 0.70 with the solvent system of ethyl acetate/formic acid/acetic acid/water (100:11:11:26) (Fig. 23.b). The previous results reported the R_f values were 0.20, 0.32, 0.39, 0.45, 0.69, 0.79, 0.90 and 0.96 (Zafar *et al.*, 2010).



(a)



Peak	Start Rf	Start Height	Max Rf	Max Height	Max %	End Rf	End Height	Area	Area %	Assigned substance
1	0.00	1.4	0.00	37.4	6.52	0.01	23.6	804.9	4.82	AutoGenerated5
2	0.01	23.6	0.03	40.3	8.03	0.05	5.9	1142.1	8.22	AutoGenerated4
3	0.13	4.0	0.16	27.5	4.06	0.17	28.2	601.6	3.27	AutoGenerated9
4	0.17	28.4	0.19	213.4	31.47	0.22	14.0	5584.7	30.40	AutoGenerated2
5	0.28	13.4	0.31	238.8	35.21	0.34	12.8	5956.8	32.41	AutoGenerated1
6	0.50	12.8	0.62	57.8	8.52	0.65	22.1	2234.0	12.16	AutoGenerated3
7	0.65	22.3	0.66	32.2	4.75	0.68	19.3	1005.5	5.47	AutoGenerated8

(b)

Fig. 3. HPTLC chromatography results. (a) Screening of NBF-BMFE fractions by using pre-coated silica gel G60 F254 TLC plates (b) The chromatogram results showed the maximum R_f values of 0.19, 0.31, 0.62, 0.68 and 0.70

LC/MS Analysis

The LC-MS chromatogram is a liable tool, which offers soft ionization to analyze flavonoids. With this, the identification of NBF-BMFE yielded two peaks at the retention time of 6.9 min and 8.2 min with 94.7% purity (Fig. 4). As correlative with earlier report of molecular weights of butrin (596.17), isobutrin (596.17), butein (272.25), butin (272.25), coreopsin (434.39), isocoreposin (434.39), monospermoside (434.39) and isomonospermoside (434.39) compounds in NBF-BMFE (Gupta *et al.*, 1970; Zafar *et al.*, 2010), our LC-MS analysis also confirmed with the presence of above bioactive compounds (Fig. 5). Moreover, the identified compounds normally are isomers of butrin and isobutrin, not easy to distinguish through mass spectrum due to the complicity of fragment pattern, in this highly polygonal compounds m/z value is 597m/z. This chemical mixture of polygonal compounds were analysed by using Electron Spray Ionization- mass spectrum (ESI-MS), which converts the chemical structure into fragment ions from the original chemical backbone (Akiyo *et al.*, 1998; Ana *et al.*, 2009). The isomers of butrin has two unit of sugar moiety and it can easily undergo the fragmentation pattern, generally this compounds termed as flavonoids (Fig. 6.a). Finally, we obtained the isomeric butrin and isobutrin parent ion at m/z 597 and fragment ions were at m/z 434 and m/z 272 with corresponding to the loss of one and two glucopyranose units (Fig. 6.b). This results implies that the NBF-BMFE majorly contains isomers of butrin.

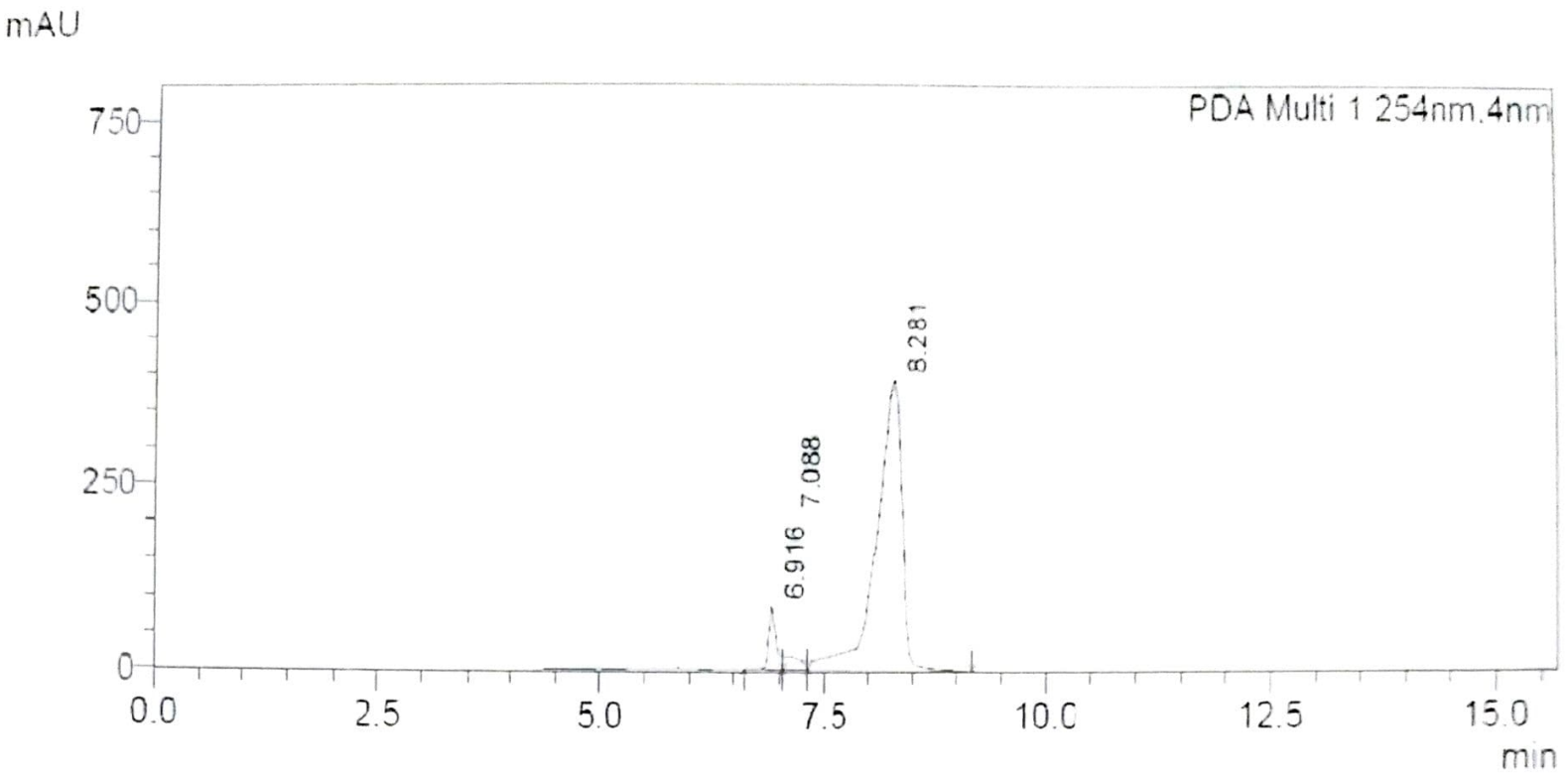


Fig. 4. HPLC chromatogram of NBF-BMFE

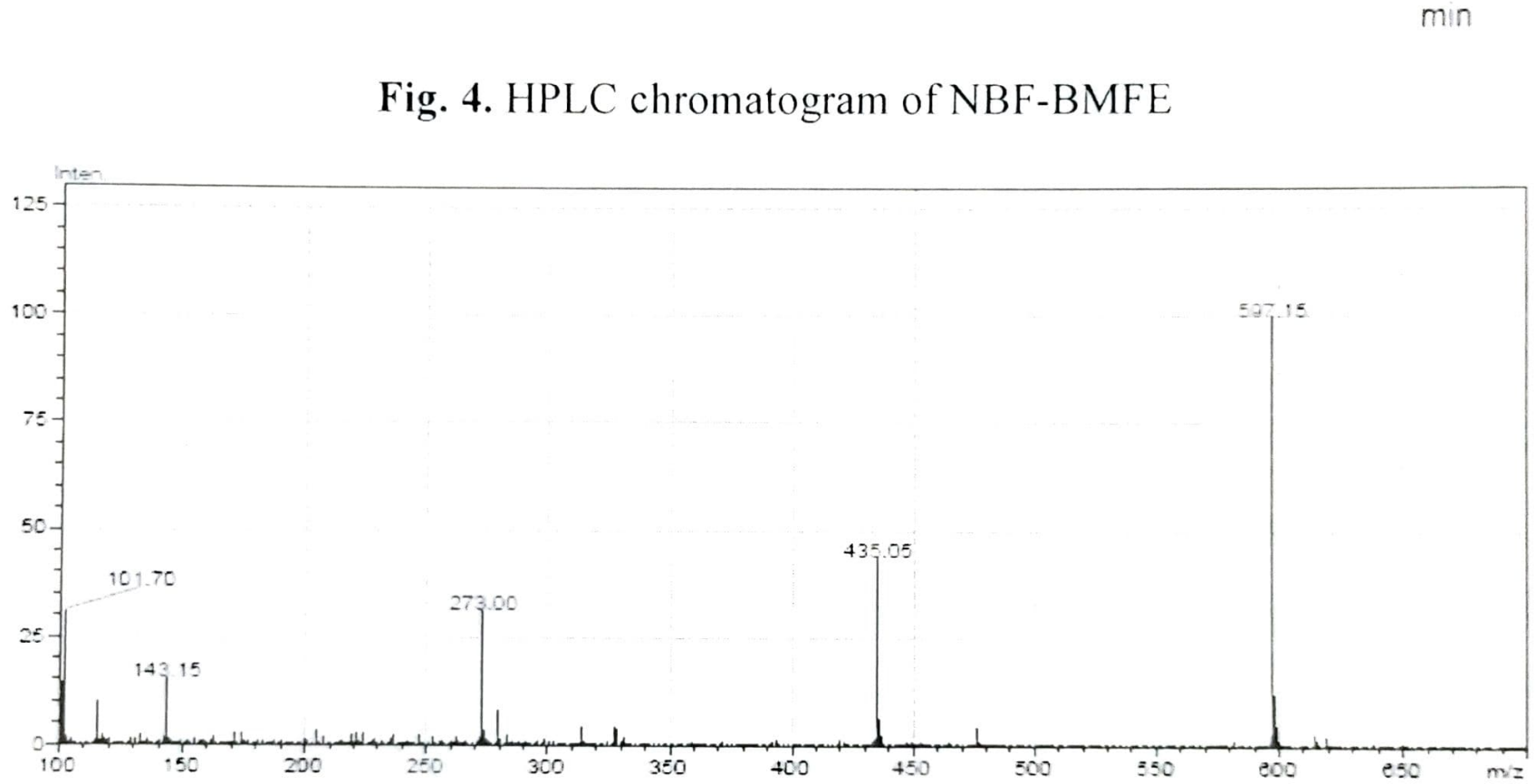
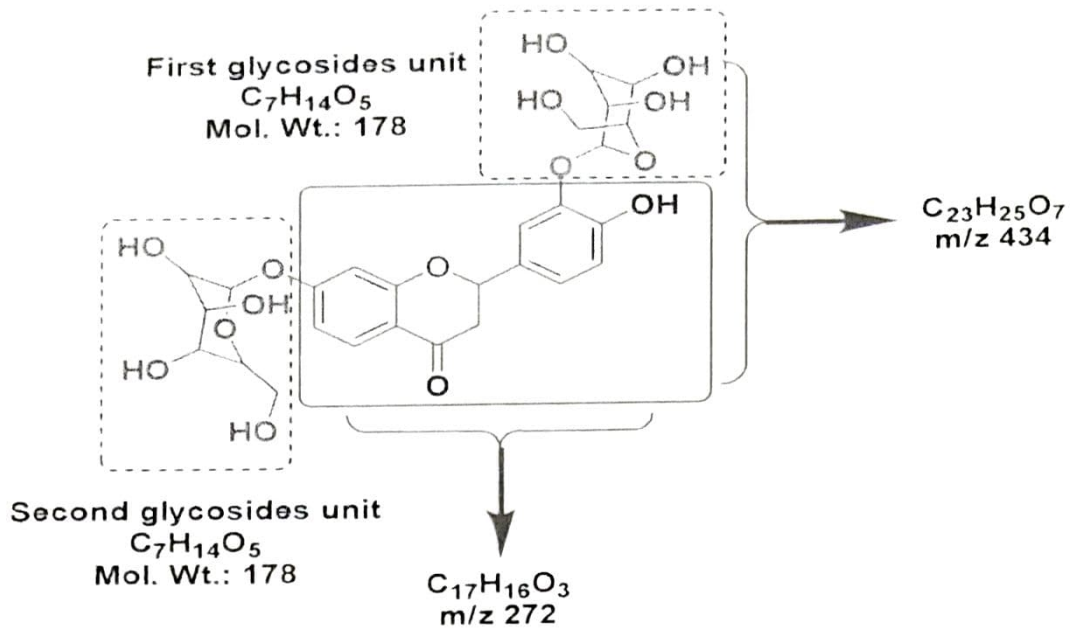


Fig. 5. Mass spectrum of NBF-BMFE

(a)



(b)

Fig. 6. Fragmentation analysis for isomers of butrin. (a) Parent ion m/z 597 and fragment ions at m/z 434 and m/z 272 (b) corresponding to the loss of one and two glucopyranose units.

Pharmacophore analysis of lead molecules

Using molinspiration, the pharmacophore analysis of eight ligand molecules with reference of quercetin were generated, had different scaffolds and activities. The parameter of Lipinski rule of five includes total polar surface area (TPSA) for NBF-BMFE and quercetin has been shown in Table 2. There were different statistically significant parameters such as LogP of -5 to 5, molecular weight (MW) $\leq$ 500, number of hydrogen bond acceptors $\leq$ 10, number of hydrogen bond donors $\leq$ 5, rotatable bonds (nRotb) $\leq$ 12, number of atoms $\leq$ 40, molecular volume $\leq$ 500 and total polar surface area $\leq$ 1000 has been demonstrated (Lipinski *et al.*, 1997).

The observed results declared that the butrin and isobutrin has not been accepted drug likeness properties, had poor permeation due to high HBA and HBD. Whereas, the resting compounds were strongly accepted since it had been strong binding affinity against complex protein structure. Moreover, it has more permeable within membrane and blood cells when compared to quercetin. By improving the pharmacological properties of butrin and isobutrin, needed molecular modification of compounds.

Table 2. Lipinski rule of five filters including TPSA for the top poses

Ligand	xlogP	TPSA	natoms	MW	HBA	HBD	RB	MV	Violations
Butrin	-2.092	245.293	42.0	596.538	15	9	7	494.504	3
Isobutrin	-1.523	256.287	42.0	596.538	15	10	9	498.166	3
Butein	2.28	97.983	20.0	272.256	5	4	3	233.924	0

Butin	1.711	86.989	20.0	272.256	5	3	1	230.261	0
Coreopsin	0.493	177.135	31.0	434.397	10	7	6	366.045	1
Isocoreposin	-0.076	166.141	31.0	434.397	10	6	4	362.383	1
Monospermoside	0.264	177.135	31.0	434.397	10	7	6	366.045	1
Isomonospermoside	-0.305	166.141	31.0	434.397	10	6	4	362.383	1
Quercetin	1.683	131.351	22.0	302.238	1	5	1	240.084	0

LogP = logarithm of the octanol/water partition coefficient; TPSA = topological polar surface area; natoms = number of atoms; MW = molecular weight; HBA = number of hydrogen bond acceptors; HBD = number of hydrogen bond donors; RB = number of rotatable bonds; MV = molecular volume; Violations = number of violations of the Lipinski's rule of five.

ADMET Analysis

Normally, many compounds failed at early stage due to poor pharmacokinetics properties and toxicity problems. If these problems could be analyzed early, it will be great advantageous for drug development process. In view of these, computer based methods like ADMET tool plays a vital role in the studies of molecular descriptors and drug likeness properties (Lipinski *et al.*, 1997; Lombardo *et al.*, 2003). The pharmacokinetic properties results herein reported in Table 3. The efficiency of the blood/brain partition co-efficient (logB/B) used as a predictor for central nervous system (CNS). This predicted CNS activity was calculated from -3 (inactive) to +1 (active) scale, displayed all the molecules were came within the acceptable range. This clearly demonstrates butrin showed good results as compared to quercetin. The blood-brain barrier plays a main role to maintain homeostasis of CNS by separating the circulating blood from the brain (Fidele *et al.*, 2013). The drug's intestinal permeability has been estimated through the Caco-2 cell permeability (PCaco), whereas the recommended range for this parameter is from -1(poor) to +1 (good), explicit all the molecules had good absorption in the intestinal membrane. Also, the human intestinal absorption (HIA) of drug metabolism and drug permeability with intestinal membrane was measured using Log_{HIA} and formed the range from 0 (poor) to 1 (great). Rather than quercetin, the molecules butein and butin was completely metabolized and easily permeable within HIA. These findings suggested that the functional groups of these compounds had great absorption within human intestine and also had strong involvement in drug metabolism. The understanding of P-glycoprotein (P-gp) substrate and inhibitors study were based on drug-drug interaction within different tissues. Commonly, P-gp offers main role to trash out the xenobiotics from various organs to reduce the drug intestinal absorption and enhance the elimination of drugs, where pass in to bile (liver) and urine (kidney) (Lin *et al.*, 2003). The

recommended range for Pgp substrate was from -5 (poor) to +1(good) and Pgp inhibitor was from 0 to 1. In which, the results of these molecules displayed within the acceptable range when compared with quercetin. The aqueous solubility prediction was mainly calculate the drug concentration, present in the target area for the establishment of therapeutic level and prevention of toxicity (Ailan *et al*., 2003). The recommended range for aqueous solubility (PlogS) was -6.5 to -0.5 and probability of Caco-2 cell permeability was -1 to 1. Indeed, the PlogS results showed all the molecules had good solubility and logpapp stated that butein and butin had good permeability on lipid absorption and metabolism. While resting compounds were came within acceptable range. On the whole, the NBF-BMFE recorded strong drug likeness properties.

Table 3. ADME and pharmacological parameters prediction for the selected ligands using admetSAR toolbox

S.No	Ligand	PlogBB[a]	PCaco[b]	log_{HIA}[c]	logpGI (substrate)[d]	logpGI (non-Inhibitor)[e]	PlogS[f]	logpapp[g]
1	Butrin	0.724	0.932	0.701	0.634	0.869	-2.196	-0.921
2	Isobutrin	0.539	0.881	0.621	0.617	0.859	-1.264	-0.577
3	Butein	0.586	0.607	0.970	0.536	0.919	-3.371	0.167
4	Butin	0.539	0.819	0.979	0.546	0.880	-2.275	0.472
5	Coreopsin	0.597	0.865	0.513	0.581	0.779	-1.528	-0.519
6	Isocoreposin	0.697	0.939	0.785	0.590	0.878	-2.448	-0.858
7	Monospermoside	0.597	0.865	0.513	0.581	0.779	-1.528	-0.519
8	Isomonospermoside	0.697	0.939	0.785	0.590	0.878	-2.448	-0.858
9	Quercetin	0.571	0.895	0.965	0.562	0.929	-2.994	0.224

[a]Predicted blood/brain barrier partition coefficient (concern value is -3.0 to 1.0)

[b]Predicted Caco-2 cell permeability in nm/s (acceptable range: -1 is poor, 1is great)

[c]Predicted Human intestinal absorption in nm/s (acceptable range: 0 poor, >1 great)

[d]Predicted P-glycoprotein substrate in nm/s (acceptable range of -5 is poor, 1 is great)

[e]Predicted P-glycoprotein inhibitor in nm/s (accepted range: 0 to 1)

[f]Predicted aqueous solubility (Concern value is − 6.5 to − 0.5)

[g]Predicted probability of Caco-2 cell permeability in cm/s (Concern value is -1 to 1)

The LD_{50} values of ligands were detected the cumulative potential acute toxicity that has administered through oral, intraperitoneal, intravenous and subcutaneous on mouse

models. The comparative analysis of LD_{50} mouse revealed that the NBF- BMFC had higher LD_{50} on oral and lower LD_{50} on subcutaneous when compared with quercetin (Fig. 7). The overall results suggested that all compounds had less toxic effect on internal tissues and no side effect were observed in the tested dosages.

The toxicity were tested with different organs to check adverse effects of organs and their systems (blood, cardiovascular system, gastrointestinal system, kidneys, liver and lungs) within the therapeutic dose range. The probability of health effects revealed that butein and butin had very less toxic effect on blood, cardiovascular, gastrointestinal, kidney, liver and lung. While other compounds had moderate toxic effect on all tissues except lung (Table 4). Hence, these inclusive results together suggested that the NBF-BMFE had no side effects with corresponding organs.

Table 4. LD_{50} and probability of health effects of selected ligands using ACD/ I-Lab 2.0

ADME-TOX Parameters	1	2	3	4	5	6	7	8	9
LD_{50} mouse[a] (mg/kg^{-1}, intraperitoneal)	1100	500	290	700	820	1500	610	800	450
LD_{50} mouse[a] (mg/kg^{-1}, oral)	2200	1200	880	2200	570	880	1200	1700	670
LD_{50} mouse[a] (mg/kg^{-1}, intravenous)	1800	630	200	300	250	580	580	1200	350
LD_{50} mouse[a] (mg/kg^{-1}, subcutaneous)	440	310	170	190	170	170	300	340	160
Prob. of blood effect[b]	0.95	0.86	0.2	0.34	0.88	0.99	0.94	0.99	0.69
Prob.of cardiovascular system effect[b]	0.98	0.97	0.57	0.78	0.98	0.89	0.91	0.85	0.27
Prob.of gastrointestinal system effect[b]	0.97	0.71	0.36	0.48	0.98	0.99	0.71	0.99	0.45
Prob. of kidney effect[b]	0.77	0.44	0.23	0.82	0.53	0.86	0.28	0.83	0.54
Prob. of liver effect[b]	0.94	0.97	0.25	0.32	0.85	0.8	0.68	0.8	0.09
Prob. of lung effect[b]	0.13	0.11	0.15	0.28	0.56	0.47	0.74	0.66	0.38

a- Estimates LD_{50} value in mg/kg after intraperitoneal, oral, intravenous and subcutaneous administration to mice.

b- Estimates probability of blood, gastrointestinal system, kidney, liver and lung effect at therapeutic dose range.

1-8 represents the Bm derivatives (butrin, isobutrin, butein, butin, coreopsin, isocoreposin, monospermoside, isomonospermoside) and 9 represents quercetin.

The drugs with moderate effect on reliability index (>0.5)

The drugs with border line effect on reliability index (>0.3, <0.5)

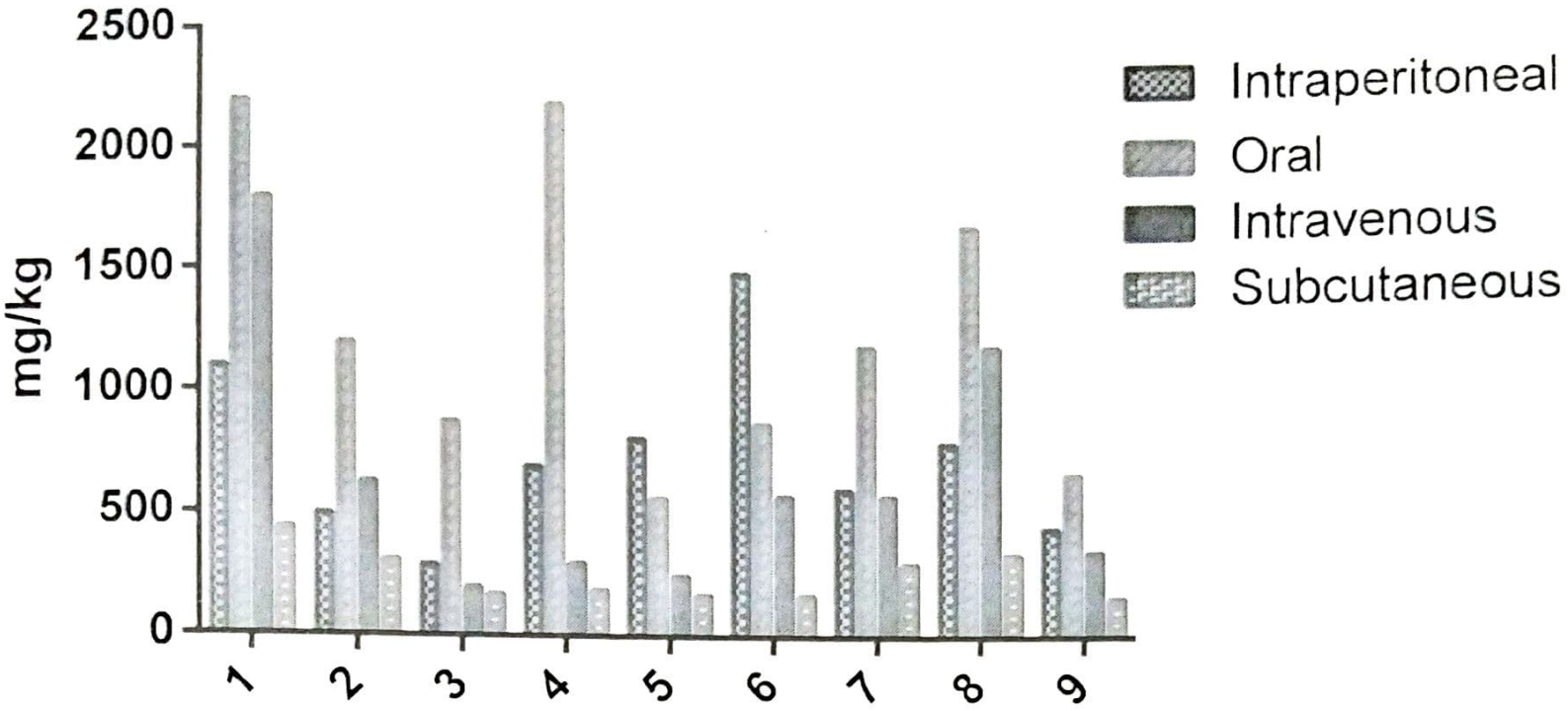


Fig. 7. Comparative analysis on $LD_{50\ mouse}$ (intraperitoneal, oral, intravenous, subcutaneous) for NBF-BMFE (1-8) and reference standard Quercetin (9). 1-8 represents the *Bm* derivatives (butrin, isobutrin, butein, butin, coreopsin, isocoreposin, monospermoside and isomonospermoside).

Cell growth inhibition property

The cytotoxic effects of NBF-BMFE and commercially available silibinin (Sigma Aldrich) was determined on SW480 human colon cancer cells at time and dose dependent manner for 24 h and 48 h. The effectiveness of compounds were measured by half maximal inhibitory concentration (IC_{50}) of cell death. The silibinin compound exhibited the IC50 values of 185 µg/ml and 100 µg/ml for 24 h and 48 h study. Similarly, the NBF-BMFE had significant antiproliferative effects with the IC_{50} value of 370 µg/ml and 200 µg/ml for 24 h and 48 h study (Fig. 8). The subsequent experiments were performed with this concentration.

The colorectal carcinoma cell lines SW480 had expressed both mutated β-catenin and APC regions (Kaur *et al.*, 2010). Moreover β-catenin mutations were detected in approximately 50% of the colorectal cancer (Sparks *et al.*, 1998). The above findings suggested that disrupting the β-catenin mutation in wnt signaling pathway may be one of the possible therapeutic approaches for treating colorectal cancer. Interestingly, our data clearly showed that the NBF-BMFE had effective cell growth inhibition against β-catenin expressing SW480 human colon cancer cells.

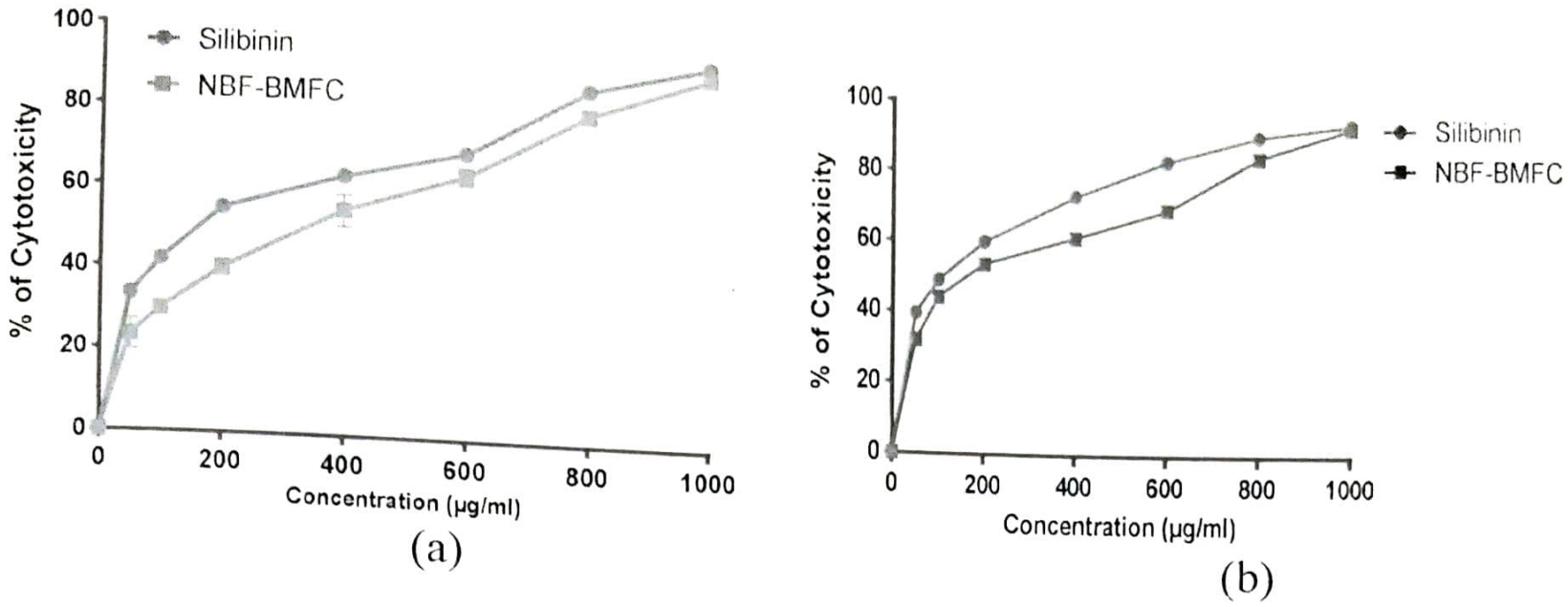
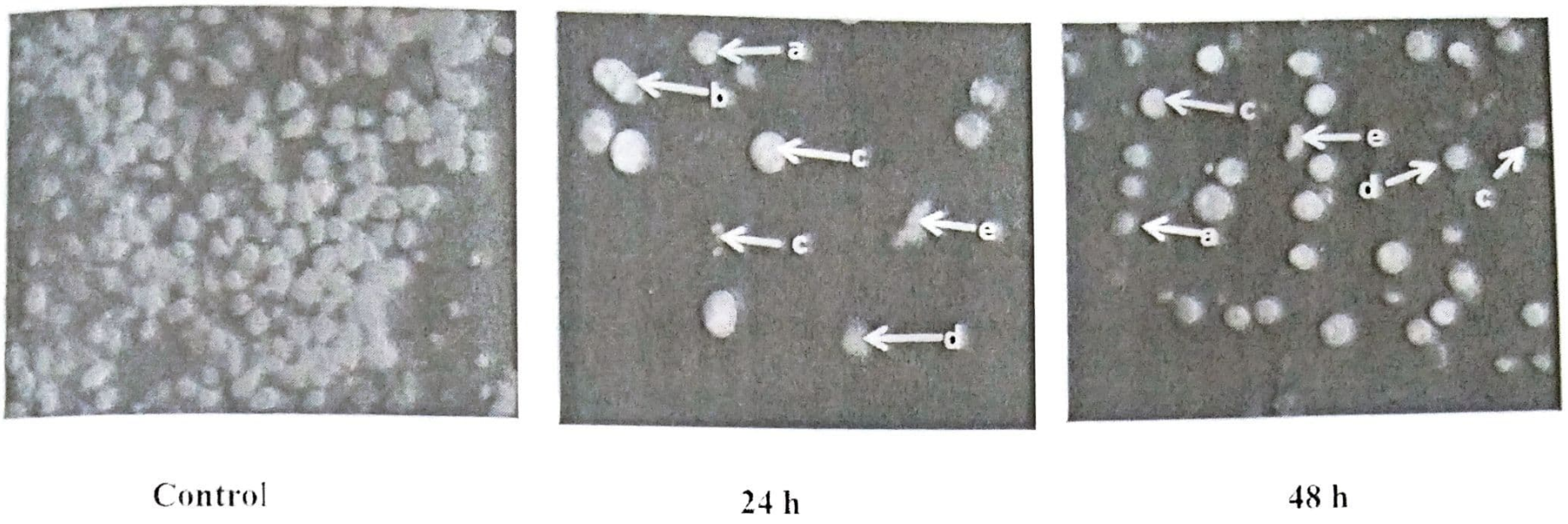


Fig. 8. Growth Inhibition effect of NBF-BMFE (50-1000 µg/ml) on SW480 human colon cancer cells at (a) 24 h and (b) 48 h with a dose and time dependent manner.

NBF-BMFE induced changes in cells reflecting morphological features of apoptosis

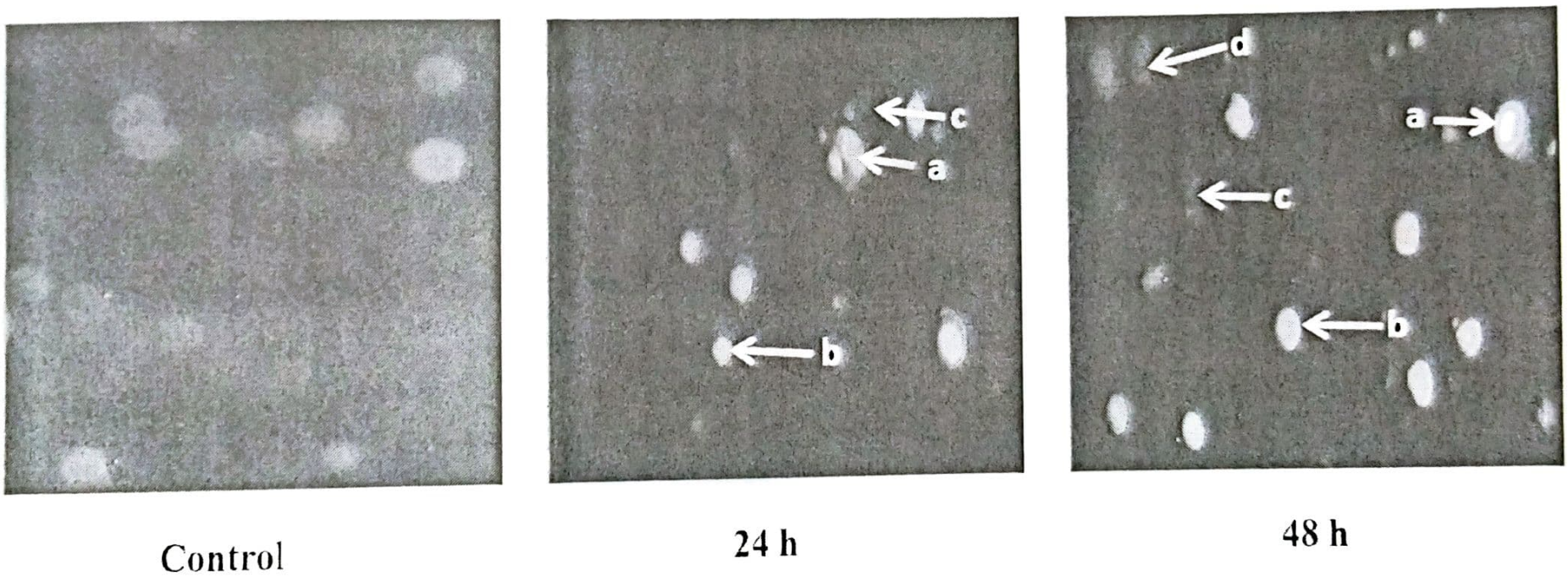
The morphological changes in SW480 cells treated with NBF-BMFE were determined by adopting AO&EB fluorescent staining and the results are represented in Fig. 36. After NBF-BMFE treatment, early apoptotic cells showed condensed nuclei and appeared bright green in color. Late apoptotic cells appeared in red color with condensed and fragmented nuclei. In necrotic cells, ethidium bromide penetrated into the membrane of dead cells and stains their nuclei which appear red. Whereas, the control cells did not undergo these morphological changes and the nuclei as well as cytoplasm fluoresced uniformly in green (Fig. 9). From the data it was clear with increasing timing of NBF-BMFE, the number of apoptotic cells also increased tremendously.

Further, we examined the nuclear morphology of NBF-BMFE treated and control cells using Hoechst 33258 staining. Hoechst dye stains the DNA and distinguishes brightly stained, condensed apoptotic nuclei from dimly stained healthy nuclei (Sudipa *et al.*, 2011). After treatment with NBF-BMFE, the control cells were seen with uniformly light blue nuclei under fluorescence microscope. While treated cells exhibited bright blue colour due to chromatin condensation and apoptotic bodies formation (Fig. 10). It indicates that apoptosis was induced on treatment with NBF-BMFE. Since DNA fragmentation is a hallmark characteristic feature of apoptosis, we performed the DNA fragmentation assay on IC_{50} value of NBF-BMFE treated SW480 human colon cancer cells. As a result, the noticeable fragmentation of DNA was observed at 24 h and 48 h respectively (Fig. 11).



Control 24 h 48 h

Fig. 9. Effect of NBF-BMFE on cell and nuclear morphology (AO/EB staining) in SW480 cancer cells. a) Early apoptotic cell, b) Blebbing, c) Chromatin condensation, d) Necrosis and e) Late apoptotic



Control 24 h 48 h

Fig. 10. Effect of NBF-BMFE on cell and nuclear morphology (Hoechst staining) in SW480 cancer cells. a) Mitotic cell, b) Flattened live cell, c) Apoptotic bodies and d) Chromatin condensation.

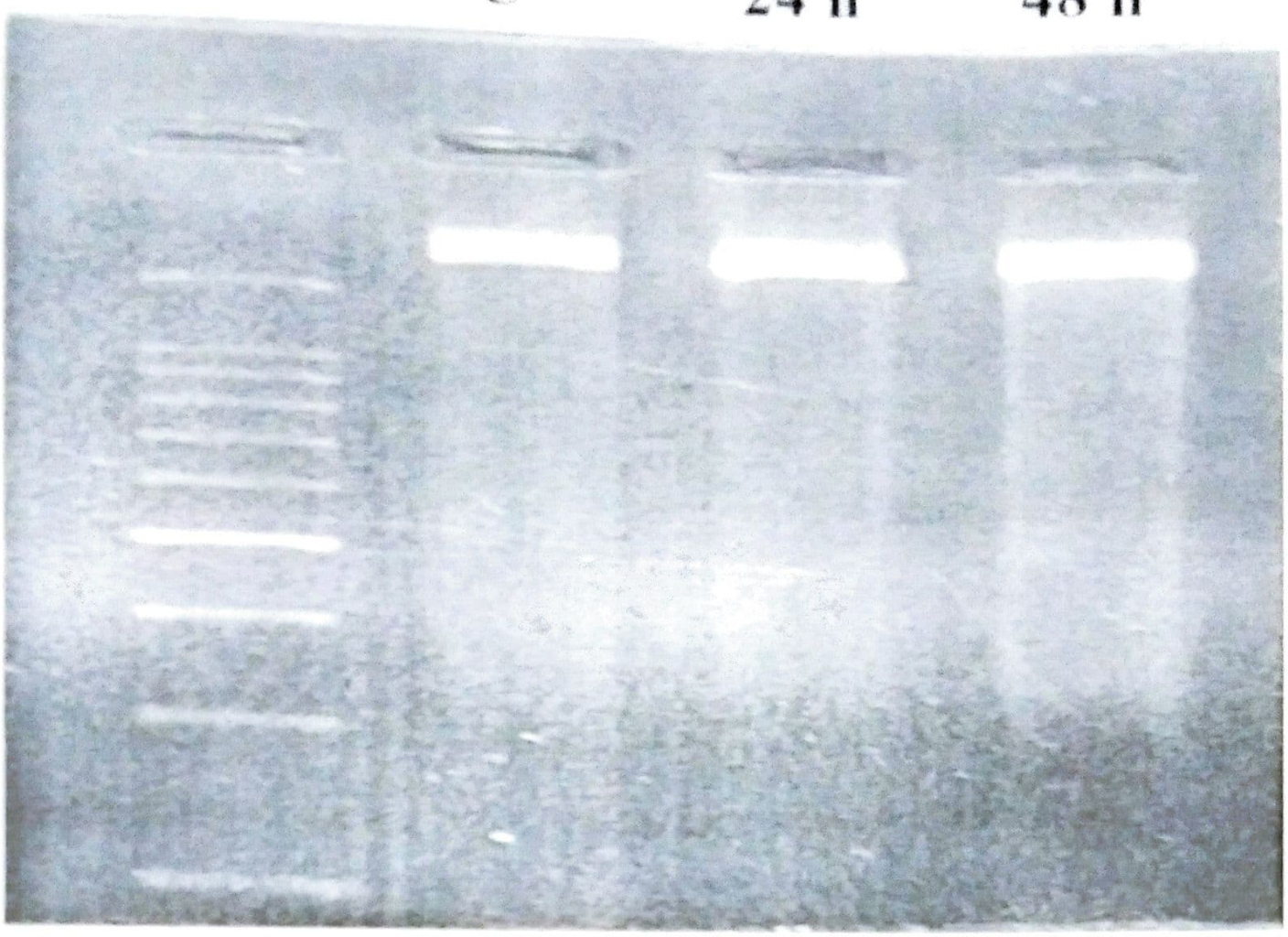


Fig. 11. Effect of NBF-BMFE on internucleosomal DNA fragmentation in SW480 cancer cells. M – Marker (100 - 1000 b) , C – Untreated Control

Effects of NBF-BMFE on mitochondrial membrane potential (ΔΨm)

The alteration in mitochondrial membrane potential (ΔΨm) was determined using the 5, 5', 6, 6'-tetra-chloro-1, 1', 3, 3' – tetraethyl benzimidazolyl carbocyanine iodide (JC-1) which is commonly used to detect mitochondrial depolarization that occurs during the early stages of apoptosis. In healthy cells, JC-1 accumulates in the mitochondria as JC-1 aggregates (fluorescence of which is red) and also in the cytoplasm as JC-1 monomers (fluorescence of which is green). During early apoptosis, the ΔΨm collapses and consequent JC-1 was aggregated, do not accumulate within the mitochondria and dissipate as JC-1 monomers leading to loss of red fluorescence. Therefore, collapse of the ΔΨm is indicated by decrease in the ratio of red to green fluorescence.

The assessment of NBF-BMFE induced apoptosis, we performed JC-1 probe at 24 and 48 h interval. It is a fluorescent cationic dye that can selectively aggregate into mitochondria by electrochemical gradient and changes color from red to green as ΔΨm decrease and untreated cells exhibited red fluorescence. After NBF-BMFE treatment in

SW480 human colon cancer cells. the apoptosis induction was clearly observed at 24 and 48 h intervals (Fig. 12).

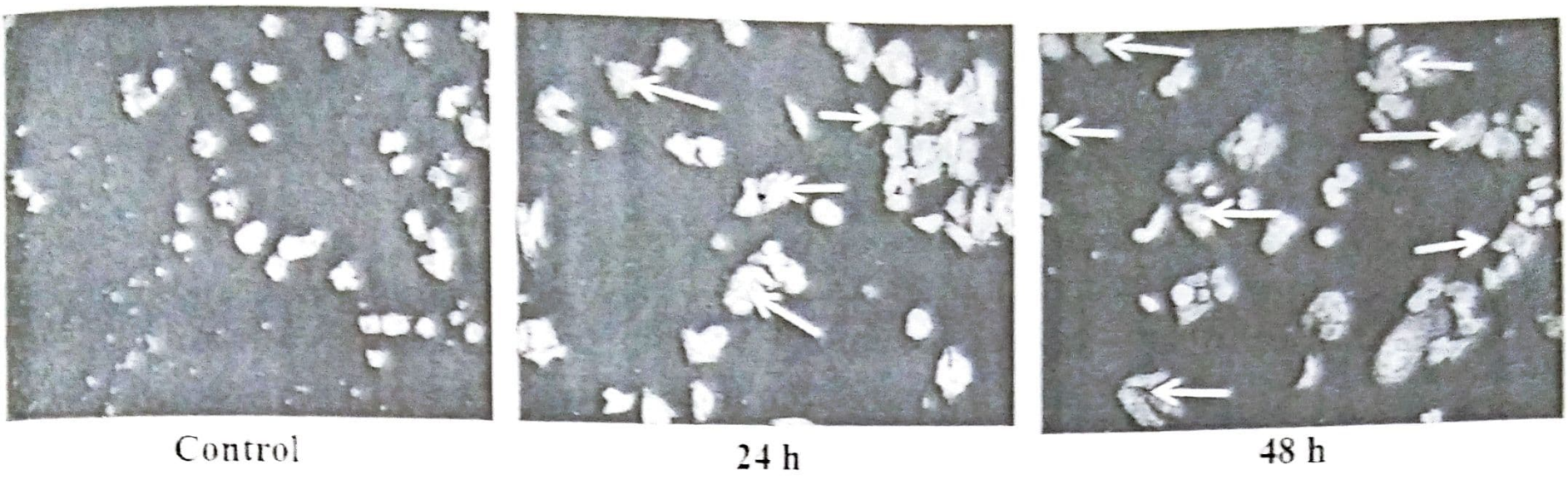
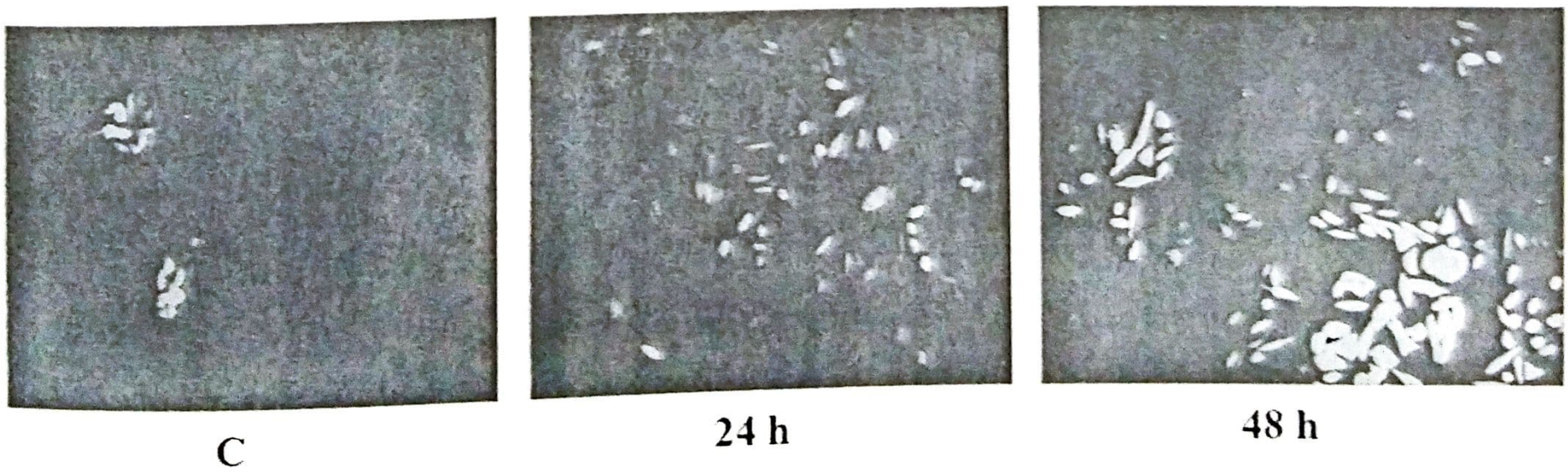


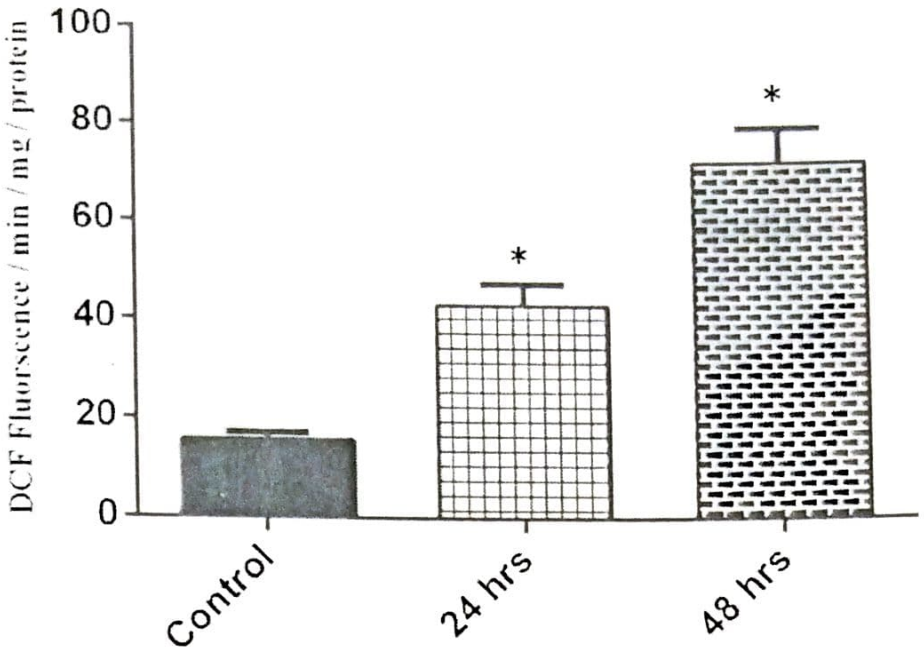
Fig. 12. Alterations in mitochondrial membrane potential on NBF-BMFE treated SW480 cells. Arrows indicate cells with reflections of mitochondrial depolarization.

ROS analysis

Reactive oxygen species (ROS) in small quantities is produced during normal cellular physiological processes but the level increase during stress and pathological conditions. The intracellular level of ROS is significantly higher in NBF-BMFE treated cells for 24 h and 48 h intervals, when compared with the control cells (Fig. 13). This propose that the ROS is involved in the triggering of apoptotic signaling and increased levels of ROS can induce depolarization of the mitochondrial membrane which eventually leads to an increase in the level of pro-apoptotic molecules in cells (Circu *et al.*, 2010).



(a)

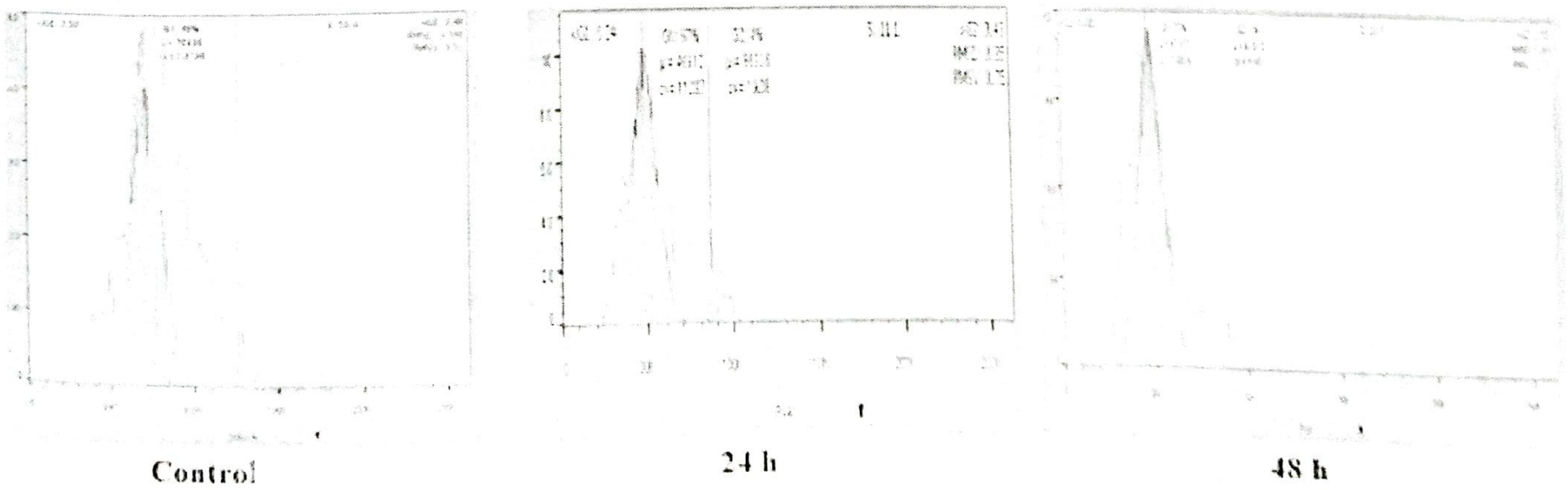


(b)

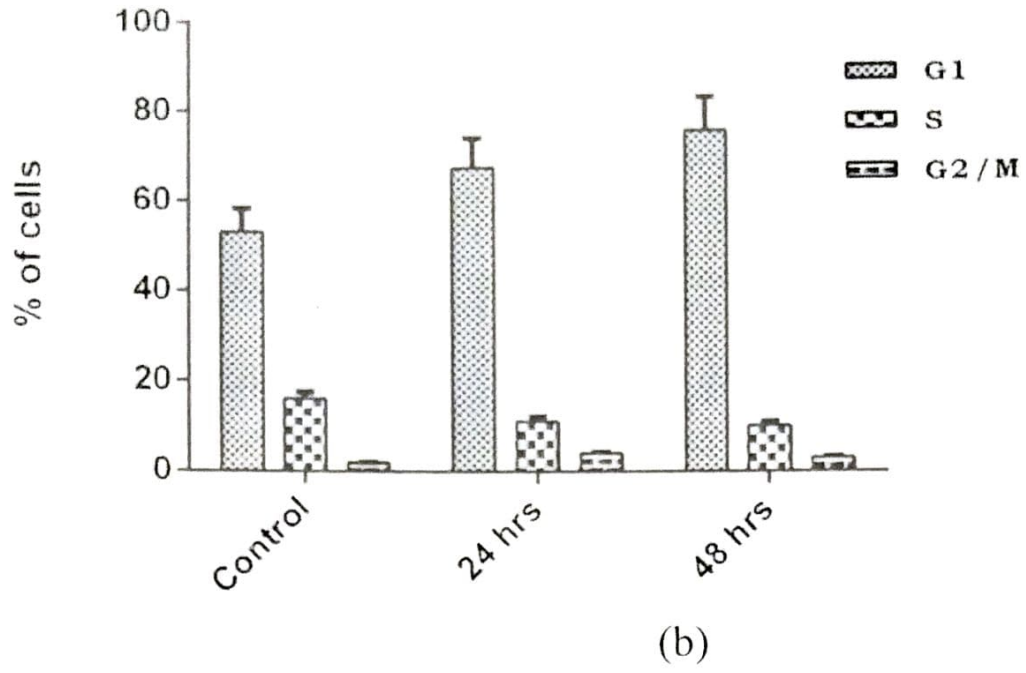
Fig. 13. Effect of NBF-BMFE on levels of ROS in SW480 cells. Values is expressed as unit mg/protein. Values represent mean ± SD. Values are statistically significant at *P< 0.05 (n=3)

NBF-BMFE changes in cell cycle distribution

The mechanism underlying NBF-BMFE mediated cell growth inhibition, cell cycle distribution was evaluated by flow cytometry. The SW480 cells treated with NBF-BMFE suffered a significant inhibition of cell cycle progression in an IC_{50} value with time dependent manner (Fig. 14). Particularly, there was a clear increase of the percentage of cells in the G1 phase, suggesting that the cells were inhibited in the progression of cell cycle. Since deregulation of the cell cycle machinery has been associated with cancer initiation and progression (Jiang *et al.*, 2012). Moreover, inhibition of cell cycle progression is an appreciated target for management and treatment of cancers with cytotoxic agents. Inteestingly, our data also suggested that NBF-BMFE, in bring about cell cycle arrest at G1 phase in the SW480 human colon cancer cells.



(a)



(b)

Fig. 14. Flow cytometric analyses of NBF-BMFE treated cells. (a) SW480 cells were treated with IC_{50} concentration of NBF-BMFE for 24 and 48 h, stained with propidium iodide and the DNA content was analysed by Flow cytometry (b) Bar chart showing the percentage of cells at each phase.

NBF-BMFE inhibits β-Catenin/Tcf Pathway in human colon cancer cells

Colon cancer cells frequently possess mutations in APC or β-catenin and activated Wnt signaling resulting the cellular accumulation of β-catenin. To investigate the inhibitory effect of NBF-BMFE on the transcriptional activity of β-catenin/Tcf in colon cancer cells, the TOP flash reporter gene assay was performed in SW480 (truncated mutation of APC and wild-type β-catenin) human colon cancer cells. The cells transfected with TOP flash reporter showed the highest transcriptional activity, further its transcription efficiency was normalized by using steaylite plus reagent sample (Perkin Elmer). As shown in Fig. 15, After 48 h of treatment, the NBF-BMFE showed two fold reduction of Tcf-dependent luciferase activity (TOP flash) in SW480 human colon cancer cells, when compared with control group.

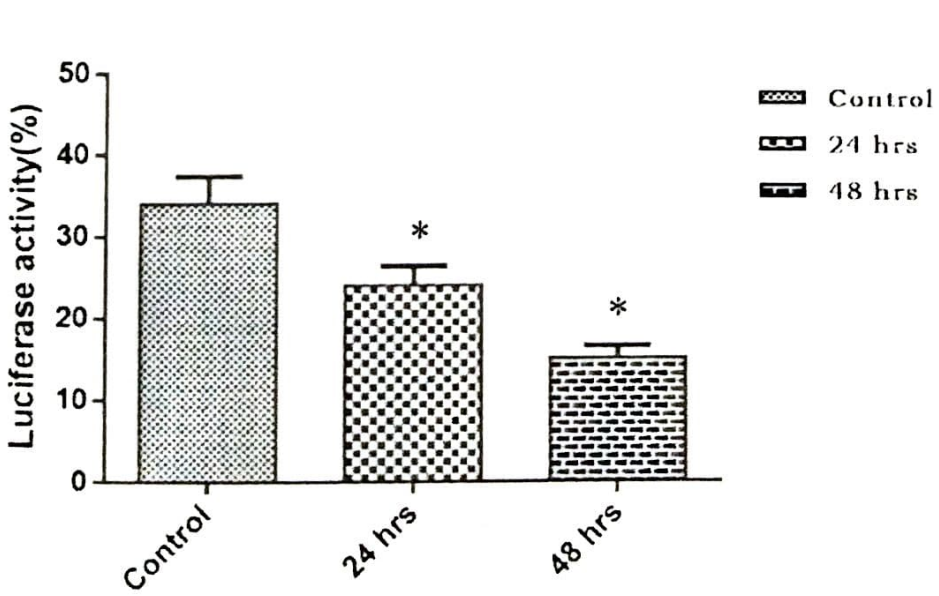
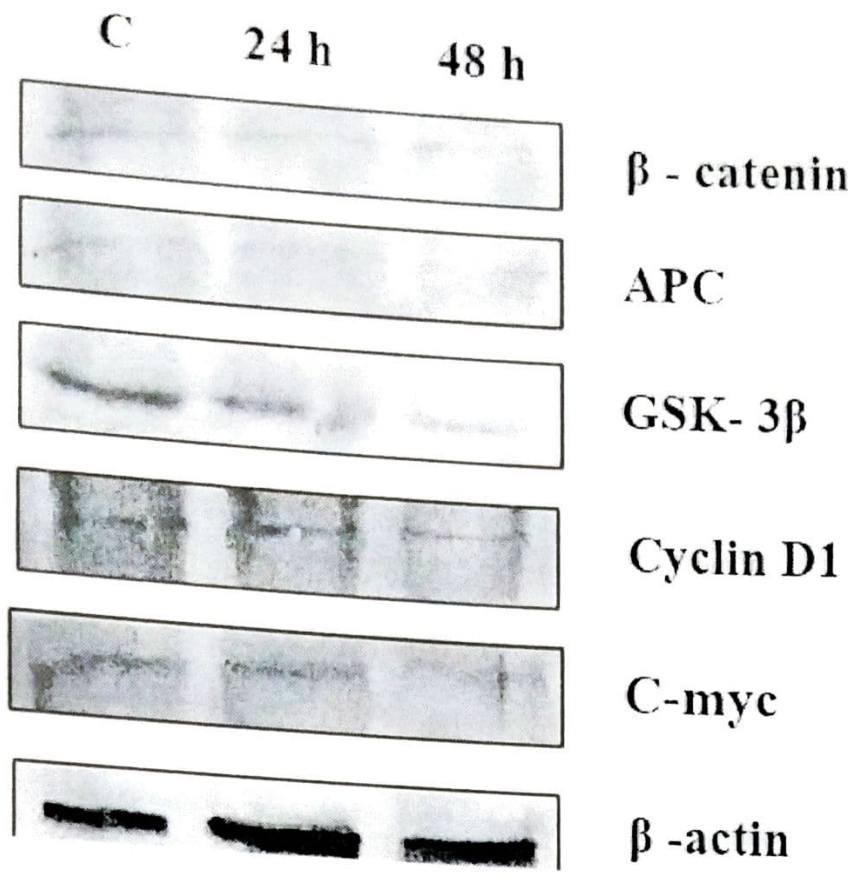


Fig. 15. NBF-BMFE inhibited the transcriptional activity of β-catenin/Tcf in SW480 cells. Values represent mean ± SD. Values are statistically significant at *$P < 0.05$ (n=3)

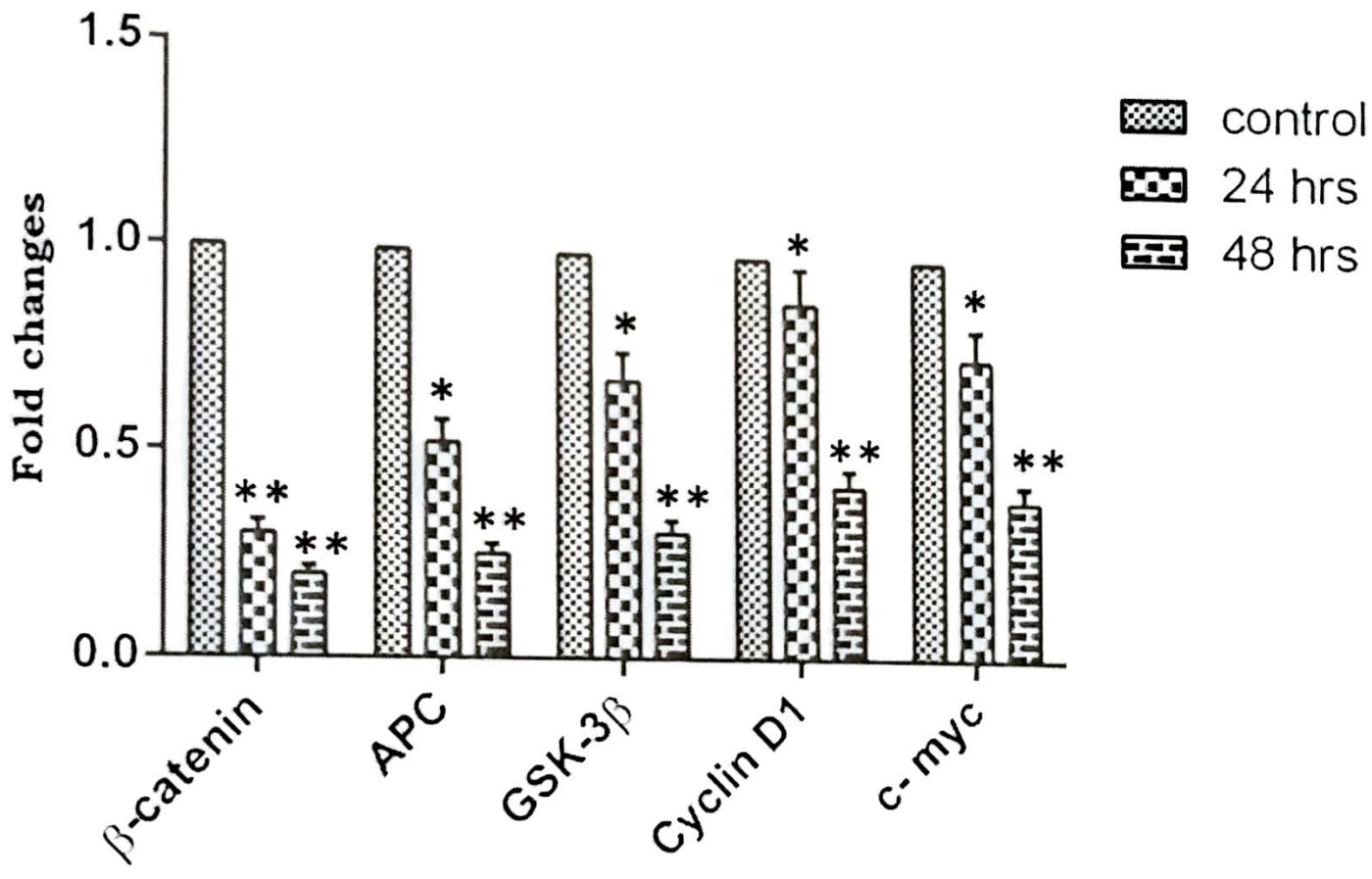
NBF-BMFE suppresses the expression of β-Catenin/Tcf signaling pathway proteins and its downstream target genes

The Wnt signalling proteins β-Catenin, APC, GSK-3β and its downstream target genes such as cyclin D1 and *c-myc* plays an important role in the regulation of Wnt signaling pathway as well in the progression of CRC. Henceforth, we examined whether NBF-BMFE could downregulate those genes in SW480 human colon cancer cells. The IC_{50} value of NBF-BMFE was significantly down regulated the expression of β-Catenin, APC and GSK-3β with two fold reduction when compared to control, after 48 h treatment. Similarly, the β-Catenin/Tcf signaling pathway downstream target genes such as cyclin D1 and *c-myc* were also significantly down regulated with one fold reduction when compared to control (Fig. 16).

The level of β-catenin in cells is tightly controlled by its degradation complex composed of Axin, APC, GSK-3β and β-catenin, in which GSK-3β phosphorylates β-catenin and thus triggers its ubiquitination and subsequent proteasomal degradation. Inactivation of this destruction complex, leading to accumulation of β-catenin in the cytoplasm and translocate into the nucleus. In the nucleus, β-catenin plays as a transcriptional activator in association with Tcf/Lef family and other transcriptional cofactors, by which a variety of target genes including *c-myc* and cyclin D1 was subsequently activated (Roose and Clevers, 1994; Polakis, 2007). Dysregulation of β-catenin signaling is believed to be critical to the early stages of human colorectal carcinogenesis (Kobayashi *et al.*, 2000). Also, most mutated APC proteins existed in colorectal tumors (Senda *et al.*, 2007). Taken together, the above findings is clearly stated that the NBF-BMFE was significantly down regulate the expression of β-catenin/Tcf signaling proteins in SW480 cells.



(a)



(b)

Fig. 16. Effect of NBF-BMFE on (a) the expression levels of β-catenin, APC, GSK-3β, cyclin D1 and *c-myc* in SW480 colon cancer cells at 24 h and 48 h treatment. (C-Control - untreated cells) (b) Values represent mean ± SD. Values are statistically significant at *P< 0.05 **P<0.01 (n=3)

Summary of the Findings:

In the present study, we demonstrated the anticancer properties of NBF-BMFE on CRC with specific to suppressing the transcriptional activity of β-catenin/Tcf signaling pathway together with ACF inhibition on AOM induced colon cancer male albino rat models.

Solubility is one of the important parameters to achieve desired concentration of the drug in systemic circulation for achieving required pharmacological response (Vemula *et al.*, 2010). Low aqueous solubility is the major problem encountered with formulation development of new chemical entities as well as generic development. The poorly water soluble drugs having slow drug absorption leads to inadequate and variable bioavailability and gastrointestinal mucosal toxicity (Sharma *et al.*, 2009). In our study the NBF-BMFE had showed complete solubility in water, it indicates that this fraction is used effectively for pharmacological studies. In previous study, there is no reports about the elucidation of isomers in NBF-BMFE. However, we evidently represented the presence of compounds having isomeric properties in NBF-BMFE by using LC/MS.

the *in vitro* results showed that the NBF-BMFE was significantly down regulated the expression of β-catenin, APC, GSK-3β, cyclin D1 and *c-myc* genes in SW480 cells after 48 h of treatment. Cell cycle deregulation associated with cancer, occurs through mutation of proteins important at different levels of the cell cycle progression and repair mechanism. Also deregulated cell cycle progression, driven by activation of growth-stimulating oncogenes and fundamental alterations in the genetic control of cell division, resulting in an uncontrolled cell proliferation (Massague, 2004). From these studies it implies that the antitumor effect of NBF-BMFE by suppressing the Wnt/β-catenin signaling pathway through inhibiting the transcriptional activity of β-catenin/Tcf in SW480 cancer cells. In the past several studies reported that Epigallocatechin gallate (EGCG) inhibited β-catenin/Tcf activity in HEK293 cells transiently transfected with a constitutively active mutant β-catenin gene (Dashwood *et al.*, 2002). Besides, in HT-29 cells, treatment with EGCG increases protein levels of E-cadherin by 27% to 58%, induces the translocation of β-catenin from nucleus to cytoplasm and plasma membrane and then decreases *c-myc* and cyclin D1 protein expression (Ju *et al.*, 2005). In addition, Jaiswal *et al*., 2002 reported that the curcumin inhibited the transcriptional activity of β-catenin/Tcf complex to induce growth arrest and apoptosis in HCT116 colon cancer cells.

Next we observed cell population arrest in G1 phase through down regulation of cyclin D1 after treatment with NBF-BMFE on SW480 colon cancer cells in a time dependent manner. Similarly, Luteolin was reported to induce cell cycle arrest at G1 phase in HT-29 colon cancer cells. It leads to the down regulation of several genes including cyclin D1 and cyclin B1 that are required for CDK4/6 activation and progression through the G1 checkpoints. The negative regulation of these positive regulators of cell cycle progression would impair CDK activities and contribute to the increase in G1 arrest following addition of luteolin (Lim *et al.*, 2007). Moreover, in HCT116 colon cancer cells, esculetin induced cell cycle arrest in the G1 phase through the down-regulation of cyclin D1/CDK4 and cyclin E/CDK2, which are regulating factors governing the G1-S phase cell cycle progression (Park *et al.*, 2011). These findings further suggest that NBF-BMFE might have synergistic effect on inducing cell cycle arrest at G1 phase and apoptosis through down regulating cyclin D1 and other Wnt/β-catenin downstream targets.

In summary, these studies provide a better understanding about the chemopreventive efficacy of NBF-BMFE on human colon cancer cells (SW480) by down regulating the key molecules of Wnt/β-catenin signaling proteins.

Conclusion

The experimental data presented here showed that NBF-BMFE had significant antiproliferative effect on SW480 human colon cancer cell line in time and dose dependent manner. Further, this extract was significantly down regulate the expression of Wnt signaling proteins such as β-catenin, APC, GSK-3β, cyclin D1 and c-myc on SW480 cells. Moreover, the in silico studies demonstrated that the NBF-BMFE compounds having good binding interaction on β-catenin, APC and GSK-3β proteins. This paper reveals the molecular mechanism underlying the anti-tumor effect of NBF-BMFE by suppressing β-catenin/Tcf signaling via decreasing its downstream target genes for first time. Finally, our results concluded that the NBF-BMFE could be a potential therapeutic regimen for colon cancer.

References:

The details of References were given in the two attached research publications.

Annexure-III

Contribution to the society

In this present study clearly illustrate the significant antiproliferative effect of NBF-BMFE and its active molecules on colorectal cancer cell line and that it may be facilitated to target the subtype specific cancer treatments in future. Further investigation on molecular pathways of cancer targeted genes will be a worthy endeavor for better understanding of the bioactive compounds from *B. monosperma*.

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Original Article

Isocoreopsin: An active constituent of n-butanol extract of *Butea monosperma* flowers against colorectal cancer (CRC)☆

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ABSTRACT

The herb *Butea monosperma* constitutes several human health beneficial components, which are mostly studied for their anticancer effects. In this study, the activity of n-butanol fractions of *B. monosperma* floral extract was examined on inhibiting aberrant crypt foci (ACF) formation in azoxymethane induced Wistar albino rats. The n-butanol extracts (150 mg/kg) decreased the ACF formation (per rat) by 92% and 78% in short- and long-term in vivo treatments, respectively. All the compounds in the n-butanol extract were isolated and purified using column and reverse-phase high pressure liquid chromatography (HPLC). Their structures were characterized using UV–visible spectroscopy, nuclear magnetic resonance (NMR) and electrospray–ionisation mass spectrometry (ESI–MS) to determine important flavonoids, namely isocoreopsin, butrin and isobutrin. These compounds were studied for their free radical scavenging and anticancer activities. The compound isocoreopsin showed significantly greater efficacy in cell death on human colon and liver cancer cell lines (50 μg/mL in HT-29 and 100 μg/mL in HepG2) than butrin (100 μg/mL in HT-29 and 500 μg/mL in HepG2) and isobutrin (80 μg/mL in HT-29 and 150 μg/mL in HepG2). These results suggest that isocoreopsin, butrin and isobutrin are the important key compounds for the chemoprevention of colon cancer and isocoreopsin can be considered as a promising novel drug.

1. Introduction

In humans, colorectal cancer (CRC) and hepatocellular carcinoma (HCC) rank the second and third most common causes of cancer-related death in most developed countries around the world [1,2]. The incidence of liver cancer is steadily increasing due to obesity, diabetes, alcohol intake, cirrhosis and rise of hepatitis C virus infection [3]. Surgery and chemotherapy are available for HCC treatment but the multidrug resistance (MDR-1) gene properties and their related protein expression always pose a challenge for the treatment of cancer [4].

Aberrant crypt foci (ACF) in colon has been proposed as precancerous lesions for CRC and an appropriate biomarker for identifying modulatory effects of xenobiotic on colon carcinogenesis [5]. The simple interpretation is that the identification of preneoplastic lesions may lead to the execution of successful chemoprevention studies in cancer treatment [6]. Interestingly, nutrients derived from plant source play a vital role in prevention and treatment of many diseases by stimulating antioxidants. Over several years, the accumulating evidence suggested that phenolic compounds have potential antioxidant activity with the presence of flavonoids [7]. Earlier reports of medicinal plants and their prospective effects on many diseases and disorders have revealed a growing interest in the herbal drug for its divergent medicinal purpose [8].

Several studies suggest that the methanolic and acetone extract of *Butea monosperma* (*B. monosperma*) flowers have antibacterial, antifungal, anti-inflammatory and wound healing properties [9–11]. In addition to this, petroleum ether extracts and ethanolic extracts of *B. monosperma* flowers possess antidiabetic, antipyretic and radical scavenging activities with respect to in vivo and in vitro models [12,13].

Ethanolic extract of *B. monosperma* leaves increased the anticancer activity against Ehrlich ascites carcinoma (EAC) in Swiss albino mice [14]. Similarly, aqueous extract of *B. monosperma* flowers was found to be effective on HBV-related X15-*myc* mouse model of HCC [15]. Further, it was reported that the aqueous extract of *B. monosperma* flowers has anticancer activity by stimulating proapoptotic function and inhibiting cell growth, anti-oxidant and free radical scavenging activities in vitro study [16]. As mentioned previously, extracts of *B. monosperma* flowers exhibit potential anticancer activity due to its flavonoids such as butin, butein, butrin, isobutrin, palasitrin, coreopsin, isocoreopsin,

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sulphurein, monospermoside and isomonospermoside [17]. Moreover, the earlier reports indicated that the overexpression of Wnt signaling pathway played a vital role in CRC and HCC development through the upregulation of early growth response genes [18–20]. However, the reports suggested that *B. monosperma* may have a suppressive effect on early growth response genes and thus mechanism needs further investigation for better understanding.

This paper details the effect of the n-butanol extract of *B. monosperma* flowers on colon cancer in azoxymethane (AOM) induced rat model and the isolation of all the three active compounds, namely isocoreopsin, butrin and isobutrin, by chromatography methods and structural validation using nuclear magnetic resonance (NMR), Fourier transform infrared spectroscopy (FTIR) and electrospray–ionisation mass spectrometry (ESI–MS). Further, it strived a clear idea on the n-butanol extract (NBE) of *B. monosperma* flowers and their individual effect on hepatocarcinoma and adenoma cancer cell lines.

2. Experimental

2.1. Chemicals and reagents

Naringenin (5,7-dihydroxy-2-(4-hydroxyphenyl)-2,3-dihydrochromen-4-one), ellagic acid, 2,2-diphenyl-1-picrylhydrazyl (DPPH) and AOM were obtained from Sigma–Aldrich Co (Saint Louis, MO, USA). Sodium carbonate, aluminium chloride, potassium acetate, silica gel (60–120 mesh) and HPLC grade acetonitrile were obtained from Merck & Co (USA). Dulbecco's modified Eagle's medium (DMEM), 10% fetal bovine serum (10% FBS), penicillin/streptomycin and DMSO (cell culture grade) were purchased from Hi Media Laboratories, Mumbai, India. All other chemicals and solvents used were of analytical grade/better.

2.2. Instrumentation

The UV spectra were recorded with double beam UV-visible spectrophotometer. The FTIR spectra of the separated compounds were confirmed on JASCO FTIR Spectrophotometer (USA). NMR spectrum was revealed in DMSO-d_6 on a Bruker Avance 500 or 600 spectrometer (Switzerland). ESI–MS was recorded on Waters UPLC-TQD (USA). Silica gel (60–120 mesh) was used to separate the various compounds by column chromatography against methanol and ethyl acetate of analytical grade (Merck & Co, USA). Purification of the compounds was achieved by pre-coated silica gel F254 TLC. Adsorption data for the free radical scavenging activity was measured by using Shimadzu 800 spectroscopy (Japan).

2.3. Plant material

Butea monosperma (Lam.) Taub. flowers were collected from the Palkalai Perur campus of Bharathidasan University, Tiruchirappalli, Tamil Nadu, India. A voucher specimen was confirmed and deposited in the Department of Plant Science, Bharathidasan University (BDUT 1001).

2.4. Extraction of B. monosperma flowers

Air dried *B. monosperma* flowers were finely powdered and extracted with methanol in soxhlet apparatus for 24 h. Then the extract was concentrated under reduced pressure in a rotator evaporator, which was partitioned with water and ethyl acetate (EtOAc). After partition, the persistent water phase was treated with n-butanol (3 times) and dried with sodium sulphate. Subsequently, the NBE was concentrated by rotator evaporator and stored at 4 °C [21].

2.5. Isolation of active compounds from B.monosperma flowers by column chromatography

The dried NBE of *B. monosperma* flowers was subjected to Silica-100 macroporous adsorption resin column. The column was rinsed with EtOAc (discarded), then EtOAc with various concentrations of MeOH (5%, 15%, 30%, 50%, 70%, and 95%, v/v) was used as mobile phase to acquire three fractions successively.

2.5.1. High performance thin layer chromatography (HPTLC)

The collected fractions were dissolved in HPLC grade methanol (1 mg/mL) and subjected to HPTLC. The sample was spotted on pre-coated silica gel F254 TLC plates using CAMAG Linomat V automatic sample Spotter and the plates were developed in EtOAc methanol (30:70, v/v) solvent system. The plates were scanned in TLC scanner 3 (CAMAG) at 254 nm. The Rf values, spectra, Lambda max and peak areas of the resolved bands were recorded and relative percentage area of each band was calculated from peak areas.

2.5.2. High pressure liquid chromatography (HPLC)

Among the three collected fractions, fraction II was analyzed on a Waters 2545 HPLC-photodiode array system (Quaternary gradient module) with symmetry C_{18} column (250 mm × 4.6 mm, 5 µm) at a column temperature of 28 °C. Two mobile phases, A and B, were used. Mobile phase A was 0.1% (v/v) phosphoric acid in water, while mobile phase B was acetonitrile. A ratio of 70% A and 30% B was applied in the first 15 min. After 15 min, a ratio of 50% A and 50% B was used for the next 25 min. Finally, 5% A and 95% B were used after 30 min for an additional 15 min. The solvent flow rate was 1 mL/min and the sample injection volume was 20 µL. The UV–visible spectra were recorded in the wavelength range of 200–800 nm.

2.5.3. Spectrometric identification

2.5.3.1. Isocoreopsin (2-(3, 4-Dihydroxy-phenyl)-7-(3, 4, 5-trihydroxy-6-hydroxymethyl-tetrahydro-pyran-2-yloxy)-chroman-4-one (1)).

UV: 217, 348, 367, 408 nm. ESI–MS: Isocoreopsin compound displays a peak at *m/z* $(M + H)^+$ 435.36 (calcd m/z 434.39). ^1H and ^{13}C NMR data (400 and 100 MHz, DMSO- d_6) (Table 1).

2.5.3.2. Butrin (2-[4-Hydroxy-3-(3, 4, 5-trihydroxy-6-hydroxymethyl-tetrahydro-pyran-2-yloxy)-phenyl]-7-(3, 4, 5-trihydroxy-6-hydroxymethyl-tetrahydro-pyran-2-yloxy)-chroman-4-one (1)).

UV: 217, 329, 368 nm. ESI–MS: Butrin compound displays a peak at *m/z* $(M + Na)^+$ 619.2 (calcd *m/z* 619.16). ^1H and ^{13}C NMR data (400 and 100 MHz, DMSO- d_6) (Table 1).

2.5.3.3. Isobutrin (3-[4-Hydroxy-3-(3, 4, 5-trihydroxy-6-hydroxymethyl-tetrahydro-pyran-2-yloxy)-phenyl]-1-[2-hydroxy-4-(3, 4, 5-trihydroxy-6-hydroxymethyl-tetrahydro-pyran-2-yloxy)-phenyl]-propenone).

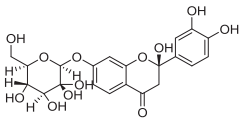
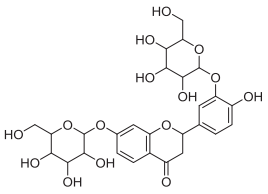
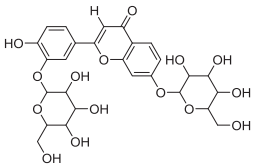
UV: 217, 348, 367, 408 nm. ESI–MS: Isobutrin compound displays a peak at *m/z* $(M+H)^+$ 597.1 (calcd *m/z* 596.17). ^1H and ^{13}C NMR data (400 and 100 MHz, DMSO- d_6) (Table 1).

2.6. Quantification of bioactive compounds

2.6.1. Determination of total flavonoids content

The total flavonoids content of different extracted compounds of *B. monosperma* flowers were determined according to $AlCl_3$ method. Different concentrations of each compound (2000 µg/mL) were separately mixed with 0.1 mL of 10% $AlCl_3$, 0.1 mL of 1 M potassium acetate to a final volume of 3 mL with distilled water, followed by incubation for 30 min at room temperature. The

Table 1.
^{13}C and ^{1}H NMR spectroscopic data for compounds 1,2,3 with ESI–MS fragmentation ions.

Compound	$\delta^{1}H$ (J, Hz)	$\delta^{13}C$	Structure
Isocoreopsin	7.720 (2.0)	190.08	 Molecular Weight : 434.39338 Molecular formula : $C_{21}H_{22}O_{10}$
	7.681 (1.0)	164.61	
	6.898 (4.0)	163.09	
	5.427 (6.0)	145.13	
	3.112 (2.0)	128.36	
	0.979 (7.0)	115.30	
		113.49	
		102.57	
		77.10	
		75.94	
		73.26	
		73.04	
		60.73	
		39.88	
		38.73	
		33.63	
		31.23	
		28.96	
		28.49	
		22.04	
		13.87	
Butrin	7.225 (3.0)	190.08	 Molecular Weight : 596.537 Molecular formula : $C_{27}H_{32}O_{15}$
	6.979 (3.0)	192.00	
	6.796 (3.0)	165.01	
	6.776 (3.0)	164.61	
	4.945 (5.0)	163.05	
	4.668 (5.0)	149.09	
	3.655 (7.0)	145.06	
	2.644 (3.0)	145.01	
		132.44	
		132.03	
		128.36	
		115.30	
		113.49	
		102.57	
		77.10	
		75.94	
		73.26	
		73.04	
		60.73	
		33.63	
		31.23	
		29.00	
		28.96	
		28.64	
		28.49	
		22.04	
		13.87	
Isobutrin	7.680 (3.0)	192.00	 Molecular Weight : 596.53398 Molecular formula : $C_{27}H_{30}O_{15}$
	6.983 (3.0)	190.05	
	6.796 (3.0)	165.01	
	6.760 (3.0)	163.05	
	4.980 (5.0)	149.09	
	4.810 (3.0)	147.08	
	4.668 (2.0)	145.08	
	3.655 (7.0)	145.06	
	2.644 (3.0)	145.01	
		137.04	
		132.03	
		129.69	
		128.36	
		127.88	
		121.69	
		115.75	
		110.90	
		103.52	
		99.69	
		79.23	
		77.17	
		76.36	
		73.30	
		69.88	
		60.49	
		60.05	
		42.97	

absorbance was recorded at 415 nm. The amount of distilled water was substituted by the same amount of fraction in the blank. Naringenin (100, 200, 300, 400, 500 and 1000 μg/mL) as a standard was used to make a calibration curve. The total flavonoids content of the compound was expressed as milligram acid equivalents (Naringenin) per gram extract [22].

2.6.2. Evaluation of DPPH radical scavenging activity

The reaction mixture was prepared by dissolving dried flower extracts of 3 mg in 50% aqueous ethanol. About 100–500 μL of the mixture was taken and made up to 750 μL with distilled water; 0.1 mM ethanolic DPPH solution was added to achieve a final volume of 1.5 mL. Then the mixture was incubated in the dark at room temperature for 30 min, and reduction of purple color was read at 517 nm. Ascorbic acid present in solution A was used as a standard and absolute ethanol as a blank. Radical scavenging activity of isolated phenolic compounds was expressed as the percentage of inhibition of free radical and calculated using the following formula [23].

$$\text{DPPH scavenged (\%)} = \left[(\text{Abs control} - \text{Abs test})/\text{Abs control}\right] \times 100\%$$

Where the Abs control is the absorbance of the control reaction, whereas Abs test is the absorbance of the compound/standard.

2.7. Experimental animals

Five-week-old male Wistar albino rats were obtained from Indian Institute of Science (IISC), Bangalore, India, and maintained in our animal house in a ventilated, temperature-controlled room at 25 °C with 12/12 h light/dark cycle for 1–2 weeks prior to the actual commencements of the experiment. They were provided with standard food pellets and drinking water (ad libitum). The protocol for the study was approved by Institutional Animal Ethics Committee (IAEC), Bharathidasan University, Tiruchirappalli, Tamil Nadu, India.

2.7.1. In vivo experimental protocol

Two protocols were designed for the evaluation of potential chemo preventive agents against AOM induced colon cancer in wistar albino rats [24]. In the first protocol, wistar albino rats were injected (subcutaneously) twice with AOM at the dosage of 15 mg/kg during the first two weeks of a five-week experimental period, and the NBEs were administered (intraperitonial) during the fourth week (two weeks after the last injection of AOM). This protocol was used to evaluate the effects of the test compound during the initiation phase in order to understand the carcinogen metabolism. The second protocol was similar to the first in most respects except that the animals would not be exposed to the test compounds until aberrant crypt foci (ACF) has grown in the colon of the rats until the end of fifth week. During the second two-week period, the NBE was administered intraperitoneally to evaluate the effects on outgrowth and progression of ACFs. Six groups of Wistar albino rats (6 rats/group) were classified for treatment regimen (Table 2). After the treatment period, the experimental animals were sacrificed by intramuscular administration of ketamine (90 mg/kg).

2.7.2. Histological observation of ACF formation

After treatment, 10–15 pieces of colonic tissues containing the nodule shape of ACF were dissected from the mid-distal part of each colon. They were placed in a petri dish containing saline solution and were washed and dried to be embedded in paraffin wax. These samples were sectioned parallel using a microtome and stained with hematoxylin and eosin (HE). Histopathological identification of ACF was based on the criteria of multiplicity of aberrant crypts. Multiplicity was determined as the number of crypts in each focus and categorized as containing up to two, three or more aberrant crypts/focuses. Fixed colorectal tumors were processed and stained, and crypts were identified as described.

2.8. Cell viability assay

The human liver cancer cell line HepG2 and the human colon

adenocarcinoma cell line HT 29 were purchased from National Centre for Cell Science (NCCS), Pune, India and cultured in DMEM medium with supplementation of 10% FBS, penicillin, streptomycin and fungizone. After 24 h of incubation in serum-free medium, the cells were exposed to isolated pure compounds at the indicated concentrations for 48 h for MTT assay. The cell viability of different test compounds was determined using the equation: (OD_{570} of the treated sample/OD_{570} of the untreated sample) × 100. All test samples mentioned above were dissolved in DMSO. The final concentration of DMSO was < 0.1% and 0.1% DMSO was used as control.

2.9. Data analysis

All the experiments were conducted in triplicates. The results were depicted as mean ± SD (standard deviation). One way ANOVA was done by using SPSS version 16 and graph pad prism software through Duncan's multiple range tests for comparing means with significance levels of $p < 0.05$ and $p < 0.01$.

3. Results and discussion

3.1. NBE inhibiting property on ACF formation in AOM induced animal model

To explicate the overall performance of isocoreopsin, butrin and isobutrin, a well-established short- and long-term protocol was used to determine NBE efficacy on inhibiting ACF formation in AOM induced rats. In terms of body weight, NBE did not show any considerable changes among the various groups. During necropsy, no pathologic alterations were found in any organs including the liver, lungs, and kidneys. Moreover, the colonic ACF formation due to AOM treatment was confirmed under the observation of light microscope after HE staining (Fig. 1). The inhibitory effect of different doses of NBE on AOM-induced colonic ACF formation is shown in Fig. 2. Male Wistar albino rats injected with 50, 100 and 150 mg/kg NBE showed significant decrease in the number of total mean ACF/colon (70%–80%; $p < 0.01$) compared with the rats injected with AOM alone (Fig. 2). Whereas AOM induced rats contained three or more aberrant crypts, which were reduced significantly after the treatment with NBE at 150 mg/kg when compared to silibinin (positive control) treated group. These studies implied that NBE exhibited a protective effect on AOM induced CRC.

3.2. Identification of isocoreopsin, butrin and isobutrin from NBE

The NBE was subjected to 60–120 mesh size silica gel column chromatogram to furnish two fractions and their purity was confirmed by HPTLC. The fraction I obtained from column chromatography contained only one compound (Compound 1). The HPTLC results clearly indicated that the fraction Ⅱ was confirmed to be isomerized compounds, butrin and isobutrin, with the Rf values of 0.68 and 0.65 min, respectively, when developed with methanol: EtoAC (30:70, v/v). Further, the fraction Ⅱ was subjected to reverse-phase HPLC C_{18} column with acetonitrile as mobile phase. Initially, it was shown that two inseparable peaks were very close to each other at a retention time of 16 min, and then were separated by using 0.1% phosphoric acid in mobile phase solution with the retention time of 19.9 and 26.2 min. Surprisingly, these two purified compounds were later found to be isomers (Compound 2 and 3) (Fig. 3).

Table 2.
Grouping of animals and dose of test agents against AOM induced colon cancer animals.

Groups	Test agent	Dose (mg/kg)
Group 1	Saline only	0
Group 2	AOM only	15
Group 3	AOM+Silibinin	100
Group 4	AOM+(n-butanol) extracts	50
Group 5	AOM+(n-butanol) extracts	100
Group 6	AOM+(n-butanol) extracts	150

Compound 1 (Fig. 4) was obtained as a pale yellow amorphous solid and had a molecular formula of $C_{21}H_{22}O_{10}$ based on ESI–MS. The IR spectrum of compound 1 indicated the presence of hydroxyl (3400 cm^{-1}) and carbonyl (1645 cm^{-1}) groups. The ^{1}H NMR spectrum evidenced signals assignable to seven multiple protons at ä 0.70 and 1.05, a methylene group adjacent to ketone at ä 2.05 and 2.25, all six D2O-exchangeable phenolic protons at ä 4.76 and 5.53, five aromatic protons appeared as multiplets at ä 6.34 and 7.46 and another aromatic proton adjacent to ketone appeared as multiplet at ä 7.69 and 7.77. Similarly, the ^{13}C NMR spectrum of compound 1 contained 21 signals indicating one methylene (ä 28.9), one acyclic oxymethine (ä 60.7), four cyclic oxymethines (ä 73.0 and 77.1), one oxygen-attached tertiary carbon and two aromatic olefinic methines (ä 102.5), six aromatic methines (ä 113.4, 115.3, 128.3 and 145.1), two aromatic tertiary carbons (ä 163.0 and 164.6) and a ketone carbonyl carbon (ä 190.0). In addition, the ESI–MS fragmentation of compound 1 also yielded the structural information. A fragment ion at *m/z* 273.07 (M – 162.05) occurred from loss of 2-(hyroxymethyl) tetrahydro-2 h-pyran-3, 4, 5-triol group in compound 1. Our results indicated that ^{1}H and ^{13}C NMR data of compound 1 significantly resembled those in Table 1. Thus, the structure of compound 1 was identified as isocoreopsin (2-(3, 4-Dihydroxy-phenyl)-7-(3, 4, 5-trihydroxy-6-hydroxymethyl-tetrahydro-pyran-2-yloxy)-chroman-4-one (1)).

Compounds 2 and 3 were obtained as pale yellow thin film and the molecular formula were assigned to be $C_{27}H_{32}O_{15}$ based on the ion peak at 597 $(M+Na)^{+}$ in the ESI–MS spectrum. The ESI–MS spectrum of compound 2 gave two fragment ions as those of compounds 1 and 3. The ^{1}H and ^{13}C NMR data of compound 2 significantly resembled those in Table 1. Moreover, these results coincided with the early report by Gupta et al. [25]. Thus, the structure of compound 2 was identified as butrin (2-[4-Hydroxy-3-(3,4,5-trihydroxy-6-hydroxymethyl-tetrahydro-pyran-2-yloxy)-phenyl]-7-(3, 4, 5-trihydroxy-6 hydroxymethyl-tetrahydro-pyran-2-yloxy)-chroman-4-one (1)). Further, the yellow amorphous solid of compound 3 was obtained and the ion peak at 597 $(M+H)^{+}$ in the ESI–MS spectrum indicated that this molecular formula could be $C_{27}H_{32}O_{15}$. Also, the ESI–MS spectrum of compound 3 imparted two fragment ions. Interestingly, the ^{1}H and ^{13}C NMR data of compound 3 considerably resembled those of the compounds in Table 1. On the basis of the above results, the structure of compound 3 was identified as isobutrin (3-[4-Hydroxy-3-(3, 4, 5-trihydroxy-6-hydroxymethyl-tetrahydro-pyran-2-yloxy)-phenyl]-1-[2-hydroxy-4-(3,4,5-trihydroxy-6-hydroxymethyl-tetrahydro-pyran-2-yloxy)-phenyl]-propenone).

Our results demonstrated that three compounds in NBE were identified and the most prominent among them was compound 1. It would be interesting to know that the greater yield of compound 1 from NBE may be a better source than the earlier report of EtoAC extract. However, significant amounts of compounds 2 and 3 were isolated as mentioned previously by Gupta et al. [25]. Surprisingly, isocoreopsin closely resembles silybin, silymarin and quercetin, which show promising effects in treatment of various diseases. This indicates that the full elucidation of extracted compounds of *B. monosperma* flowers focuses better insight into its nutritional and medicinal properties.

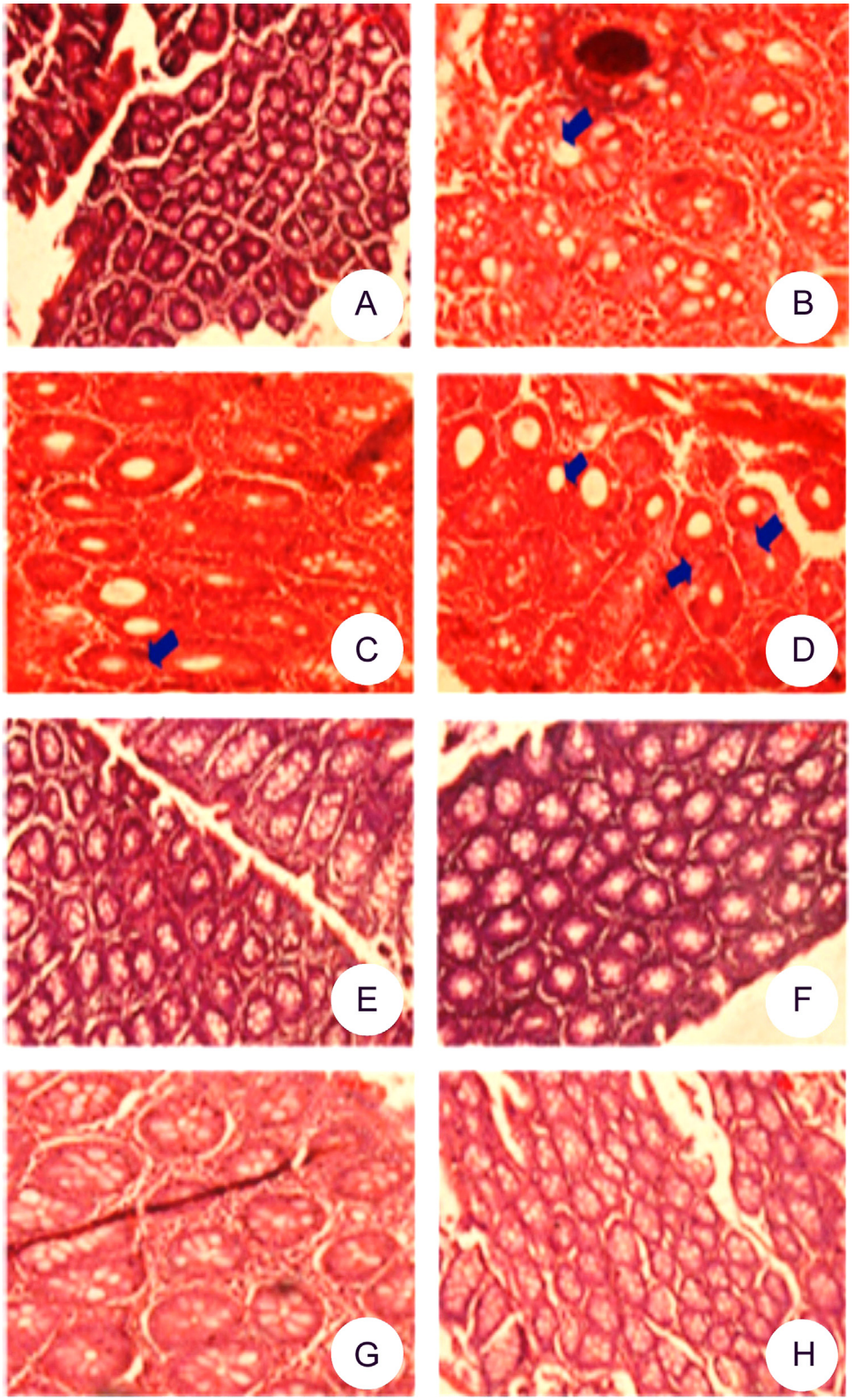


Fig. 1. Histopathological examination of ACF formation in experimental groups (hematoxylin-eosin staining). (A) Normal colon, (B) ACF with single crypt, (C) ACF with dual crypts, (D) ACF with three crypts, (E) Silibinin treated group (short term), (F) Silibinin treated group (long term), (G) NBE treated group (short term) and (H) NBE treated group (long term).

3.3. *Characterization of bioactive contents from NBE*

Previous studies have reported that the maximum concentration of flavonoid (25.29%) is present in NBE rather than in the absolute ethanol, methanol and other aqueous extracts [26,27]. In this study, for the first time, we have shown the flavonoid contents of all three compounds from NBE to access their bioactivity of each individual. The flavonoid contents of three different compounds of

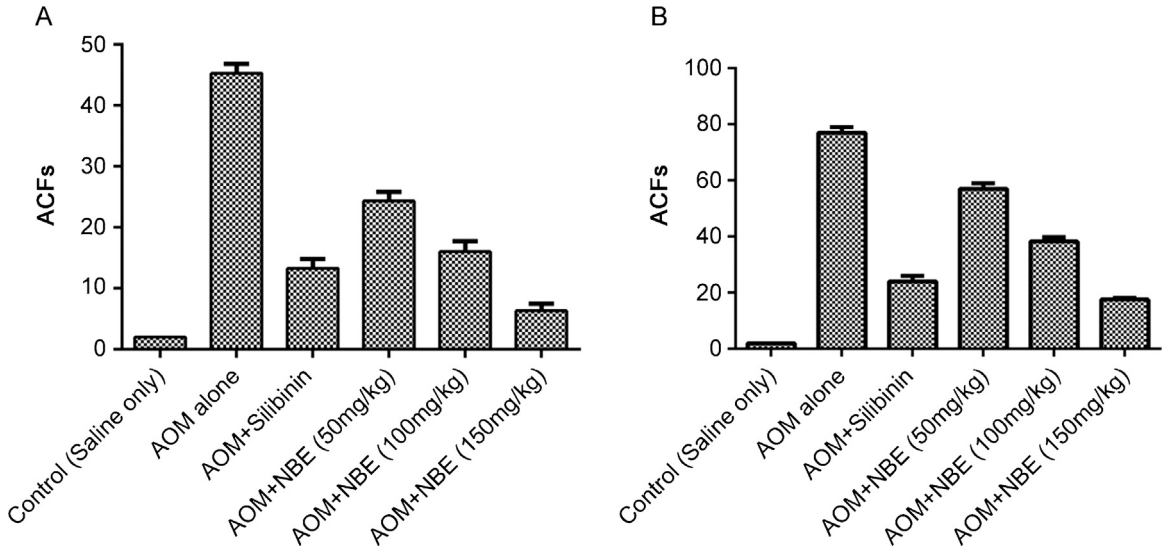


Fig. 2. Effects of n-butanol extract (NBE) on aberrant crypt formation: (A) NBE effects on AOM induced short-term and (B) NBE effects on AOM induced long-term experiment.

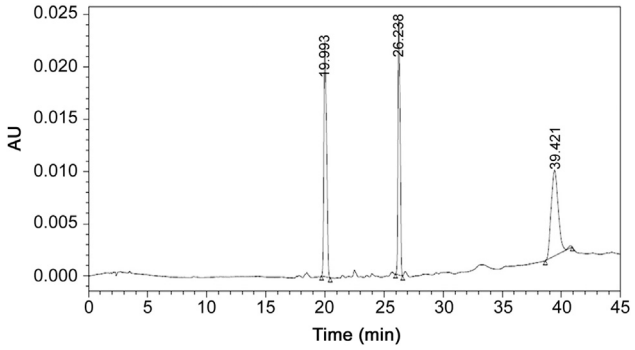


Fig. 3. HPLC results of butrin and isobutrin with retention time of 19.9 and 26.2 min, respectively.

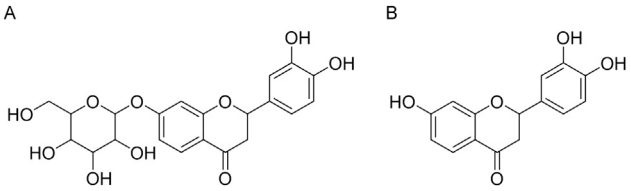


Fig. 4. (A) Structure of isocoreopsin and (B) its fragmentation ion at *m/z* 272.

NBE (isobutrin > isocoreopsin > butrin) are shown in Table 3. The HPLC fraction of isobutrin showed higher flavonoid content (96.00 ± 0.130) than those of other two compounds in NBE.

3.4. Free radical scavenging activity of NBE

Fig. 5 depicts the dose-dependent DPPH radical scavenging activity of isolated compounds from NBE. Antioxidant substance inhibited the spread of free radicals in biological systems by undergoing a redox reaction with the phenolic compounds, which might restrain the polymerization of free radicals and other oxidizing reactions [28]. Based on the concentration, the phenolic compounds established a dual mode of mechanism in antioxidant (low concentration) and pro-oxidant (high concentration) effects [29]. The EC50 values of extracts ranged from 10 to 50 μg/mL and isocoreopsin of flower extracts furnished the highest DPPH radical scavenging activity with EC50 values of 10 μg/mL. The earlier results interpreted that butein had a strong antioxidant activity (87% at 1 mg/mL) with EC50 value of 0.36 mg/mL when compared to butrin (22%) [30]. These findings clearly indicate that the isolated compounds from n-butanol have significant activity at the minimum dose. Since it has been suggested that flavonoids are potential antimutagenic, antiallergenic, antiviral, anti-inflammatory, antioxidant, anticarcinogenic agents and are reported to induce apoptosis, inhibit cell proliferation and angiogenesis [31]. From these studies, it implies that these three active compounds may have broader medical values, especially chemopreventive efficacy against human cancer.

Table 3.
Flavonoid contents of *B. monosperma* flower extract compounds.

Extract of *B. monosperma* flowers	Total flavonoid (μg naringenin equiv/mg extracts)
Isocoreopsin	79.82 ± 0.295^{a}
Butrin	5.68 ± 0.286^{b}
Isobutrin	96 ± 0.130^{c}

[a,b,c] $p < 0.05$ between the values.

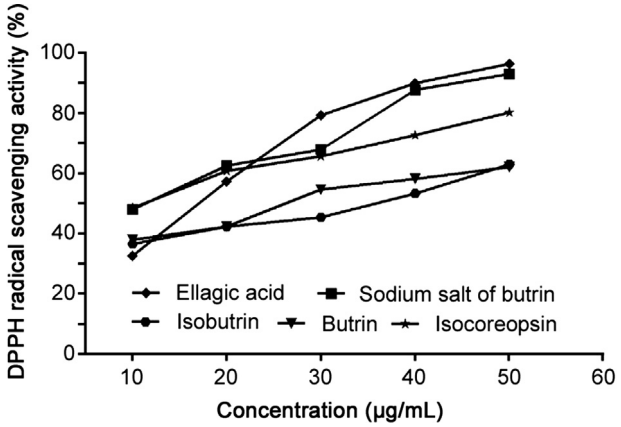


Fig. 5. Dose-dependent DPPH free radical scavenging activity of compounds in NBE.

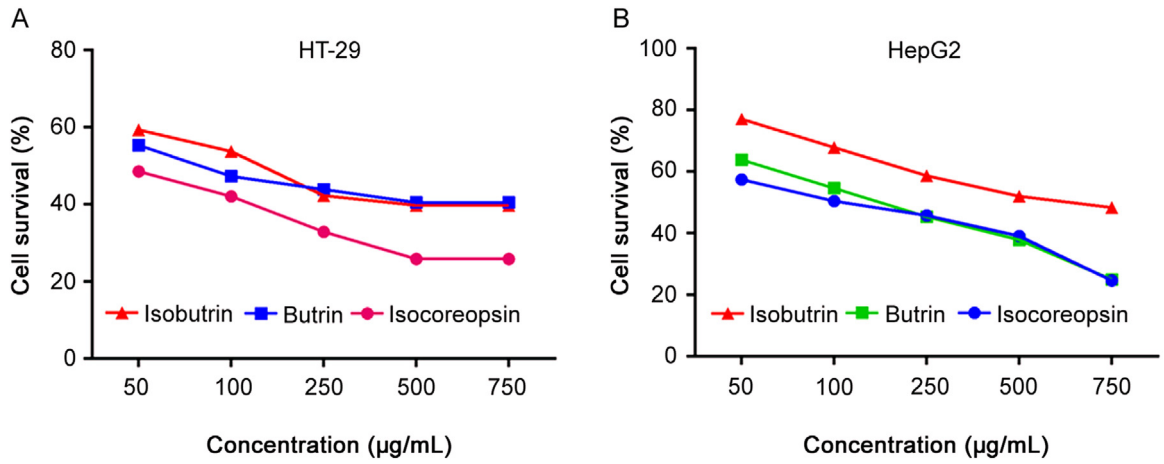


Fig. 6. Anticancer activity of three compounds in NBE on (A) human colon cancer and (B) liver cancer cell lines at 48 h.

3.5. Anticancer activity

All three compounds isolated in this study showed antiproliferative activity against HT-29 and HepG2 cell lines (Fig. 6). However, significant response was achieved for isocoreopsin with the concentration of 50 µg/mL, followed by butrin and isobutrin (100 µg/mL and 500 µg/mL) at 48 h. Our results also indicated that isocoreopsin against HT-29 cell line revealed interesting differential antiproliferative activities with more than two fold effects when compared to that for HepG2 cell line. Whereas, the butrin (150 mg/mL) produced approximately similar results in both cell lines and isobutrin (500 mg/mL) was significantly less effective in HepG2 cell line. Nevertheless, these results revealed that the compound isocoreopsin has potential cytotoxicity in suppressing colon and hepatocarcinoma cancer cell lines.

Wnt signaling gene is responsible for malignancies in colon, rectum, skin, liver, brain and prostate cells, due to the activation of β-Catenin [32]. Moreover, abnormal expression of GSK-3β (Glycogen synthase kinase-3β), APC (Adenomatous polyposis coli) and β-Catenin genes leads to down regulate the downstream targeted genes of tumor suppressor genes and it has been observed in >90% of colorectal cancer development. This implies that it has a possibility to inhibit Wnt/β-Catenin (HT-29) and NF-kB (HepG2) signaling pathways. Interestingly, our results clearly indicated that the individual compound exhibited cell-growth inhibition, when compared to the earlier anticancer property of butein, butrin and aqueous extracts of *B. monosperma* with different cancer cell lines [33]. Our previous studies on silico docking demonstrated that the active compounds, isobutrin, butrin and isocoreopsin, isolated from *B. monosperma* flowers have strong binding interaction with Wnt β-catenin protein which is associated with CRC. Moreover, the isocoreopsin obeyed the Lipinski rule of five parameters and fulfilled the ADMET predictions. This proposes that it may have potential anticancer activity against CRC [34,35].

4. Conclusion

In the present study, the 150 mg/kg of NBE treated group showed the strongest inhibitory effect and decreased the total number of ACF formation (per rat) by 92% (short-term) and 78% (long-term), respectively. These results suggest that the n-butanol extracts may have potential chemopreventive efficacy by inhibiting ACF formation in colon and mainly contain three bioactive compounds, namely isocoreopsin, butrin and isobutrin. The novel glucoside isocoreopsin of NBE showed a better free radical scavenging and anticancer activity. However, the flavonoid contents for these three different compounds differed significantly. Our results clearly illustrate the significant antiproliferative effect of isocoreopsin on colorectal cancer cell line and that it may be facilitated to target the subtype specific cancer treatments in future. Further investigation on molecular pathways of cancer targeted genes will be a worthy endeavor for better understanding of the bioactive compounds from *B. monosperma*.

Acknowledgments

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References

[1] Y. Jun, Z. Wen, M. Paul, et al., Adenomatous polyposis coli (APC) differentially regulates β-Catenin phosphorylation and ubiquitination in colon cancer cells, J. Biol. Chem. 28 (2006) 17751–17757.
[2] C.S. Hung, H.H. Liu, M.T. Huang, et al., Knockdown survivin expression reduces the efficacy of curcumin treatment in hepatocellular carcinoma cells, Ann. Surg. Oncol. 19 (2012) 3547–3555.
[3] R. Cabrera, D.R. Nelson, Review article: the management of hepatocellular carcinoma, Aliment. Pharmacol. Ther. 31 (2010) 431–476.
[4] Y.J. Chang, C.J. Tai, L.J. Kuo, et al., Glucose-regulated protein 78 (GRP78) mediated the efficacy to curcumin treatment on hepatocellular carcinoma, Ann. Surg. Oncol. 18 (2011) 2395–2403.
[5] R.P. Bird, Role of aberrant crypt foci in understanding the pathogenesis of colon cancer, Cancer Lett. 93 (1995) 55–71.
[6] A.P. Fields, S.R. Calcagno, M. Krishna, et al., Protein kinase C is an effective target for chemoprevention of colon cancer, Cancer Res. 69 (2009) 1643–1650.
[7] T. Mainardi, S. Kapoor, L. Bielory, Complementary and alternative medicine: herbs, phytochemicals and vitamins and their immunologic effects, J. Allergy Clin. Immunol. 123 (2009) 283–294.
[8] A. Ghasemzadeh, N. Ghasemzadeh, Flavonoids and phenolic acids: role and biochemical activity in plants and human, J. Med. Plants Res. 5 (2011) 6697–6703.
[9] K. Avula Muralidhar, K. Sudhakar Babu, T. Ravi sankar, et al., Evaluations of wound healing properties of bioactive fractions from the extract of *Butea monosperma* (lam) stem bark, Int. J. Phytomed. 3 (2011) 41–49.
[10] K. Sharma Ashish, M. Chaudhary, R. Kumar, et al., Evaluation of antiinflammatory and analgesic activity of *Butea monosperma* gum, Nov. Sci. Int. J. Pharm. Sci. 1 (2012) 60–64.
[11] B.P. Singh, S. Swati, Antimicrobial activity and spectral characterization of flower of *Butea monosperma*, Res. J. Pharm. Bio. Chemi. Sci. 3 (2012) 598–606.
[12] B.T. Divya, S. Mini, *In vitro* radical scavenging activity of different extracts of *Butea monosperma* bark, Int. J. Curr. Pharma. Res. 3 (2011) 3.
[13] Y.L. Ramachandra, C. Ashajyothi, S. Padmalatha, Evaluation of antidiabetic and

antipyretic activity of *Butea monosperma* (lam) extracts on rats, RaiInt. J. Preclin. Pharm. Res. 3 (2012) 73.
[14] J. Banu Rekha, B. Jayakar, Anti cancer activity of ethanolic extract of Leaves of *Butea monosperma* (Lam) Taub, Curr. Pharm. Res. 1 (2011) 106–110.
[15] G. Mathan, G. Fatima, K.A. Saxena, Gupta, et al., Chemoprevention with Aqueous extract of *Butea monosperma* flower results in normalization of nucleas morphometry and inhibition of a proliferation marker in liver tumors, Phytother. Res. 25 (2011) 324–328.
[16] C. Tenzin, S. Surendra Kumar, K. Vijay, Chemopreventive and anti-cancer properties of the aqueous extract of flowers of *Butea monosperma*, J. Ethnopharmacol. 129 (2010) 208–213.
[17] A. Sehrawat, V. Kumar, Butein imparts free radical scavenging, anti-oxidative and proapoptotic properties in the flower extracts of *Butea monosperma*, Biocell 36 (2012) 63–71.
[18] J.M. Wong, K. Mafune, H. Yow, et al., Ubiquitin-ribosomal protein S27a gene overexpressed in human colorectal carcinoma is an early growth response gene, Cancer Res. 53 (1993) 1916–1920.
[19] M. Bienz, H. Clevers, Linking colorectal cancer to Wnt signaling, Cell 103 (2000) 311–320.
[20] A. Hata, R.S. Lo, D. Wotton, et al., Mutations increasing auto inhibition inactivate tumour suppressors Smad2 and Smad4, Nature 388 (1997) 82–87.
[21] H. Wagner, B. Geyer, M. Fiebig, et al., Isobutrin and butrin, the antihepatotoxic principles of *Butea monosperma* flowers, Planta Med. 52 (1986) 77–79.
[22] M. Mack, R. Ashwell, Ndhlala, F. Jeffrey, et al., Phenolic composition, antioxidant & acetylcholinesterase inhibitory activities of *Sclerocarya birrea* and *Harpephyllum caffrum* (Anacardiaceae) extracts, Food Chem. 123 (2010) 67–76.
[23] A. Hegedus, R. Engel, L. Abranko, et al., Antioxidant and antiradical capacities in apricot (*Prunus armeniaca L.*) fruits: variations from genotypes, years, and analytical methods, J. Food Sci. 75 (2010) C722–C730.
[24] M.J. Michal, Colon cancer chemoprevention with ginseng and other botanicals, J. Korean Med. Sci. 16 (2001) 81–86.
[25] S.R. Gupta, B. Ravindranath, T.R. Seshadri, The glucosides of *Butea monosperma*, J. Phytochem. 9 (1970) 2231–2235.
[26] S. Manish, Lavhale, S.H. Mishra, Evaluation of free radical scavenging activity of *Butea monosperma* Lam, Ind. J. Exp. Bio. 45 (2007) 376–384.
[27] G. Piyush, B. Bibhilesh, S. Mendhe, et al., Antioxidant activity of flavonoidal content of *Butea monosperma*, Int. J. Pharm. Sci. Res. 1 (2010) 4.
[28] A. Lilia, Conde-Hernandez, A. Jose, et al., Total phenolic and antioxidant activity of *Piper auritum* and *Porophyllum ruderale*, Food Chem. 142 (2014) 455–460.
[29] S. Chung, M. Janelle, T. Mou, et al., Inhibition of carcinogenesis by dietary polyphenolic compounds, Annu. Rev. Nutr. 21 (2001) 381–406.
[30] R. Zafar, A. Nahid, K. Abubakar, et al., Butrin, isobutrin, and butein from medicinal plant *Butea monosperma* selectively inhibit nuclear factor-kb in activated human mast cells: suppression of tumor necrosis factor-α, interleukin (IL)-6 and IL, J. Pharm. Exp. Ther. 333 (2010) 354–363.
[31] E.C. Maria, F. Marta, S. Pilar, et al., Phenolic compounds in *Brassica* vegetable, Mol 16 (2011) 251–280.
[32] Y. Takigawa, A.M. Brown, Wnt signaling in liver cancer, Curr. Drug. Targets 9 (2008) 1013–1024.
[33] P.V. Prasad, P.K. Subhaktha, A. Narayana, et al., Palasa (*Butea monosperma* (Lamk.) Taub.) and its medico-historical study, Bull. Ind. Inst. Hist. Med. 36 (2006) 117–128.
[34] P. Navaneethakrishnan, C.N. Prashantha, S. Boopathi, et al., In silico design of *Butea monosperma* floral derived compounds and its inhibitory effect on β-Catenin, GSK-3β and APC complex proteins in colorectal cancer, Int. J. Drug. Dis. 5 (2013) 191–197.
[35] P. Navaneethakrishnan, C.N. Prashantha, S. Boopathi, et al., Antiproliferative activity of N-butanol floral extract from Butea monosperma against hct 116 colon cancer cells; drug likeness properties and in silico evaluation of their active compounds toward glycogen synthase kinase-3β/axin and β-catenin/T-cell factor-4 protein complex, Asian J. Pharm. Clin. Res. 8 (2015) 134–141.

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Extract from *Butea monosperma* inhibits β-catenin/Tcf signaling in SW480 human colon cancer cells

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ABSTRACT

The colorectal cancer (CRC) is the fourth leading cause of cancer death in worldwide. It has been found that > 90% of CRC is caused by aberrant activation of canonical Wnt/β-catenin signaling pathway. Continuous activation of this pathway believes to be an initiating event in colorectal carcinogenesis. There are growing evidences suggest that traditional herbal plant medicines are being raised as a complementary alternative treatment for cancer. In this series, *Butea* (*B*) *monosperma* has been illustrated as a valuable traditional medicinal plant with > 45 medicinal traits. Therefore, by targets this pathway using *n*-butanol fraction of *B. monosperma* flower extract (NBF-BMFE) could be a better therapeutic strategy for treating CRC. In this present study, we evaluate the inhibitory effect of NBF-BMFE against over activated Wnt signaling mediated colon cancer cells (SW480). Interestingly, the *in vitro* finding described that the NBF-BMFE had good antiproliferative effect against SW480 human colon cancer cells. Moreover, it showed significant level of down regulated expression in Wnt signaling proteins such as β-catenin, adenomatous polyposis coli (APC), glycogen synthase kinase 3β (GSK-3β), cyclin D1 and *c-myc* in time-dependent manner. Further, the *in silico* results of NBF-BMFE derived compounds have shown good binding interaction with target sites of β-catenin, APC and GSK-3β protein. In conclusion, NBF-BMFE may be used as an effective inhibitor for Wnt signaling targeted combined chemotherapeutic agents against CRC.

1. Introduction

The colorectal cancer (CRC) is diagnosed as third most common form of cancer in men (6,63,000 cases, 10% of the total cancers) and second in women (5,70,000 cases, 9.4% of the total cancers) (Jemal et al., 2011). Aberrant activation of Wnt/β-catenin signaling pathway is a hallmark of the majority of CRCs, results in increased stability of protein β-catenin (Barker and Clevers, 2006). Continuous activation of Wnt/β-catenin signaling is believing to be an initiating event in colorectal carcinogenesis (Fodde et al., 2001; Giles et al., 2003).

In the absence of Wnt signaling, the destruction complex consisting of adenomatous polyposis coli (APC), glycogen synthase kinase 3β (GSK-3β), casein kinase 1 (CK1) and scaffolding protein axin catalyses the phosphorylation of cytoplasmic β-catenin leading to its proteosomal degradation at key amino-terminal Serine (SER) and Threonine (THR) residues (Miller et al., 1999). In the presence of Wnt, transmembrane receptors Frizzled and low density lipoprotein receptor- related protein (LRP5/6) recruit the cytoplasmic protein dishevelled (Dvl) and Axin to the receptor complex, which results in the disruption of the β-catenin destruction complex (Bilic et al., 2007). Then, it allows β-catenin to

Abbreviations: CRC, colorectal cancer; NBF-BMFE, *n*-butanol fraction of *B. monosperma* flower extract; APC, adenomatous polyposis coli; GSK-3β, glycogen synthase kinase 3β; CK1, casein kinase 1; LRP5/6, low density lipoprotein receptor- related protein; Dvl, dishevelled; Tcf, T-cell factor; LEF, lymphoid enhancer factor; DMEM, Dulbecco's modified Eagle's medium; PBS, phosphate buffered saline; FBS, fetal bovine serum; DMSO, dimethyl sulfoxide; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; DCFH-DA, dichlorodihydro-fluorescein diacetate; PVDF, polyvinylidine difluoride; HRP, horse radish peroxidase; NCCS, National Centre for Cell Science; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; DAB, 3,3′-diaminobenzidine; Nacl, sodium chloride; TBS-T, Tris buffered saline – Tween 20; PDA, photodiode array; LC/MS, liquid chromatography/mass spectrometry; PDB, protein data bank; LGA, Lamarckian Genetic Algorithm; IC_{50}, half maximal inhibitory concentration; ROS, reactive oxygen species; NSAIDs, nonsteroidal anti-inflammatory drugs; ASN, asparagine; GLY, glycine; CYS, cysteine; GLU, glutamic acid; ALA, alanine; GLN, glutamine; LYS, lysine; HIS, histidine; LEU, leucine; SEP, serine phosphatase; ILE, isoleucine; MET, methionine; TYR, tyrosine; ASP, aspartic acid; TPO, thyroid phosphatase; H, hour; mg, milligram; ng, nanogram; mM, millimolar; nM, nanomolar; mL, millilitre; μL, microlitre

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accumulate in the cytoplasm and subsequently translocate to the nucleus, where its directly binds with the T-cell factor (Tcf)/lymphoid enhancer factor (LEF) family, and these interactions stimulate transcription of Wnt target genes (Mao et al., 2001).

Recently, Herbal medicines and their derived phytocompounds are being raised as a complementary treatment for cancer. In this series, *Butea monosperma* Lam. Kuntze (Fabaceae), commonly known as flame of the forest, have been illustrated as a valuable traditional medicinal plant with > 45 medicinal attributes. Among its different parts, flower is the one which is associated with several pharmaceutical effects (Burlia and Khadeb, 2007; Choedon et al., 2010). Phytochemical analysis of *B. monosperma* flowers constituents showed the presence of flavonoid glucosides such as butrin, isobutrin, butein, butin, sulfurein, coreopsin, isocoreopsin, monospermoside and isomonospermoside (Wagner et al., 1986; Gupta et al., 1970). Ethanol extract of *B. monosperma* flowers have been shown as anti-hyperglycemic, anti-diabetic and anti-fertility activity (Somani et al., 2006; Razdan et al., 1970). The Same extract has also possess hepatoprotective and anti-tumorigenic effect (Sehrawat and Sultana, 2006; Mathan et al., 2011). Whereas, the petroleum ether extract of *B. monosperma* flowers has been showed to exhibit anticonvulsive activity, due to the presence of triterpenes (Kasture et al., 2002). These constructive effects were attributed to several class of compounds identified in *B. monosperma* flowers extracts.

The preliminary reports of *B. monosperma* flower extract had showed the cytotoxic effect against human colon cancer cells. So far there are no reports of this extract against wnt/β-catenin signaling mediated CRC on SW480 cells. Based on these reports, this present study was aimed to assess the *n*-butanol fraction of *B. monosperma* floral extracts (NBF-BMFE) inhibitory effects on Wnt/β-catenin signaling proteins against CRC in SW480 cells.

2. Materials and methods

2.1. Preparation of plant extracts and fractions

The *B. monosperma* flowers were collected from the Bharathidasan University Campus, Tiruchirappalli. The voucher specimen was deposited in the Department of Plant Science, Bharathidasan University (Tiruchirappalli, Tamil Nadu, and India). The *B. monosperma* flowers were dried and ground into a fine powder and their preparation of extract has described previously (Wagner et al., 1986). In brief, the dried powder (500 g) of *B. monosperma* flower was extracted with methanol in a Soxhlet extractor for 20 h and solvent was removed under reduced pressure in a rotatory evaporator. From that, we obtained 130 g of an orange powder. Further, the 20 g of total methanol extract was partitioned between water and Ethyl acetate (3 times). Then after removal of the ethyl acetate fraction, the remaining water phase was treated with *n*-butanol (3 times). Both the ethyl acetate and the *n*-butanol fractions were dried using sodium sulfate.

2.2. Chemicals and antibodies

Dulbecco's modified Eagle's medium (DMEM) and phosphate buffered saline (PBS) were purchased from Sigma-Aldrich, USA. The Fetal bovine serum (FBS), Trypsin-EDTA, Lipofectamine 3000 and penicillin-streptomycin antibiotic were obtained from Invitrogen, USA. Dimethyl sulfoxide (DMSO), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), Dichloro-dihydro-fluorescein diacetate (DCFH-DA) and Silibinin was purchased from Sigma Aldrich, USA. The polyvinylidine difluoride (PVDF) membrane was purchased from Pall life sciences, USA. Antibodies to β-catenin, APC, GSK-3β, cyclin D1, *c-myc* and β-actin were obtained from Santa Cruz Biotechnology, USA. The secondary antibodies, horse radish peroxidase (HRP) conjugated anti-mouse IgG and anti-rabbit IgG were obtained from Santa Cruz Biotechnology, USA.

2.3. Cell culture

Human colon cancer cells SW480 was obtained from American Type Culture Collection (ATCC), Rockville, USA. HCT-116, HT-29 and SW-620 cells were obtained from National Centre for Cell Science (NCCS), Pune, India. The cells were maintained in DMEM medium supplemented with 10% FBS and antibiotics (100 U/mL penicillin and 100 µg/mL streptomycin) in a humidified atmosphere of 5% CO_2 and 95% air in a CO_2 incubator.

2.4. MTT assay

Cell growth was assessed by using 3-(4,5-dimethyl-thiazol-2yl)-2,5-diphenyl-tetrazolium bromide (MTT) as described previously (Reddy et al., 2006). At first, 5×10^4 cells per well was seeded in 96-well plate and after 24 h the cells were incubated with different concentrations of NBF-BMFE and Silibinin up to 1000 µM for 24 and 48 h. After incubation, the medium containing compounds were removed and 100 µL MTT (5 mg/ml in PBS) was added to each well. After 3 h incubation in dark, MTT was discarded and 150 µL DMSO was added to each well. Absorbance at 570 nm was measured with an ELIZA micro plate reader. The percent cell viability was then calculated.

2.5. Measurement of intracellular reactive oxygen species (ROS)

The measurement of intracellular ROS formation was based on the oxidative conversion 2′,7′-dichlorofluorescein- diacetate (DCFH-DA) to fluorescent compound dichloroflouorescin (DCF) (Jia et al., 2006; Lin et al., 2006). After treatment, SW480 cells were harvested and suspended in 0.5 ml PBS containing 10 µl DCFH-DA for 15 min at 37 °C in the dark. DCFH-DA was taken up by the cells and deacetylated by cellular esterase to form a non-fluorescent product DCFH, which was converted to a green fluorescent product DCF by intracellular ROS produced by treated SW480 cells. Fluorescent measurements were made with excitation and emission filters set at 488 and 530 nm, respectively. Fluorescence microscopic images were taken using blue filter (450–490 nm).

2.6. Cell cycle analysis

The SW480 cells were cultured in T25 flasks and incubated with the respective IC50 doses of NBF-BMFE for 24 and 48 h. After incubation, cells were harvested and fixed in 70% (v/v) ice-cold ethanol. Fixed cells were washed with PBS, incubated with 1 mg RNase/ml for 30 min before they were stained with propidium iodide. Finally, cell cycle distributions were analysed in a FACS caliber flow cytometer (BD BioSciences) and the percentage of cells in each phase was determined (Palanivel et al., 2013).

2.7. β-Catenin/Tcf transcription reporter assay

The SW480 cells were plated in 6-well plates, grown to 80–90% confluence and transiently transfected with the plasmids of TOPflash and FOPflash, respectively. It has 3 copies of the Tcf/Lef binding sites in the upstream of a thymidine kinase (TK) promoter and the firefly luciferase gene. Fop flash has mutated copies of Tcf/Lef sites and is used as control for measuring nonspecific activation of the reporter. All transfections were performed with Lipofectamine 3000 reagent and 1.8 µg of TOPflash or FOPflash plasmids. After transfection, cells were incubated in medium the IC50 value of NBF-BMFE for 24 h and 48 h and then lysed with reporter lysis buffer at harvest. A luciferase activity assay was conducted in a white 96-well plate and detected with a Victor × 2 multimode microplate reader (PerkinElmer, USA). Finally, steadylite plus reagent (PerkinElmer) was added to each well. Equal volumes of cell culture medium and steadylite plus reagent were mixed well and waited 10 to 15 min for complete cell lysis and to allow full

signal generation and measure the luminescence. The luciferase activity was normalized as luminescence intensity divided by the protein concentration of the same lysate sample (Zhang et al., 2012). The experiments were performed in triplicate and the results were reported as folds of induction when compared with control group.

2.8. Western blot analysis

Western blot analysis was performed essentially according to our standard protocol.(Yu et al., 2001) Briefly, the cells were solubilized in lysis buffer and the following centrifugation at 10,000 rpm for 15 min, the supernatant was used for Western blot analysis. In all the analyses, the protein concentration was determined by Bio-Rad protein assay kit (Bio-Rad, USA). Aliquots of cell lysates containing 50 μg of protein was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Following electrophoresis, the proteins were transferred electrophoretically onto PVDF membranes (Pall Life sciences, USA). The membranes were incubated for 1 h at room temperature with blocking buffer, TBS-T (20 mM Tris, pH 7.6, 100 nM NaCl, 0.1% Tween-20) and 5% non-fat dry milk with gentle agitation. After washing the membranes with TBS-T, they were incubated overnight at 4 °C in TBS-T buffer containing 2.5% milk and with one of the antibodies (1:1000 dilution): β-catenin, APC, GSK-3β, cyclin-D1, c-myc and β-actin. After, the membranes were washed three times with TBS-T, it subsequently incubated with secondary horse raddish peroxidase conjugated antibodies (1:5000 dilution) in TBS-T containing 2.5% milk for 2 h at room temperature with gentle shaking in a rocker. Then membranes were again washed with TBS-T and the protein bands were visualized by using the 3,3′-diaminobenzidine (DAB) chromogen system. All the Western blots were performed at least three times for each experiment.

2.9. LC/MS analysis

The NBF-BMFE was performed on LC system (SHIMADZU), equipped with Photo diode array (PDA) detector and electron spray ionization source. The mobile phase consists of 0.1% formic acid in water (Pump A) and acetonitrile (pump B). The sample was dissolved in 1 ml of water and filtered by using 0.22 μ syringe filter with the injection of 20 μl into C18 reversed phase column (Phemomenax, Luna, 5 μm particles size, 250 mm length, 4.6 mm internal diameter) by ambient conditions. A linear gradient has been used at 0 min,10% B to 26% B at 20 min and then 65% B at 35 min and finally with 100% B at 36 min (Table 3). The flow rate at 0.5 ml/min and the HPLC flow was directed into mass spectrophotometer, operated in both positive and negative mode. The fall out spectra was recorded from 200 to 800 nm and scanned over a mass range of 100 to 800.

2.10. Identification of protein target

The structural and functional characteristics of Wnt/β-catenin signaling proteins such as GSK-3β, APC and β-catenin are important to determine the cellular sensitivity of CRC. Three dimensional structures of these proteins were predicted using X-ray crystallography, which incurred from RCSB Protein databank such as GSK-3β (PDBID: 1IO9 with resolution of 2.05 Å), β-catenin/APC complex (PDBID: 1TH1 of resolution 2.50 Å) and β-catenin (PDBID: 2BCT of 2.90 Å).

2.11. Protein structure validation and active site prediction

The validation of 3D modelled structures over stereo chemical quality with residue-by-residue geometry by using Procheck. The residues of loop dictionaries and geometry statistics of non-bonded interactions with different atom types, error functions and plots score was predicted using ERRAT and WHAT_CHECK. Ramachandran plot helps to understand the stereo chemical quality of residue-by-residue geometry, calculated Z-score and volume that predicted the best quality of 3D complex structures. The Ligand binding sites and active sites of 3D modelled protein structures were analysed by using CastP calculation server (Binkowski et al., 2003).

Table 1
NBF-BMFE compounds obtained from PubChem compound data base.

Compound name	PubChem ID	Molecular formula
Butrin	164630	$C_{27}H_{32}O_{15}$
Isobutrin	5281256	$C_{27}H_{32}O_{15}$
Butein	5281222	$C_{15}H_{12}O_5$
Butin	92775	$C_{15}H_{12}O_5$
Coreopsin	25245366	$C_{21}H_{22}O_{10}$
Isocoreopsin	193124	$C_{21}H_{22}O_{10}$
Monospermoside	42607524	$C_{21}H_{22}O_{10}$
Isomonospermoside	42607822	$C_{21}H_{22}O_{10}$

2.12. Ligand preparation

The medicinally important Indian traditional plant *B. monosperma* floral extracted compounds such as butrin, isobutrin, butein, butin, coreopsin, isocoreposin, monospermoside, and isomonospermoside ligand molecules were retrieved from PubChem compound database (http://pubchem.ncbi.nlm.nih.gov/search/search.cgi) (Table 1). The ligand optimization was performed by using Hyperchem Professional 7.0 (Froimowitz, 1993).

2.13. Molecular docking

Molecular docking studies were carried out using AutoDock 4.2 and AutoDock Tools 1.5.4 from the Scripps Research Institute (http://www.scripps.edu/mb/olson/doc/autodock). The Lamarckian Genetic Algorithm (LGA) was used for ligand conformational searching and the local search algorithm which builds a population of individuals (genes), each being a different random conformation of the docked molecule (Morris and Lim-Wilby, 2008). The grid were generated around the active site at 80 × 80 × 80 to calculate molecular simulation using AMBER tools, showed auto grid of active site residues around the complex structure. There were 150 populations with mutation rate of 0.02, crossover rate of 0.8 and default grid spacing 0.375 Å were used as parameter settings for docking. Consequently, these simulations were performed using up to 2.5 million energy evaluations with a maximum of 27,000 generations and each simulation was performed by 10 times, that yielded 10 docked conformations. Finally, the lowest energy conformations were regarded as the binding conformations between ligands and the protein.

2.14. Statistical analysis

All experiments were performed in triplicate and the data are presented as Mean ± SD. Statistical analysis was performed adopting one-way ANOVA by using graph pad prism software. The significance level was set at $^*P < 0.05$ and $^{**}P < 0.01$.

3. Results and discussion

3.1. Cell growth inhibition property

The cytotoxic effects of NBF-BMFE and commercially available silibinin (Sigma Aldrich) were determined on SW480 human colon cancer cells at time and dose dependent manner for 24 h and 48 h. The effectiveness of compounds was measured by half maximal inhibitory concentration (IC_{50}) of cell death. The silibinin compound exhibited the IC_{50} values of 185 μg/ml and 100 μg/ml for 24 h and 48 h study. Similarly, the NBF-BMFE had significant antiproliferative effects with the IC_{50} value of 370 μg/ml and 200 μg/ml for 24 h and 48 h study

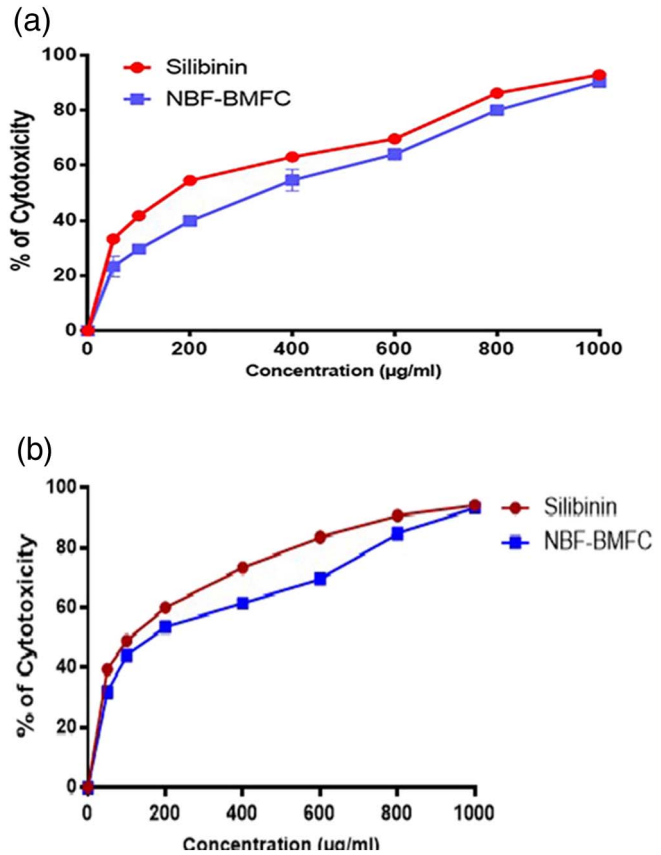

Fig. 1. Growth Inhibition effect of NBF-BMFE (50–1000 µg/ml) on SW480 human colon cancer cells at (a) 24 h and (b) 48 h with a dose and time dependent manner.

(Fig. 1). The subsequent experiments were performed with this concentration.

The colorectal carcinoma cell lines SW480 had expressed both mutated β-catenin and APC regions (Kaur et al., 2010). Moreover β-catenin mutations were detected in approximately 50% of the colorectal cancer (Sparks et al., 1998). The above findings suggested that disrupting the β-catenin mutation in Wnt signaling pathway may be one of the possible therapeutic approaches for treating colorectal cancer. Interestingly, our data clearly showed that the NBF-BMFE had effective cell growth inhibition against β-catenin expressing SW480 human colon cancer cells. In HCT-116 cells, NBF-BMFE and butein exhibited the IC50 value at $^{>}$100 µM (Supplementary data), previously butein showed 79% cell death at 100 µg on AML-12 cells (Anuradha and Kumar, 2012) after 48 h treatment. In another study, the silibinin and NBF-BMFE exhibited the cytotoxic effect at approximately 100 µM in HT-29 cells after 48 h treatment (Supplementary data). In SW620 cells the cytotoxic effect of NBF-BMFE was > 100 µM (data not shown). Overall, the NBF-BMFE had good anti-proliferative effect against human colon cancer cells.

3.2. ROS analysis

Reactive oxygen species (ROS) in small quantities is produced during normal cellular physiological processes but the level increase during stress and pathological conditions. The intracellular level of ROS is significantly higher in NBF-BMFE treated cells for 24 h and 48 h intervals, when compared to the control cells (Fig. 2). This propose that the ROS is involved in the triggering of apoptotic signaling and increased levels of ROS can induce depolarization of the mitochondrial membrane which eventually leads to an increase in the level of pro-apoptotic molecules in cells (Circu and Aw, 2010).

3.3. NBF-BMFE changes in cell cycle distribution

The mechanism underlying NBF-BMFE mediated cell growth inhibition and cell cycle distribution were evaluated by flow cytometry. The SW480 cells treated with NBF-BMFE suffered a significant inhibition of cell cycle progression in an IC_{50} value with time dependent manner (Fig. 3a & b). Particularly, there was a clear increase of the percentage of cells in the G1 phase, suggesting that the cells were inhibited in the progression of cell cycle. Since deregulation of the cell cycle machinery has been associated with cancer initiation and progression (Jiang et al., 2012). Moreover, inhibition of cell cycle

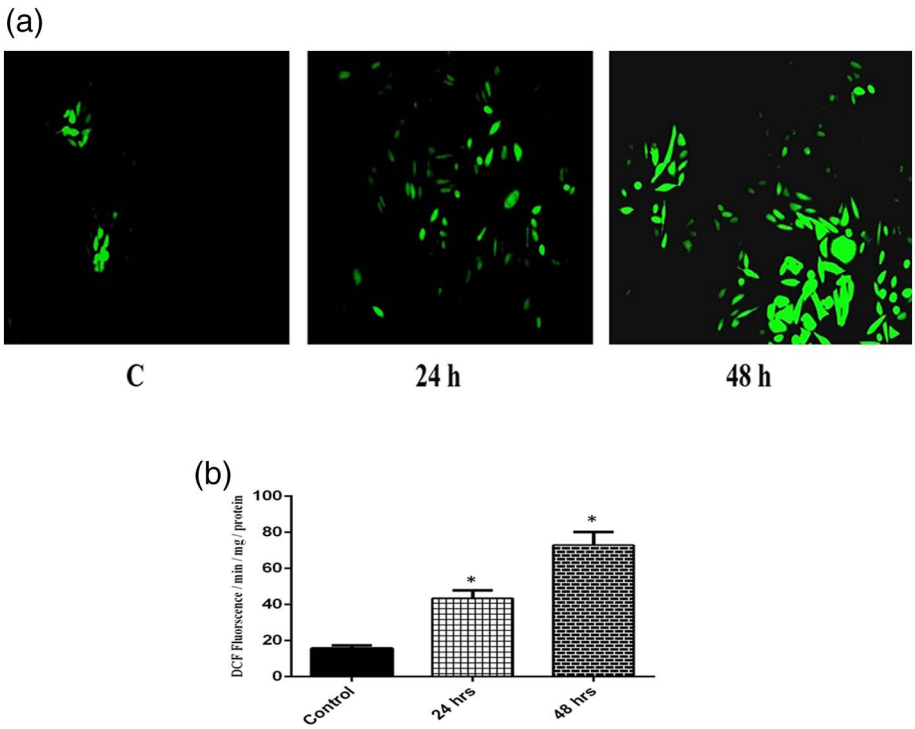

Fig. 2. Effect of NBF-BMFE on levels of ROS in SW480 cells. (a) SW480 cells were treated with IC_{50} concentration of NBF-BMFE for 24 and 48 h, stained with DCFH-DA and fluorescent measurements were made with excitation and emission filters set at 488 and 530 nm, respectively. (b) Values is expressed as unit mg/protein. Values represent mean ± SD. Values are statistically significant at *P < 0.05 (n = 3).

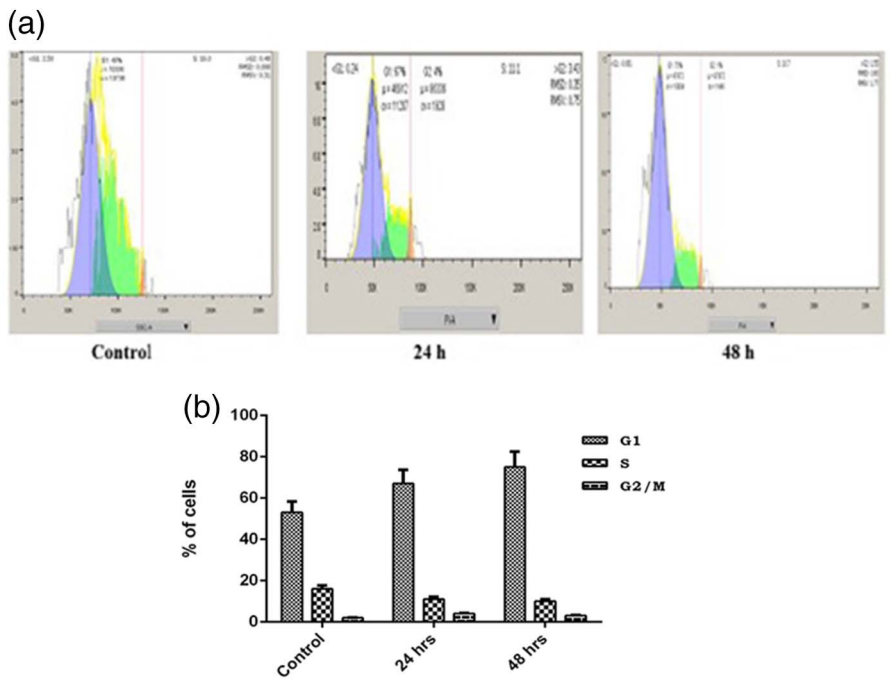

Fig. 3. Flow cytometric analyses of NBF-BMFE treated cells. (a) SW480 cells were treated with IC_{50} concentration of NBF-BMFE for 24 and 48 h, stained with propidium iodide and the DNA content was analysed by Flow cytometry. (b) Bar chart showing the percentage of cells at each phase.

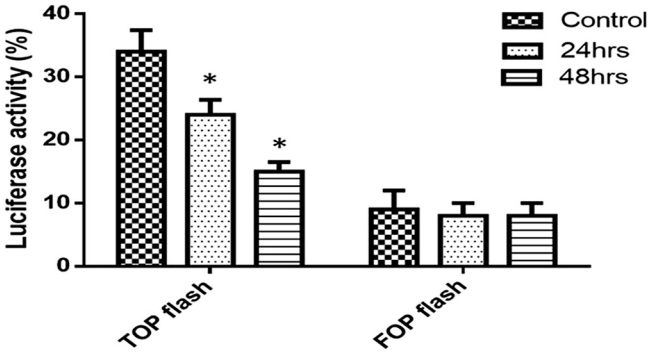

Fig. 4. NBF-BMFE inhibited the transcriptional activity of β-catenin/Tcf in SW480 cells. Values represent mean ± SD. Values are statistically significant at *P < 0.05 (n = 3).

progression is an appreciated target for management and treatment of cancers with cytotoxic agents. Interestingly, our data also suggested that NBF-BMFE has bring about cell cycle arrest at G1 phase in the SW480 human colon cancer cells.

3.4. NBF-BMFE inhibits β-catenin/Tcf pathway in human colon cancer cells

Colon cancer cells frequently possess mutations in APC or β-catenin and activated Wnt signaling resulting in the cellular accumulation of β-catenin. To investigate the inhibitory effect of NBF-BMFE on the transcriptional activity of β-catenin/Tcf in colon cancer cells, the TOP/FOPflash reporter gene assay was performed on SW480 (truncated mutation of APC and wild-type β-catenin) human colon cancer cells. The cells transfected with TOP/FOPflash reporter showed the highest transcriptional activity, further its transcription efficiency was normalized by using steadylite plus reagent sample (PerkinElmer). As shown in Fig. 4, After 48 h of treatment, the NBF-BMFE showed two fold reduction of Tcf-dependent luciferase activity (TOPflash) in SW480 human colon cancer cells, whereas FOPflash activity, a mutant of β-catenin/Tcf binding, remains unchanged. Aspirin and other non-steroidal anti-inflammatory drugs (NSAIDs), being used as chemopreventive agents against CRC, had been found to inhibit the Wnt/β-catenin signaling pathway (Takahashi-Yanaga and Sasaguri, 2007). Both

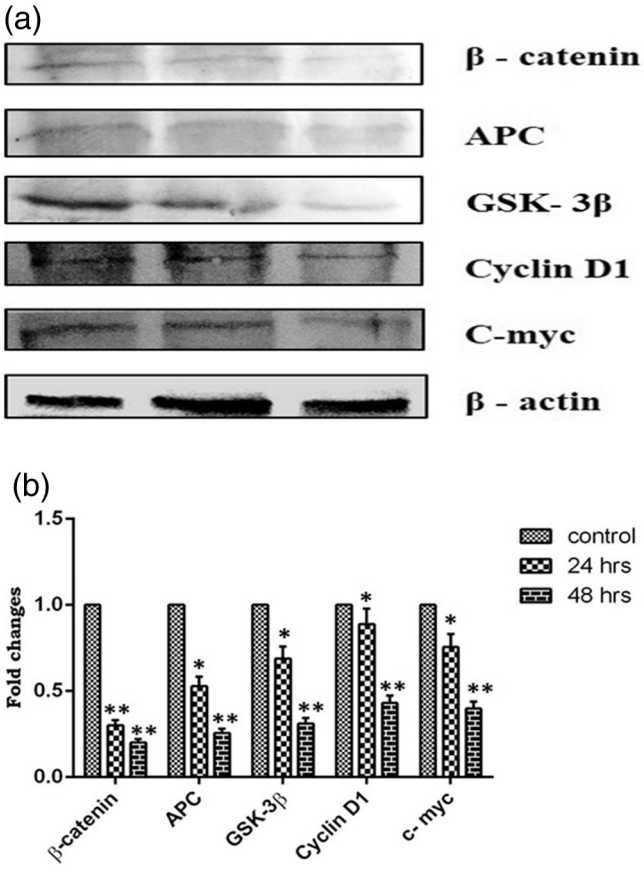

Fig. 5. Effect of NBF-BMFE on (a) the levels of β-catenin, APC, GSK-3β, cyclin D1 and c-myc in SW480 colon cancer cells. (b) Values represent mean ± SD. Values are statistically significant at *P < 0.05 **P < 0.01 (n = 3).

aspirin and indomethacin could decrease the transcriptional activity of β-catenin/Tcf-responsive genes (Dihlmann et al., 2001). Similarly, NBF-BMFE also found to reduce the transcriptional activity of Wnt/β-catenin signaling pathway.

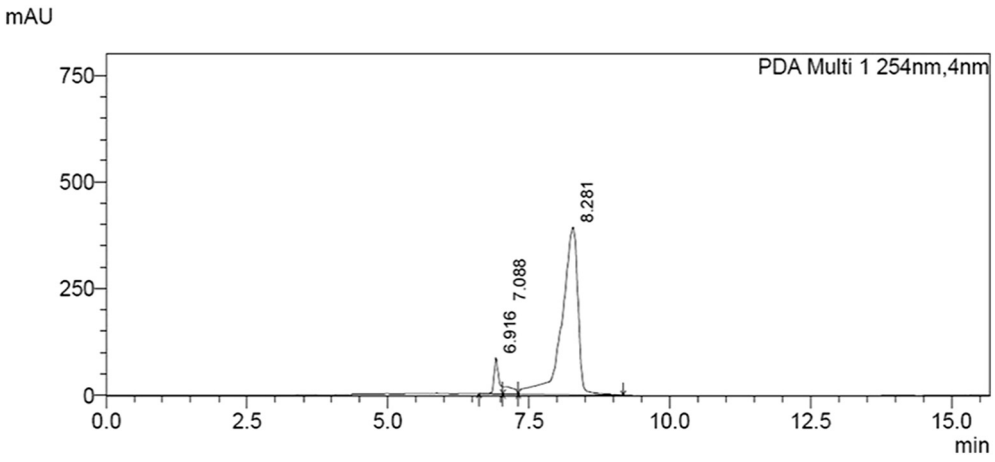

Fig. 6. HPLC chromatogram of NBF-BMFE.

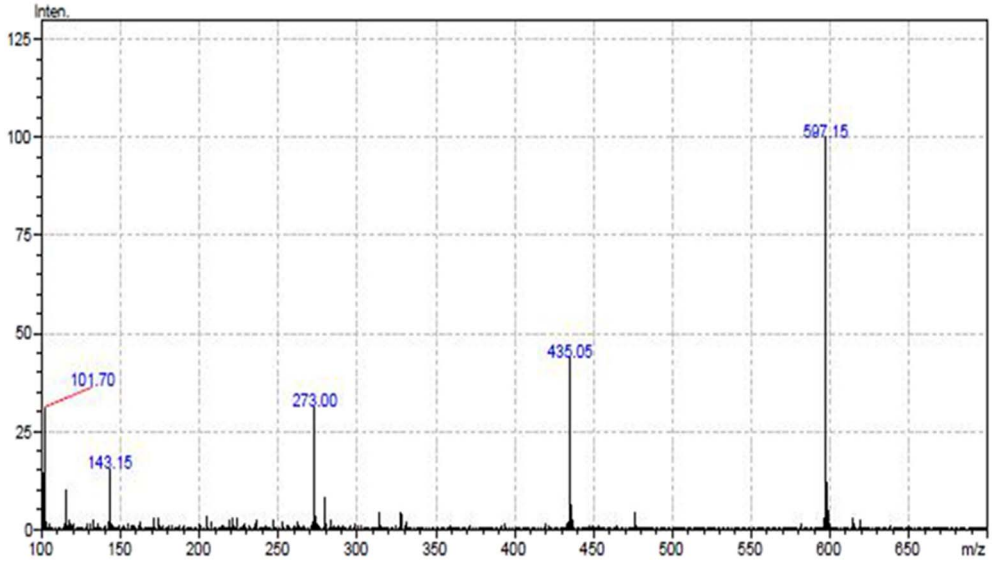

Fig. 7. Mass spectrum of NBF-BMFE.

Table 2
n-Butanol fraction of *B. monosperma* floral extracted compounds docked with β-catenin protein.

Ligand	Binding energy (Kcal/mol)	H bond	Amino acids
Butrin	− 8.98	3	CYS419, ASN426, THR418
Isobutrin	− 9.54	5	ASN426, GLY422, CYS419, THR418, GLU462
Butein	− 12.95	4	ALA392, GLN395, ASN434, LEU427
Butin	− 5.30	4	ALA392, GLN395, ASN434, ASN430
Coreopsin	− 10.14	7	ASN430, ASN431, LYS435, ARG469, LYS508, SER473, THR428
Isocoreopsin	− 10.04	3	GLU568, GLU571, LYS508
Monospermoside	− 4.26	6	LYS435, SER473, ASN516, ASN430, SER473, HIS470
Isomonospermoside	− 6.57	6	GLN395, ASN431, ASN434, ASN426, LEU427, CYS429

3.5. NBF-BMFE suppresses the expression of β-catenin/Tcf signaling pathway proteins and its downstream target genes

The destruction complex proteins β-catenin, APC, GSK-3β and its downstream target genes such as cyclin D1 and *c-myc* plays an important role in the regulation of Wnt signaling pathway as well in the progression of CRC. Henceforth, we examined the effect of NBF-BMFE on Wnt signaling proteins by using SW480 human colon cancer cells. After 48 h treatment, the IC_{50} value of NBF-BMFE was significantly down regulate the expression of β-Catenin, APC and GSK-3β with two fold reduction as well as cyclin D1 and *c-myc* were also significantly down regulate with one fold reduction when compared to control group (Fig. 5).

Dysregulation of β-catenin signaling is believed to be critical to the early stages of human colorectal carcinogenesis (Kobayashi et al., 2000). Also, most mutated APC proteins existed in colorectal tumors (Senda et al., 2007). Wnt/β-catenin signaling also appears to regulate cancer cell invasion, metastasis, and angiogenesis (Wang and Ma, 2007). Many studies were demonstrated that the expression of Wnt family ligands correlated with the invasion of colon cancer (Holcombe et al., 2002). In this study, we demonstrated that the NBF-BMFE was significantly downregulate the β-catenin/Tcf signaling proteins in SW480 cells.

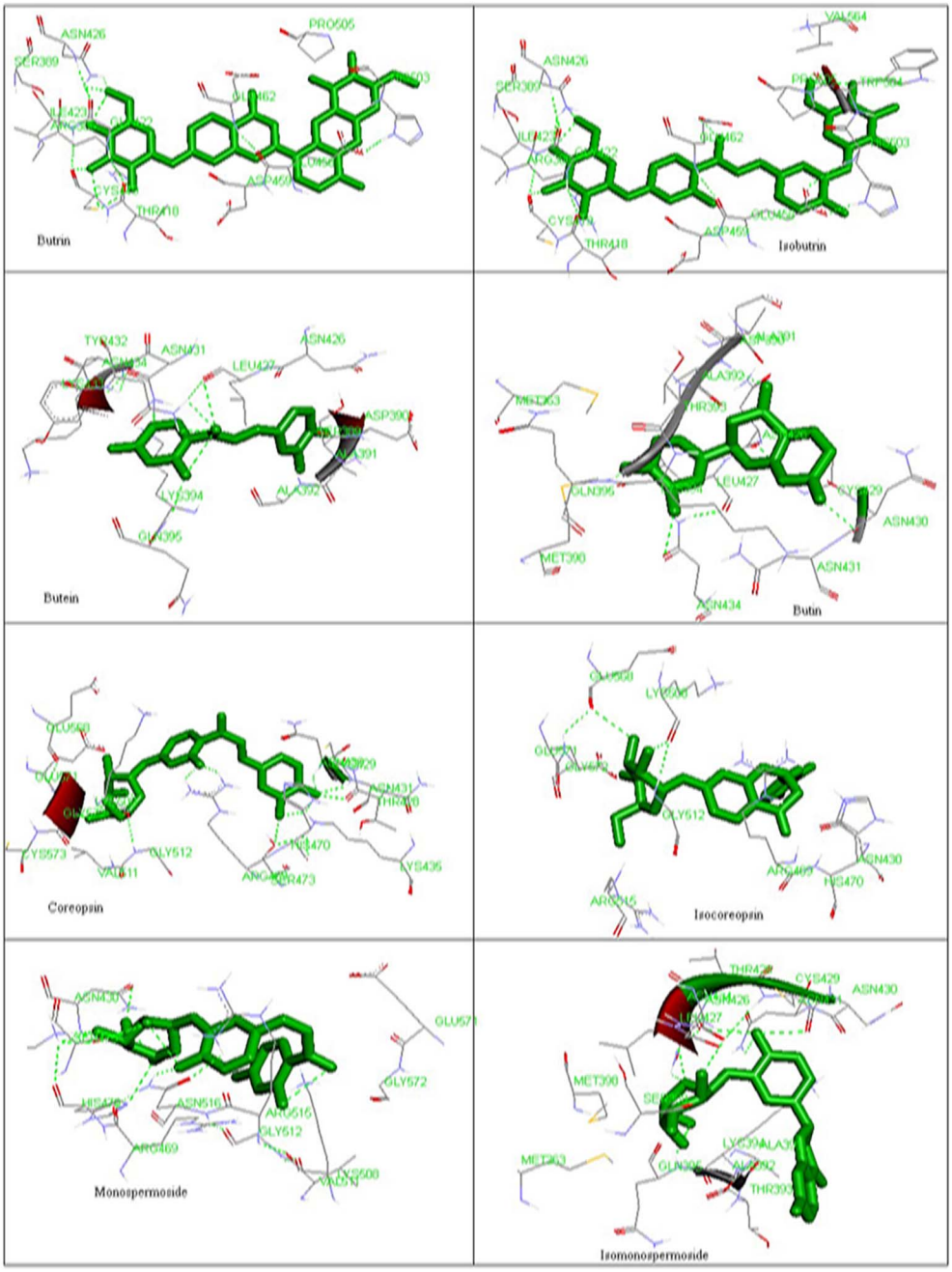

Fig. 8. Docking results of β-catenin with *n*-butanol fraction of *B. monosperma* floral extracted compounds (green dots denoted strong hydrogen bond interaction).

3.6. LC/MS analysis

The LC/MS results have showed that NBF-BMFE yielded two peaks at the retention time of 6.9 min and 8.2 min respectively, with highest purity of 94.7% (Fig. 6). Previous studies have showed molecular weights of butrin (596.17), isobutrin (596.17), butein (272.25), butin (272.25), coreopsin (434.39), isocoreposin (434.39), monospermoside (434.39) and isomonospermoside (434.39) which correspondingly existed in NBF-BMFE (Gupta et al., 1970; Rasheed et al., 2010). In related to this, our results also has confirmed with the presence of above bioactive compounds in NBF-BMFE (Fig. 7). Further we have interested on studying binding interaction of these compounds into Wnt signaling proteins by using *in silico* methods.

3.7. Molecular docking study analysis

In an attempt to rationalize the obtained *in vitro* biological results and attain insights into the possible binding modes of NBF-BMFE derived compounds to the active sites of, β-catenin, β-catenin/APC complex and GSK-3β, molecular docking study was performed. The x-ray crystallographic structures of β-catenin (PDB ID: 2BCT), β-catenin/APC complex (PDB ID: 1TH1) and GSK-3β (PDB ID: 1IO9) was retrieved from the protein data bank. The minimized free energy was calculated from electrostatics and Van-Der-Waals interactions between residues of complex proteins.

The molecular model of 3D structures for β-catenin, β-catenin/APC complex and GSK-3β were comprised of merged polar and non-polar hydrogen atoms. In ligands, non-polar hydrogen atoms were united with Gustier charges and all rotatable bonds of ligands were kept in rotation. An initial validation of the docking protocol was performed based on conformations, positions and orientations of a ligand has been obtained from docking with the experimentally determined 3D protein structures. The protein-ligand interaction energy was calculated based on the parameters such as hydrogen bond interaction, binding energy

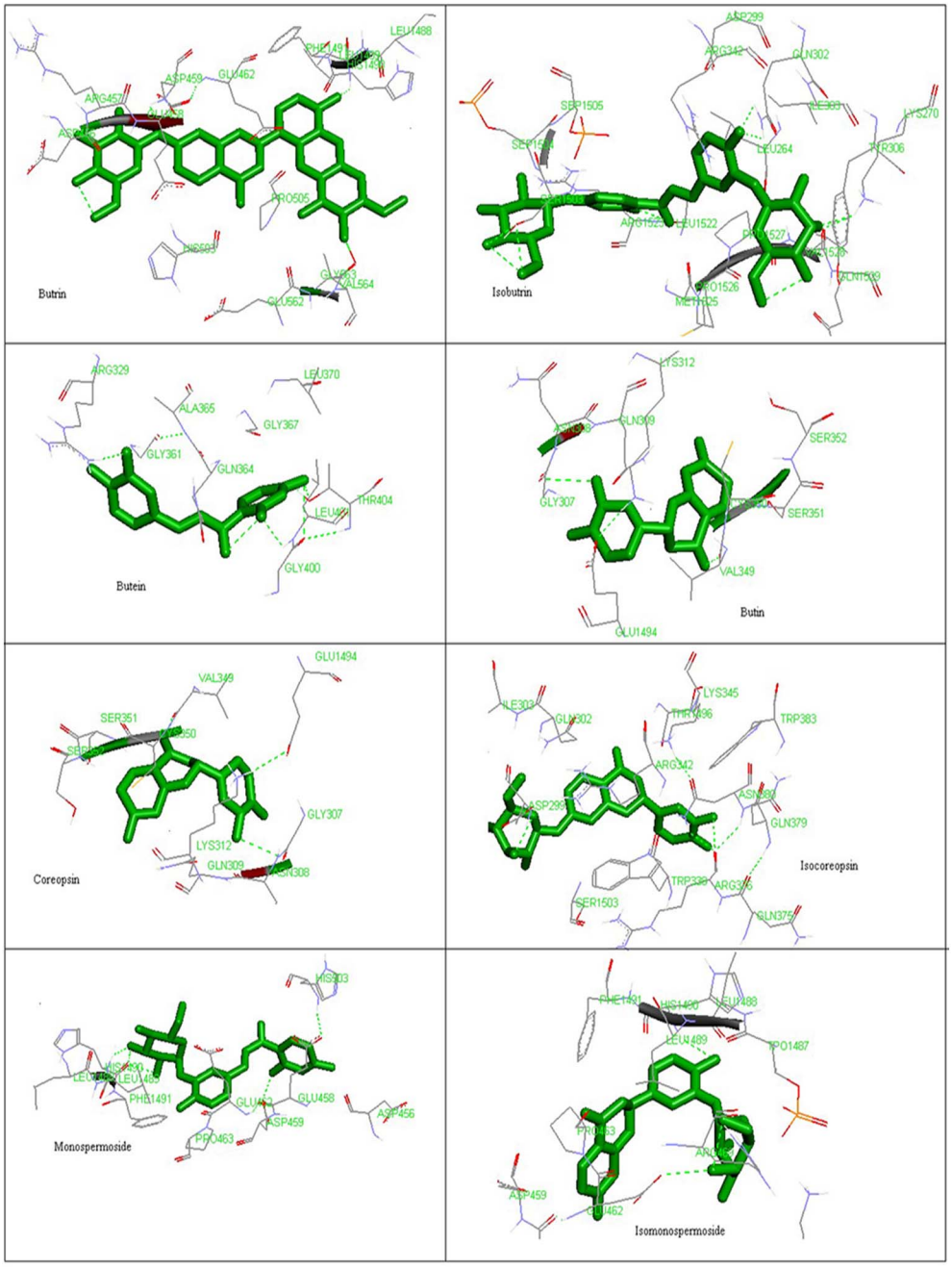

Fig. 9. Docking results of β-Catenin/APC Complex with *n*-butanol fraction of *B. monosperma* floral extracted compounds (green dots denoted strong hydrogen bond interaction).

Table 3
n-Butanol fraction of *B. monosperma* floral extracted compounds docked with β-catenin/APC complex.

Ligand	Binding energy (Kcal/mol)	H bond	Amino acids
Butrin	− 7.38	5	SER1503, SEP1504, SEP1505, LEU1506, ASN261
Isobutrin	− 10.88	7	LEU1511, ASP1512, GLU1513, LYS1518, ARG1523, SEP1505, GLN1517
Butein	− 8.05	3	LEU1509, LEU1511, GLU1513
Butin	− 5.82	1	ARG515
Coreopsin	− 4.17	6	HIS223, LEU1506, ILE1516, SEP1505, LEU1522, MET1525
Isocoreopsin	− 4.50	4	LYS1518, SEP1505, LEU1506, ILE1516
Monospermoside	− 3.18	4	ARG515, ARG612, TPO1487, GLU571
Isomonospermoside	− 2.49	4	LEU1506, LYS1518, ARG1523, SEP1505

and RMSD of active site residues (Azam et al., 2011).

The 781aminoacids (a.a) of β-catenin is a chief role in cell-cell adhesion by interacting with cadherin family proteins. It is homologous to armadillo repeats (ARM) and its N-terminal region contains 130 a.a, 550 a.a in central region and 100 a.a in C-terminal region approximately. The central region contained 12 imperfect sequence repeats of 42 a.a known as armadillo repeats vital to interact with various proteins including cadherins, APC and TCF/LEF (Miyaki et al., 1994). In β-

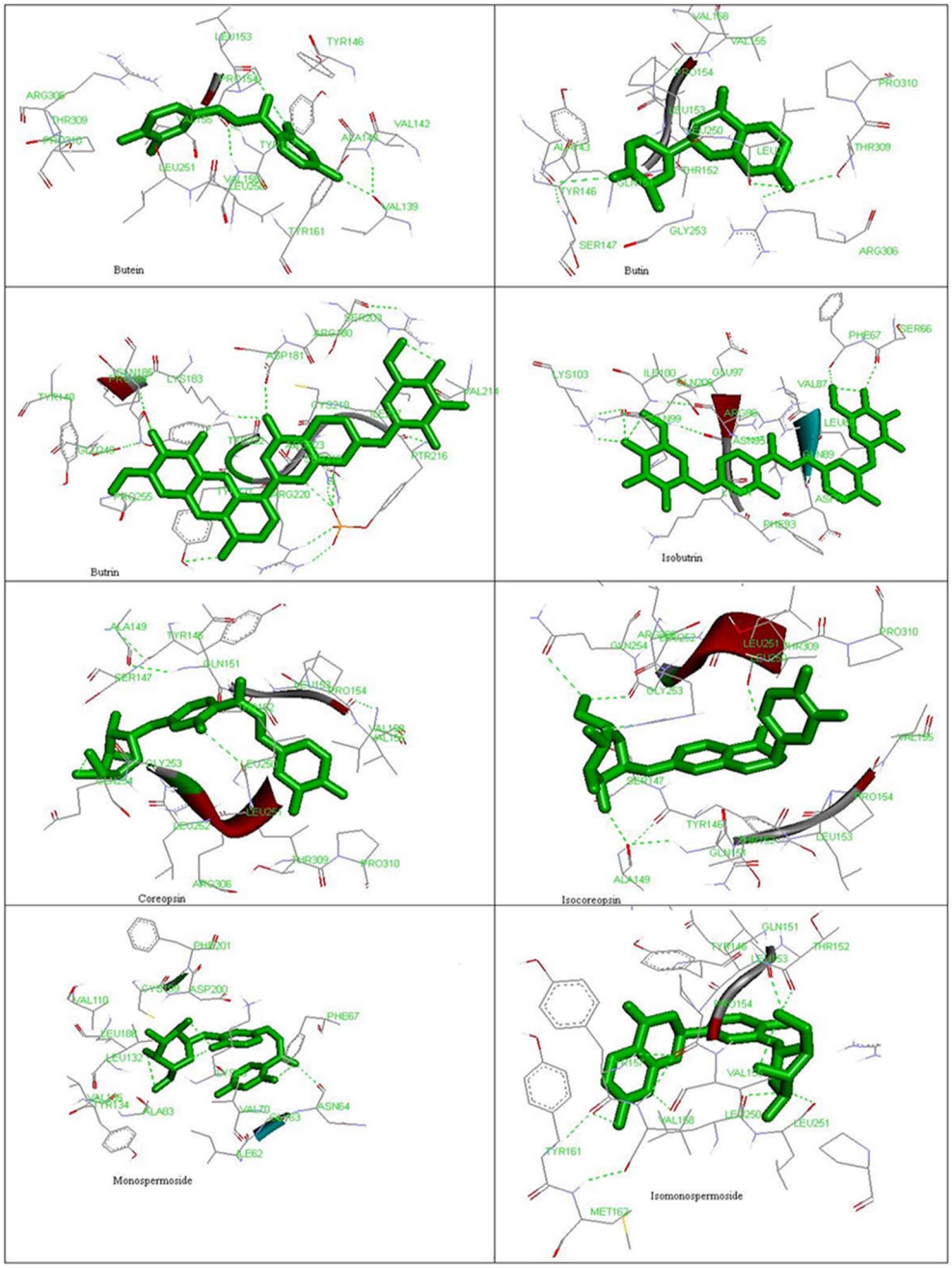

Fig. 10. Docking results of GSK-3β with *n*-butanol fraction of *B. monosperma* floral extracted compounds (green dots denoted strong hydrogen bond interaction).

catenin, the Isobutrin and coreopsin was strongly bound with the active site of ASN426, GLY422, CYS419, THR418, GLU462, ASN430, ASN431, LYS435, ARG469, LYS508, SER473 and THR428 (Table 2) which is present in armadillo repeats. It leads to form the 5 and 7 hydrogen bonds respectively with binding interaction energy of − 9.54 kcal/mol and − 10.14 kcal/mol (Fig. 8). Correspondingly, the other lead molecules also expressed good interactions with this protein. Hence, our results were showed the effective binding interaction in the central region of β-catenin in order to regulate the Wnt signaling pathway in disease conditions.

The putative APC phosphorylated sites of SER1385, THR1388, SER1389, SER1391, SER1501, SER1504, SER1505, SER1507 and SER1510 were converted to alanine (S-A mutants) from GST-APC, may lead to cause CRC (Yang et al., 2006). In β-catenin/APC complex protein, the butrin and isobutrin were strongly bound with the phosphorylation sites of SER1503, SER1504 and SER1505, with the hydrogen bonds of 5 and 7 respectively (Fig. 9). Moreover, the binding energy for butrin and isobutrin was − 7.38 kcal/mol and − 10.88 kcal/mol (Table 3).

The tumor suppressor gene APC was originally identified as a major causative genetic factor for developing numerous colorectal polyps towards familial adenomatous polyposis coli (FAP) affected patients. It possessed 2843 aa of various binding domains in its N and C-terminal regions. The N-terminal region had oligomerization domain, 7 armadillo repeats followed by 15 aa and 20 aa repeats of two β-catenin binding sites located at the residues of 1020–1169 and 1342–2075 aa respectively. Among these two binding sites, the 20 aa repeat had high affinity upon phosphorylation whereas the functional significance of 15 aa residues is still obscure (Breitman et al., 2008). Most of the tumor associated mutations in APC have been mapped in the residues of 1286–1513 (Polakis, 1995). In results, the butrin and isobutrin were strongly bound with the 20 aa β-catenin regions as well as in the tumor associated mutated regions.

The GSK-3β protein has involved in multiple signaling pathways

Table 4
n-Butanol fraction of *B. monosperma* floral extracted compounds docked with GSK-3β protein.

Ligand	Binding energy (Kcal/mol)	H bond	Amino acids
Butrin	− 12.24	5	ASP181, TYR221, LYS183, PTR216, GLN185
Isobutrin	− 9.054	5	ARG96, LYS103, SER66, GLN206, PHE67
Butein	− 9.82	2	VAL139, LEU153
Butin	− 9.76	4	ARG306, LEU251, THR309, ALA143
Coreopsin	− 10.19	1	LEU250
Isocoreopsin	− 11.80	5	ARG306, ALA149, GLY253, GLN254, LEU250
Monospermoside	− 5.64	1	ASN64
Isomonospermoside	− 10.04	5	LEU250, LEU251, THR152, GLN151, TYR157

and phosphorylates of its substrates. The unphosphorylated GSK-3β is 97% orientation with two functional domains of the activated protein kinases. It is documented that the phosphate ion is present in Arg96, Arg180 and Lys205 positions that helps to phosphorylate substrates (Edwards et al., 2005). Surprisingly in GSK-3β, the isobutrin compound was strongly bound with the phosphate ion of ARG96, with the formation of hydrogen bond of 5 and the binding energy is − 9.054. The other molecules also having good interactions with the GSK-3β protein (Fig. 10) (Table 4).

4. Conclusion

The experimental data presented here showed that NBF-BMFE had significant antiproliferative effect on SW480 human colon cancer cell line in time and dose dependent manner. Further, this extract was significantly down regulate the expression of Wnt signaling proteins such as β-catenin, APC, GSK-3β, cyclin D1 and *c-myc* on SW480 cells. Moreover, the *in silico* studies demonstrated that the NBF-BMFE compounds having good binding interaction on β-catenin, APC and GSK-3β proteins. This paper reveals the molecular mechanism underlying the anti-tumor effect of NBF-BMFE by suppressing β-catenin/Tcf signaling *via* decreasing its downstream target genes for first time. Finally, our results concluded that the NBF-BMFE could be a potential therapeutic regimen for colon cancer.

Conflict of interest

The authors declare that they have no competing interests.

Acknowledgement

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.genrep.2017.11.003.

References

Anuradha, S., Kumar, Vijay, 2012. Butein imparts free radical scavenging, anti-oxidative and proapoptotic properties in the flower extracts of *Butea monosperma*. Biocell 36, 63–71.
Azam, F., Prasad, M.V., Thangavel, N., Ali, H.I., 2011. Molecular docking studies of 1-(substituted phenyl)-3-(naphtha [1, 2-d] thiazol-2-yl) urea/thiourea derivatives with human adenosine A(2A) receptor. Bioinformation 6, 330–334.
Barker, N., Clevers, H., 2006. Mining the Wnt pathway for cancer therapeutics. Nat. Rev. Drug Discov. 5, 997–1014.
Bilic, J., Huang, Y.L., Davidson, G., Zimmermann, T., Cruciat, C.M., Bienz, M., Niehrs, C., 2007. Wnt induces LRP6 signalosomes and promotes dishevelled-dependent LRP6 phosphorylation. Science 316, 1619–1622.
Binkowski, T.A., Naghibzadeh, S., Liang, J., 2003. CASTp: Computed Atlas of Surface Topography of proteins. Nucleic Acids Res. 31, 3352–3355.
Breitman, M., Zilberberg, A., Caspi, M., Rosin-Arbesfeld, R., 2008. The armadillo repeat domain of the APC tumor suppressor protein interacts with Striatin family members. Biochim. Biophys. Acta 1783, 1792–1802.
Burlia, D.A., Khadeb, A.B., 2007. A comprehensive review on *Butea monosperma* (Lam.) Kuntze. Pharmacogn. Rev. 1, 333–337.
Choedon, T., Shukla, S.K., Kumar, V., 2010. Chemopreventive and anti-cancer properties of the aqueous extract of flowers of *Butea monosperma*. J. Ethnopharmacol. 129, 208–213.
Circu, M.L., Aw, T.Y., 2010. Reactive oxygen species, cellular redox systems and apoptosis. Free Radic. Biol. Med. 48, 749–762.
Dihlmann, S., Siermann, A., Von Knebel Doeberitz, M., 2001. The nonsteroidal anti-inflammatory drugs aspirin and indomethacin attenuate β-catenin/TCF-4 signaling. Oncogene 20, 645–653.
Edwards, B.S., Bologa, C., Young, S.M., Balakin, K.V., Prossnitz, E.R., Savchuck, N.P., Sklar, L.A., Oprea, T.I., 2005. Integration of virtual screening with high-throughput flow cytometry to identify novel small molecule formylpeptide receptor antagonists. Mol. Pharmacol. 68, 1301–1310.
Fodde, R., Smits, R., Clevers, H., 2001. APC, signal transduction and genetic instability in colorectal cancer. Nat. Rev. Cancer 1, 55–67.
Froimowitz, M., 1993. HyperChem: a software package for computational chemistry and molecular modeling. BioTechniques 14, 1010–1013.
Giles, R.H., Van Es, J.H., Clevers, H., 2003. Caught up in a Wnt storm: Wnt signaling in cancer. Biochim. Biophys. Acta 1653, 1–24.
Gupta, S.R., Ravindranath, B., Seshadri, T.R., 1970. Glucosides of *Butea monosperma*. Phytochemistry 9, 2231–2235.
Holcombe, R.F., Marsh, J.L., Waterman, M.L., Lin, F., Milovanovic, T., Truong, T., 2002. Expression of Wnt ligands and Frizzled receptors in colonic mucosa and in colon carcinoma. J. Clin. Pathol-Mol. 55, 220–226.
Jemal, A., Bray, F., Center, M.M., Ferlay, J., Ward, E., Forman, D., 2011. Global cancer statistics. CA Cancer J. Clin. 61, 69–90.
Jia, S.J., Jiang, D.J., Hu, C.P., Zhang, X.H., Deng, H.W., Li, Y.J., 2006. Lysophosphatidylcholine-induced elevation of asymmetric dimethylarginine level by the NADPH oxidase pathway in endothelial cells. Vasc. Pharmacol. 44, 143–148.
Jiang, Z., Chai, J., Chuang, H.H., Li, S., Wang, T., Cheng, Y., Chen, W., Zhou, D., 2012. Artesunate induces G0/G1 cell cycle arrest and iron-mediated mitochondrial apoptosis in A431 human epidermoid carcinoma cells. Anti-Cancer Drugs 23, 606–613.
Kasture, V.S., Kasture, S.B., Chopde, C.T., 2002. Anticonvulsive activity of *Butea monosperma* flowers in laboratory animals. Pharmacol. Biochem. Behav. 72, 965–972.
Kaur, M., Velmurugan, B., Tyagi, A., Agarwal, C., Rana, P., Singh, R.P., Agarwal, R., 2010. Silibinin suppresses growth of human colorectal carcinoma SW480 cells in culture and xenograft through down-regulation of β-catenin-dependent signaling. Neoplasia 12, 415–424.
Kobayashi, M., Honma, T., Matsuda, Y., Suzuki, Y., Narisawa, R., Ajioka, Y., Asakura, H., 2000. Nuclear translocation of beta-catenin in colorectal cancer. Br. J. Cancer 82, 1689–1693.
Lin, W., Huang, Y.W., Zhou, X.D., Ma, Y., 2006. *In vitro* toxicity of silica nanoparticles in human lung cancer cells. Toxicol. Appl. Pharmacol. 217, 252–259.
Mao, J., Wang, J., Liu, B., Pan, W., Farr, G.H., Flynn, C., Yuan, H., Takada, S., Kimelman, D., Li, L., Wu, D., 2001. Low-density lipoprotein receptor- related protein-5 binds to Axin and regulates the canonical Wnt signaling pathway. Mol. Cell 7, 801–809.
Mathan, G., Fatima, G., Saxena, A.K., Chandan, B.K., Jaggi, B.S., Gupta, B.D., Qazi, G.N., Balasundaram, C., Anandrajan, K.D., Kumar, V.L., Kumar, V., 2011. Chemoprevention with aqueous extract of *Butea monosperma* flowers results in normalization of nuclear morphometry and inhibition of a proliferation marker in liver tumors. Phytother. Res. 25, 324–328.
Miller, J.R., Hocking, A.M., Brown, J.D., Moon, R.T., 1999. Mechanism and function of signal transduction by the Wnt/beta-catenin and Wnt/Ca2þ pathways. Oncogene 18, 7860–7872.
Miyaki, M., Konishi, M., Kikuchi-Yanoshita, R., Enomoto, M., Igari, T., Tanaka, K., Muraoka, M., Takahashi, H., Amada, Y., Fukayama, M., Maeda, Y., Iwama, T., Mishima, Y., Mori, T., Koike, M., 1994. Characteristics of somatic mutation of the adenomatous polyposis coli gene in colorectal tumors. Cancer Res. 54, 3011–3020.
Morris, G.M., Lim-Wilby, M., 2008. Molecular docking. Methods Mol. Biol. 443, 365–382.
Palanivel, K., Kanimozhi, V., Kadalmani, B., Akbarsha, M.A., 2013. Verrucarin A, a protein synthesis inhibitor, induces growth inhibition and apoptosis in breast cancer cell lines MDA-MB-231 and T47D. Biotechnol. Lett. 35, 1395–1403.
Polakis, P., 1995. Mutations in the APC gene and their implications for protein structure and function. Curr. Opin. Genet. Dev. 5, 66–71.
Rasheed, Z., Akhtar, N., Khan, A., Khan, K.A., Haqqi, T.M., 2010. Butrin, isobutrin, and butein from medicinal plant *Butea monosperma* selectively inhibit nuclear factor-KB in activated human mast cells: suppression of tumor necrosis factor-α, interleukin (IL)-6, and IL-8. J. Pharmacol. Exp. Ther. 333, 354–363.
Razdan, M.K., Kapila, K., Bhide, N.K., 1970. Study of the antioestrogenic activity of alcoholic extracts of petals and seeds of *Butea frondosa*. Indian J. Physiol. Pharmacol. 14, 57–60.
Reddy, S., Rishi, A.K., Xu, H., Levi, E., Sarkar, F.K., Majumdar, A.P., 2006. Mechanisms of curcumin and EGF-receptor related protein (ERRP)-dependent growth inhibition of colon cancer cells. Nutr. Cancer 55, 185–194.
Sehrawat, A., Sultana, S., 2006. Chemoprevention by *Butea monosperma* of hepatic carcinogenesis and oxidative damage in male wistar rats. Asian Pac. J. Cancer Prev. 7, 140–148.

Senda, T., Iizuka-Kogo, A., Onouchi, T., Shimomura, A., 2007. Adenomatous polyposis coli (APC) plays multiple roles in the intestinal and colorectal epithelia. Med. Mol. Morphol. 40, 68–81.
Somani, R., Kasture, S., Singhai, A.K., 2006. Antidiabetic potential of *Butea monosperma* in rats. Fitoterapia 77, 86–90.
Sparks, A.B., Morin, P.J., Vogelstein, B., Kinzler, K.W., 1998. Mutational analysis of the APC/beta-catenin/Tcf pathway in colorectal cancer. Cancer Res. 58, 1130–1134.
Takahashi-Yanaga, F., Sasaguri, T., 2007. The Wnt/β-catenin signaling pathway as a target in drug discovery. J. Pharm. Sci. 104, 293–302.
Wagner, H., Geyer, B., Fiebig, M., Kiso, Y., Hikino, H., 1986. Isobutrin and butrin, the antihepatotoxic principles of *Butea monosperma* flowers. Planta Med. 52, 77–79.
Wang, Z., Ma, Q., 2007. β-Catenin is a promising key factor in the SDF-1/CXCR4 axis on metastasis of pancreatic cancer. Med. Hypotheses 69, 816–820.
Yang, J., Zhang, W., Evans, P.M., Chen, X., He, X., Liu, C., 2006. Adenomatous polyposis coli (APC) differentially regulates beta-catenin phosphorylation and ubiquitination in colon cancer cells. J. Biol. Chem. 281, 17751–17757.
Yu, Y., Rishi, A.K., Turner, J.R., Liu, D., Black, E.D., Moshier, J.A., Majumdar, A.P., 2001. Cloning of a novel EGFR-related peptide: a putative negative regulator of EGFR. Am. J. Physiol. Cell Physiol. 280, C1083–9.
Zhang, Y., Mu, Q., Zhou, H., Vrijens, K., Rousse, M.F., Jiang, G., Yan, B., 2012. Binding of carbon nanotube to BMP receptor enhances cell differentiation and inhibits apoptosis via regulating bHLH transcription factors. Cell Death Dis. 3, e308.