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GC-MS ANALYSIS AND *IN-VITRO* CYTOTOXICITY STUDY OF *DENDROPHTHOE FALCATA* AND *TRIDAX PROCUMBENS* EXTRACTS AGAINST HACAT CELLS LINE

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Keywords:

Cytotoxicity, HaCat, MTT, SRB assay, GC-MS, Bioactive compounds

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ABSTRACT: The present study shows the cytotoxic effect of *Dendrophthoe falcata* (L. f) and *Tridax procumbens* Linn. extracts against HaCat (Skin cancer) cell line by MTT and SRB assay method. The phytochemical screening of extracts dichloromethane: methanol (DM), methanol: water (MW), and ethanol: water (EW) revealed the presence of secondary metabolites such as alkaloids, flavonoids, saponins, tannins, sterols, and triterpenes. % Cell inhibition by MTT assay of DFDM extract shows 90.00 ± 0.09% and TPDM 91.29 ± 0.02% at 80 µg/ml. IC_{50} values of DFDM extract <20 µg/ml and TPDM extract 20 µg/ml compare to IC_{50} values of standard 5-fluorouracil <10 µg/ml by MTT assay. SRB assay method DFDM extracts shows 86.96 ± 0.07% and TPDM shows 89.14 ± 0.27 with IC_{50} values < 20 µg/ml compare standard 5-fluorouracil <10µg/ml. HPTLC fingerprint analysis of active DFDM extract showed 11 peaks at 366 nm R_f values 0.06-0.87 & TPDM shows showed 12 peaks R_f values 0.33- 0.83 at 366 nm. Active extracts were analyzed agilent 7890A GC coupled with triple quadrupole mass detector. GC–MS analysis DFDM extract shows presence of the 16 phytocompounds, major are [Diethetyl phosphonate- (RT-6.615, 166.075, 43.8.%), Benzyl oxy tridecanoic acid (RT-6.730, MW-320.235, %A-44%), 9(2-phenyl ethyl) Heptadecane (RT-10.233, 344.344,29.1 %), Hexadecanoic acid butyl ester (RT-15.099, 312.302, 65.5%), Linoleic acid, 2,3 bis (O-TMS) propyl esters (RT-23.098,26.3%)]. TPDM extracts shows 19 phytocompounds, major are [Tetradecene (RT-8.249, 198.23, 32.8%), Cyclohexasulfide (RT-9.893, 191.83, 71.4%), Cyclic octaatomic sulphur (RT-15.810, 66.7%), Squalene (RT-22.081, 24.1%). The study showed that the phytochemicals present in the leaves extracts of *D. falcata* and *T. Procumbens were* responsible for cell inhibitory activity against the HaCat cell line.

INTRODUCTION: Medicinal plants provide thousands of phytomolecule which play a major inhibitory role against different pathogens. Nowadays these phytochemical used to treat the disease, chronic infections. From ancient time in Ayurveda, plant extracts and their formulations are used to treat diseases [1].

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The WHO report shows that phytomolecule, herbal formulations were utilized by 60% of the world's population and in some countries are incorporated extensively in the public health system [2]. Pathogenic tumor cell always produces the resistance to the anticancer drugs which are used to treat the diseases [3].

Due to this problem, researchers are developing new synthetic molecules but having side effects, nowadays, new phytomolecule, secondary metabolites from plant origin are explored because of their diverse pharmacological activity [4]. Phytomolecule such as alkaloids, glycosides, saponins, steroids, and triterpenoids, fat and oils,

flavonoids, phenols, and tannins were recently used as drugs or to prevent various infections [5], few of the natural products were reported for the cytotoxic activity against various types of cancer cell lines [6]. *Dendrophthoe falcata* (L. f) Ettingsh, hemiparasitic mistletoe belonging to Loranthaceae family, commonly known as 'Banda' green plant found in Asian countries India, Srilanka, Thailand, China, and Australia. The leaves, stem, flower parts were used to treat wounds, menstrual troubles, breathing problems, psychic disorders, pulmonary tuber-culosis, in consumption, and mania by the tribal of India [7]. Phytochemical screening of *Dendrophthoe falcata* extracts reported containing several cardiac glycosides, flavonoids, and pentacyclic triterpenes [8]. Research articles report that *D. falcata* also *have* possessed contraceptive, hepatoprotective, wound healing, anti-microbial, anti-oxidant, anti-nociceptive, antihyperlipidemic, cardioprotective, and also antitumor activities [9]. *D. falcata* extracts also reported for lowering the breast tumor growth in the experimental female Wistar rats [10]. *Tridax procumbens* from the family Asteraceae, known as 'Coat buttons' plant contains maximum natural cellulose fiber. *Tridax procumbens* is native of tropical America, tropical Asia, Africa, Australia, and India. It is a wild herb distributed throughout India [11]. In the Indian traditional medicine system, *T.procumbens* used as an anticoagulant, antifungal hepatoprotective, and in dysentery [12, 13]. Recent research on *T. procumbens* reports that the methanol extract exhibited high antifungal activity against clinically important human skin pathogens [14]. The present study was done to find out the *in-vitro* cytotoxicity of *D. falcata* and *T. Procumbens* leaf extracts against HaCat (Skin cancer) cells line & carried out to profile of chemical compounds from active extracts.

MATERIALS AND METHODS:

Chemicals and Reagents: All the chemicals and reagents used in the research were of analytical grade and purchased from SD- Fine, Research-lab, Sigma-Aldrich (India), Silica gel 60 F 254 HPTLC aluminum sheets 20 × 20 cm, Merck KGaA, Germany.

Plant Material: *Dendrophthoe falcata* (L. f) a hemiparasite of *Mangifera indica* plant and *Tridax procumbens* plant leaves, were collected from the local region at flowering stage September - November from Bhor-Kapurhol road, Pune, Maharashtra, India. (Lat.-18012'51" N; Long-73054'35"E) and were taxonomically identified and authenticated by Dr. Rashami Dubey, Scientist Govt. of India, Botanical Survey of India (BSI), Pune, Maharashtra (India). The herbarium of plant specimen has been deposited at B.S.I Pune voucher specimen number- VIBTRP2 and VIBDEF3 BSI/WRC/TECH/2013.

Extraction: Shade dried leaves were powdered (1kg), Soxhlet extracted with dichloromethane: methanol (7:3), 70% (v/v) methanol & ethanol to get respective extracts. Extracts were vacuum concentrated by rotary evaporated under reduced pressure at 60 ºC ± 1 ºC. Extracts were dried in hot air oven at 40-45 ºC and then extracted was stored at - 20 ºC till bioevaluation in an air-tight container. Phytochemical screenings of extracts were carried out using phytochemical tests as described by Trease, G. E., Evans, and Harborne [15, 16].

High-Performance Thin Layer Chromatography (HPTLC) Analysis: Biologically active di-chloromethane: methanol extracts of *D. Falcate* (DFDM) and *Tridax procumbens* (TPDM) further studied by HPTLC. Fingerprint analysis performed by CAMAG HPTLC equipment consists of automatic & Linomat syringe using the Linomat applicator IV sample applicator, developing in CAMAG twin trough chamber. Evaluated by CAMAG HPTLC densitometer with win CATS planar chromatography manager software was used data collection. 5 mg of extract was dissolved in 10 ml of methanol. Silica gel 60 F254 and HPTLC aluminum sheets were used as adsorbents. 10 µl of the sample was applied as a band of 5-6 mm and at a separation of 6 mm from each other. Nitrogen gas was flushed on plates for simultaneous drying of bands. Flat bottomed CAMAG Twin chamber saturated with 10 ml of n-hexane: toluene: ethyl acetate (2: 4: 1.3) mobile phase before develop-pment. The developed plate was scanned using TLC scanner with WinCATS software.TLC plates were visualized and a fingerprint profile was photo-documented at 366 nm. R_f values were calculated & data reported [17].

GC-MS Analysis: GC-MS analysis of active extracts DFDM and TPDM studied by Agilent

7890A Gas Chromatography, Agilent 7000B Mass spectrophotometer (GC-MS) (USA) coupled with triple quadrupole mass spectrometer detector. The GC-MS system was equipped with a DB-5MS column (30 mm× 0.25 m 0.2-micron film Filter). Carrier gas Helium is used as at a flow rate of 1.0 mL/min and a splitless.

In temperature programming, initial temperature is 110 ºC (hold 2 min ramped at 15 °C / min to 150 °C (hold 1 min) ramped at 10 °C/min to 280 °C (hold 5 min), final experiments total run time 23.5 min. The injector and detector were held at 250 °C. Mass spectra were obtained by electron ionization (EI) at 70 eV, using a spectral range of m/z 50-700 amu [14, 19].

***In-vitro* Cytotoxicity Study:**

MTT Assay:

Cell Culture: The HaCat cells line (Skin cancer) was procured from National Centre for Cell Science (NCCS), Pune, India. HaCat cells were grown & maintained as a monolayer in DMEM medium supplemented with 10% fetal bovine serum containing 5% of a mixture of Gentamycin(10 ug), Penicillin (100 Units/ ml), and Streptomycin (100 µg/ml) in the presence of 5% CO_2 at 37 ºC. The cells were plated at a density of 1 × 104 cells per well in a 96-well plate and cultured for 24 h at 37 °C [20-22].

Assay: Cell viability was determined with 3-(4, 5-dimethyl thiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) with minor modifications. This assay measures the conversion of MTT to dark blue formazan precipitation by succinate dehydrogenase of the intact mitochondria of living cells. HaCat cells line were seeded into 96-well plates at a density of 1×10^4 cells per well in DMEM (10% FBS) incubated for12 h. The extracts were firstly dissolved in dimethyl sulfoxide (DMSO) and then diluted in a culture medium with a final DMSO concentration of 0.5% (v/v).

After that, the cells were exposed 10, 20, 40 & 80 (µg/ml) concentrations appropriately diluted with DMSO and incubated at 37 ºC in a humidified atmosphere of 5% CO_2. Medium containing 0.5% DMSO was used as control. After treatment, medium was replaced with a fresh culture medium without FBS containing MTT at a concentration of 0.5 mg/ml, and the cells were further incubated for 4 h at 37 °C. The optical density (OD) was measured at 490 nm using a microplate reader. The result represented the mean of three readings. Cell viability was determined by OD of treated wells divided by OD of vehicle control.

SRB Assay: For the present study, cells were inoculated into 96 well microtiter plates in 100 µl of individual cell lines by Sulforhodamine B (SRB) method. HaCat cells line maintained in DMEM medium supplemented with 10 % fetal bovine serum. The cells were consequently expose to 10, 20, 40, & 80 µg/ml concentrations of extracts. After extracts incubation, add 50 µl TCA (50%) and kept for 1 hour at 4 ºC, then plate washed with triple distilled water and dried the plate. Then add 100 µl SRB dyes in each well and kept for 30 min at room temperature. Again wash three times with 1% acetic acid and air dry the plate, then add 200 µL tris buffer, and absorbance was read at 490 nm. Percent growth was calculated on a plate-by-plate basis for test wells relative to control wells [23, 24].

Percent cytotoxicity = Reading of control - Reading of treated cells/ Reading control × 100

RESULTS AND DISCUSSION:

Extraction and Phytochemical Screening: Phyto-chemical extraction plant was performed by different polarity solvents by soxhlet apparatus. From three batches of around 1 kg of air-dried powdered leaves, mean percentage yields of DFDM -8.40% (SD = 1.34), DFMW-5.8% (SD = 1.02), DFEW-7.2% (SD = 1.11) and for TPDM-9.1% (SD = 1.07), TPMW- 5.2% (SD = 0.63), TPEW-6.4% (SD = 1.04) Most of the constituents were polar in nature. DFDM & TPDM extracts showed the presence of phytochemicals such as alkaloids, flavonoids, phenolic compound tannins, saponins, phytosterols, and diterpenes.

Alkaloids extracted from plants show biological activities such antimicrobial [25], antitumor, and antiviral activities [26]. Saponins from plants show good antioxidant, immunostimulant, anticancer activity and also act on the permeability of cell membranes by the pore formation make lysis of the cell. Triterpenoids saponins are potent cytotoxic acts through cell membrane mediated transport [27]. Phytochemicals such as steroids, alkaloids, phenols, flavonoids, saponins, tannins, anthro-quinone potent antibacterial, anticancer agents [28].

HPTLC Fingerprints Study of Extract of *Dendrophthoe falcata* (L. f) and *Tridax Procumbens* (Linn.) Plant: The HPTLC fingerprints study of DFDM extract showed 12 peaks at 366 nm with R_f values 0.06-0.87 TPDM extract showed 13 peaks with R_f values 0.33- 0.83, at 366 nm showed maximum concentration justifies the presence of phytochemicals may be responsible for biological activity.

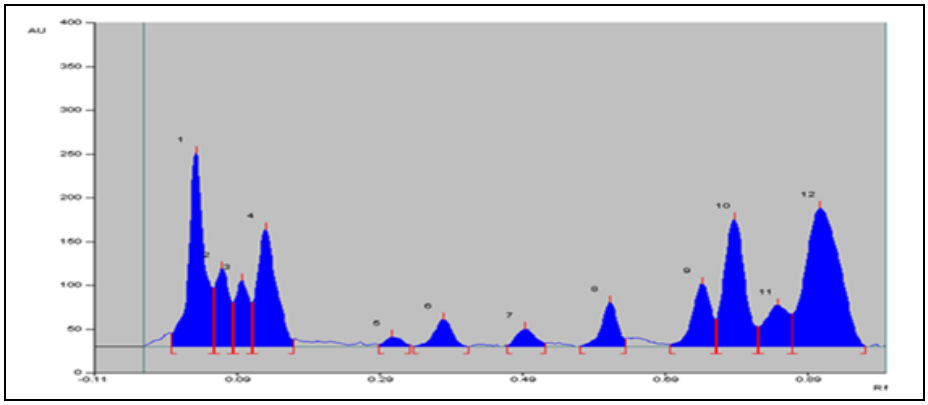


FIG. 1: HPTLC CHROMATOGRAM OF DFDM EXTRACT OF MEASURED AT 366 nm

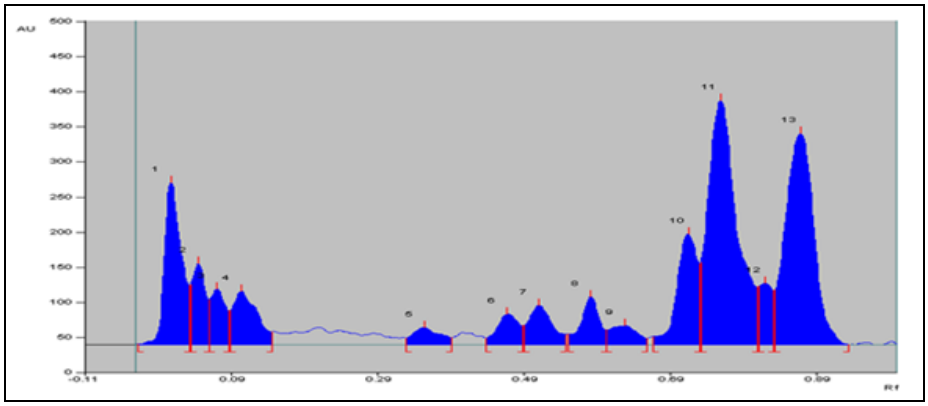


FIG. 2: HPTLC CHROMATOGRAM OF TPDM EXTRACT MEASURED AT 366 nm

GC-MS Analysis of DFDM and TPDM Extracts: GC-MS analysis of extracts of *D. falcata* (L. f) and *T. procumbens* (Linn.) revealed the presence of bioactive compounds. DFDM extract showed 16 peaks **Fig. 3, Table 1, Fig. 4** and TPDM extracts showed 19 **Fig. 5, Table**. The compounds were identified using the NIST database. The identification of the chemical compounds was confirmed based on the retention time; molecular formula, molecular weight, and peak area in percentage were detected.

The major bioactive components of DFDM extract were identified as Diethyl ethyl phosphonate (43.8%), Benzyl-oxy tridecanoic acid (44.1%), 9(2-phenyl ethyl) Heptadecane (29.1%),Phytol acetate(16.8%), 1,1-dipheyl -4-phenyl thiobut-3-en-1-ol(14.3%), Octadecane 3-ethyl-5-(2-ethyl butyl) (23.0%), Hexadecanoic acid butyl ester (65.5%), Heptacosane (24.2%), Squalene (17.5%), Linoleic acid, 2, 3 bis (O-TMS) propyl esters (26.3%). Major bioactive components of TPDM extract were Tetradecene (32.80%), Acoradiene (14.30 %), 2, 4-Di-tert-butylphenol (Antioxidant No.33%) (29.30 %), Cyclohexasulfide (Hexathiane) (71.4%), Hexadecane (26.4%), 7-methyl-Z-tetradecane-1-ol-acetate (30.3%), Cyclic Octaatomic sulfur (66.7%), Octadecane, 3-ethyl -5-(2-ethyl butyl)(28.4%), Squalene(24.1%) These bioactive compounds have been reported. Phytochemical bioactive compounds play essential roles in against diseases and general metabolisms.

Sulfur molecules involved in G_2/M arrest and apoptosis of cell and activation of p53 pathway in response to the oxidative DNA damage of cancer cell. Sulfides inhibit the growth of skin cancer cells with respect to normal keratinocyte HaCat cells [29]. GC-MS study of DFDM & TPDM reveals the presence of squalene which was reported that

antioxidant triterpene act by oxygen scavenging activities (withdraws or donate electron from molecule). Squalene was a good antitumor agent act *via* the strong inhibitory activity of HMG-COA reductase catalytic activity [30].

Review from past research indicates that major components separated from DFDM and TPDM extracts by GC-MS may be responsible for cytotoxicity.

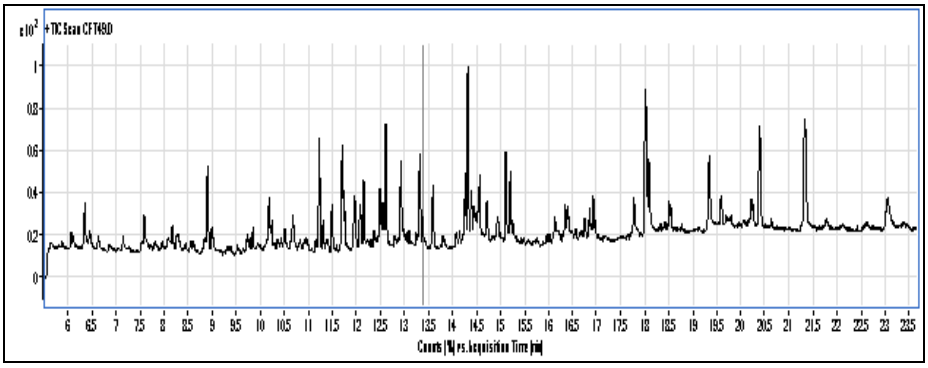
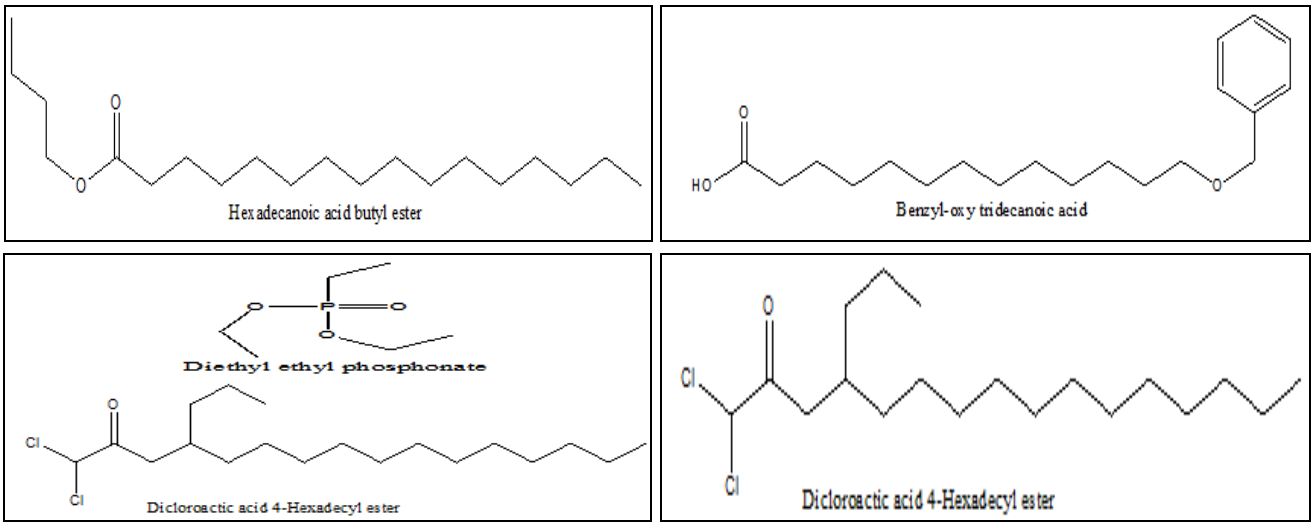


FIG. 3: GC-MS SPECTRA OF DFDM EXTRACT

TABLE 1: PHYTOCHEMICAL COMPONENTS OF DFDM EXTRACT USING GC–MS

S. no.	RT (min)	Name of Compounds	MF	MW (g/mol)	PA (%)
1	6.335	Carbonic acid ethyl hexadecyl ester	$C_{19}H_{38}O_3$	314.282	7.59
2	6.615	Diethyl ethyl phosphonate	$C_{16}H_{15}O_3P$	166.075	43.8
3	6.730	Benzyl-oxy tridecanoic acid	$C_{20}H_{32}O_3$	320.235	44.1
4	8.890	Dicloroactic acid 4-Hexadecyl ester	$C_{18}H_{34}Cl_2O_2$	352.193	5.79
5	10.233	9-(2-phenyl ethyl) Heptadecane	$C_{25}H_{44}$	344.344	29.1
6	10.825	Docosyl ethyl carbonate	$C_{25}H_{50}O_3$	398.375	7.0
7	11.693	Phytol acetate	$C_{22}H_{42}O_2$	338.318	16.8
8	12.921	1,1-dipheyl -4-phenyl thiobut-3-en-1-ol	$C_{22}H_{20}OS$	332.123	14.3
9	14.545	Octadecane 3-ethyl-5-(2-ethyl butyl)	$C_{26}H5_4$	366.422	23.0
10	15.099	Hexadecanoic acid butyl ester	$C_{20}H_{40}O_2$	312.302	65.5
11	16.343	Methyl 14-methyl eicosanoate	$C_{22}H_{44}O_2$	340.334	16.9
12	18.012	Di-n-octyl phthalate	$C_{24}H_{38}O_4$	390.2.77	19.9
13	19.338	Heptacosane	$C_{27}H_{56}$	380.438	24.2
14	20.392	Squalene	$C_{30}H_{50}$	410.391	17.5
15	21.784	1,1,3,3,5,5,7,7,9,9,11,11,13,13 tertadecamethyl Heptasiloxane	$C_{14}H_{44}O_6Si_7$	504.152	18.4
16	23.098	Linoleic acid ,2,3 bis(O-TMS)propyl esters	$C_{27}H_{54}O_7Si_2$	498.356	26.3

RT = retention time; MF = molecular formula; MW = molecular weight; PA = peak area



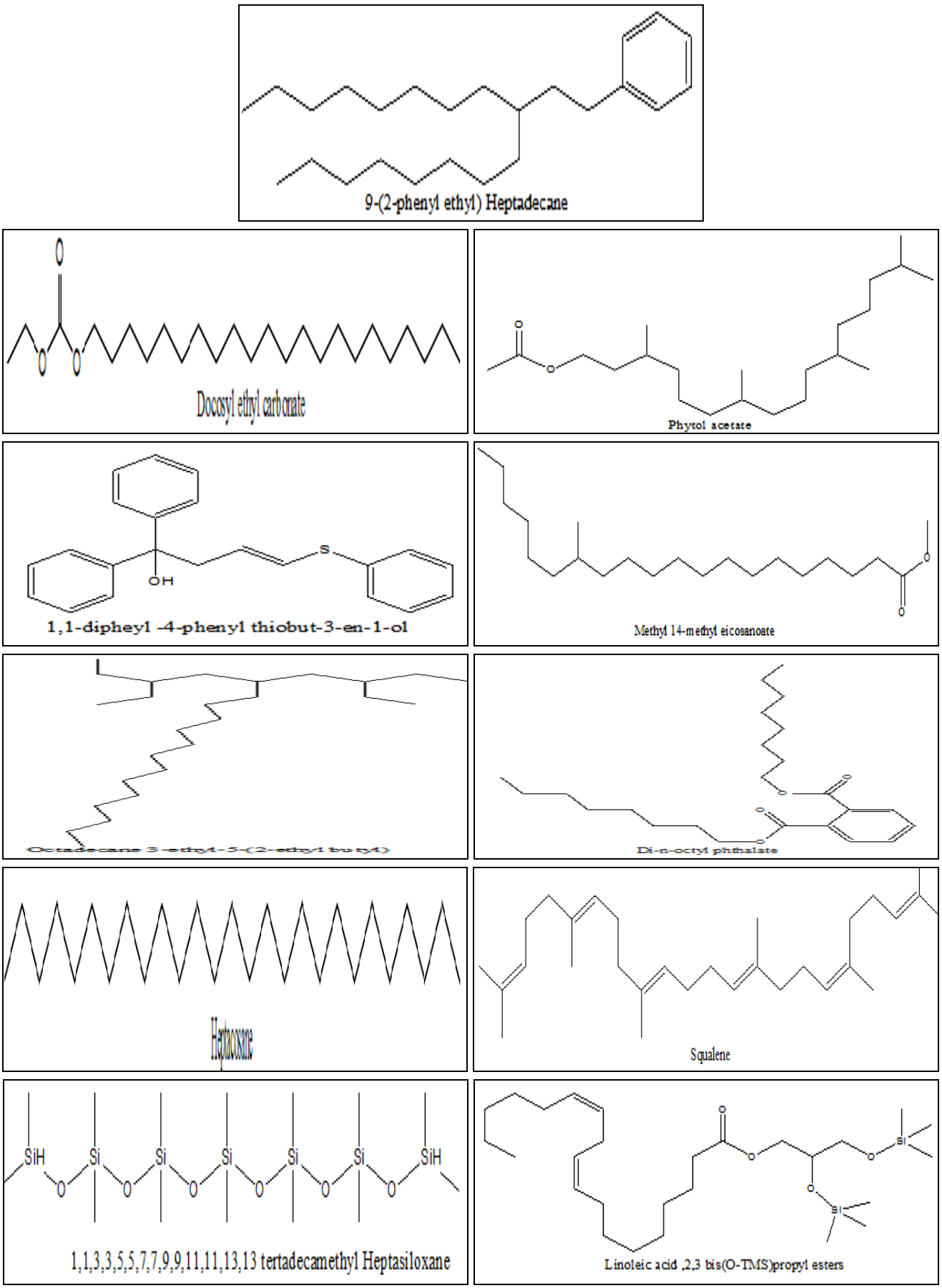


FIG. 4: PHYTOCHEMICAL COMPONENTS OF DENDROPHTHOE FALCATA DFDM EXTRACT

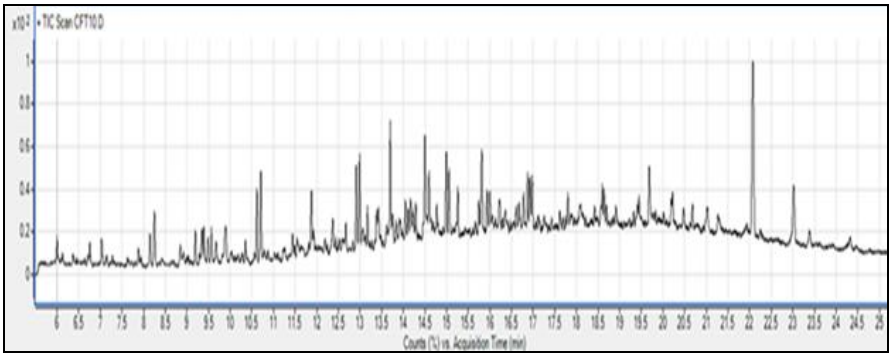
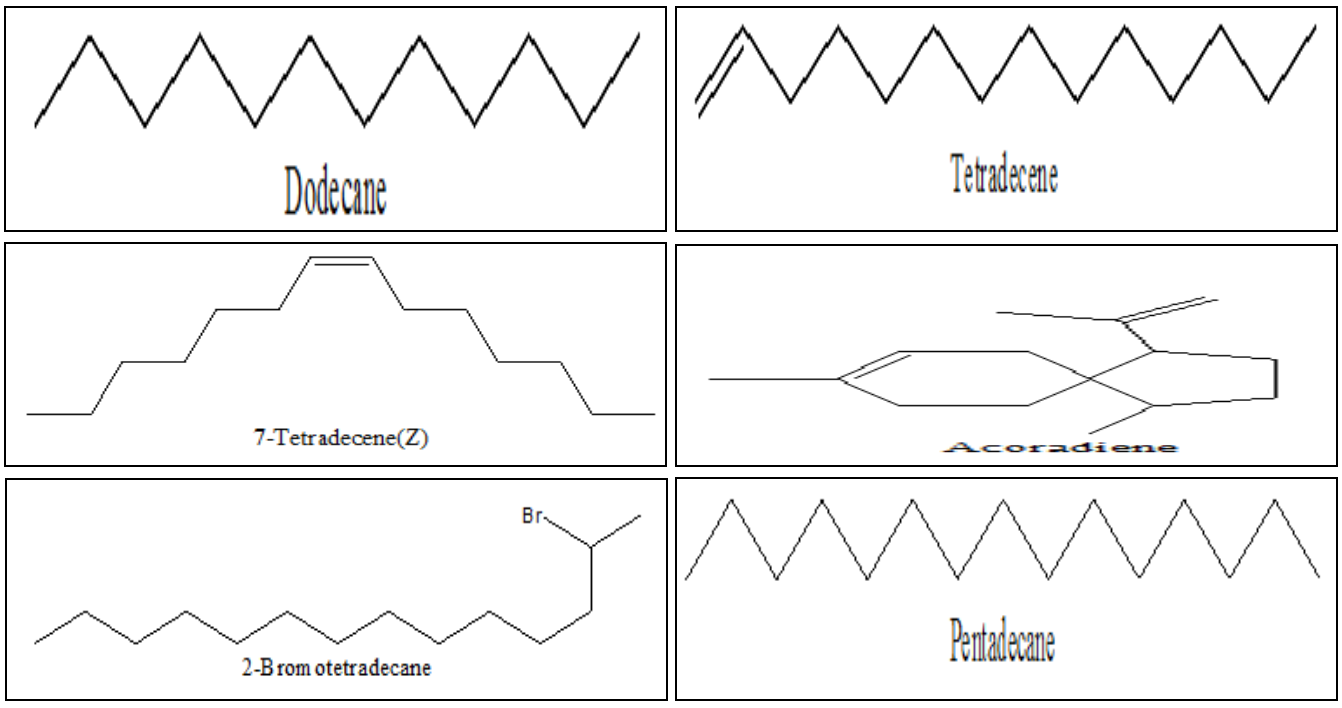


FIG. 5: GC-MS SPECTRA OF TPDM EXTRACT

TABLE 2: PHYTOCHEMICAL COMPONENTS OF TPDM EXTRACT USING GC–MS

S. no.	RT (min)	Name of compound	MF	MW (g/mol)	PA (%)
1	6.001	Dodecane	$C_{12}H_{26}$	170.20	07.94
2	8.149	7-Tetradecene(Z)	$C_{14}H_{28}$	196.21	05.03
3	8.249	Tetradecene	$C_{14}H_{30}$	198.23	32.80
4	9.192	Acoradiene	$C_{15}H_{24}$	204.18	14.30
5	9.348	2-Bromotetradecane	$C_{14}H_{29}Br$	276.39	11.2
6	9.394	7-Hexadecene(Z)	$C_{16}H_{32}$	224.25	4.77
7	9.490	Pentadecane	$C_{15}H_{32}$	212.25	10.7
8	9.564	2,4-Di-tert-butylphenol (Antioxidant No.33)	$C_{14}H_{22}$	206.16	29.30
9	9.893	Cyclohexasulfide(Hexathiane)	S_6	191.83	71.40
10	10.616	Centene(1-hexadecane)	$C_{16}H_{32}$	224.25	5.09
11	10.706	Hexadecane	$C_{16}H_{34}$	226.26	26.4
12	13.699	7-methyl-Z-tetradecane-1-ol-acetate	$C_{17}H_{32}O_2$	268.24	30.30
13	15.689	Neronine,4β,dihydro	$C_{18}H_{21}NO_6$	347.13	9.30
14	15.810	Cyclic Octaatomic sulfur	S_8	255.76	66.7
15	16.230	Octadecane,3-ethyl -5-(2-ethyl butyl)	$C_{26}H_{54}$	366.42	28.4
16	16.877	17-Pentatricontene	$C_{35}H_{70}$	490.54	27.4
17	19.695	9-(2',2'-Dimethyl propanoilhydrazono) -3,6-dichloro-2,7-bis-[2-(diethylamino)-ethoxy]fluorene	$C_{30}H_{42}Cl_2N_4O_3$	576.22	47.8
18	22.081	Squalene	$C_{30}H_{50}$	410.39	24.1
19	23.019	Heptacosane,1cholro	$C_{27}H_{55}Cl$	414.39	11.5



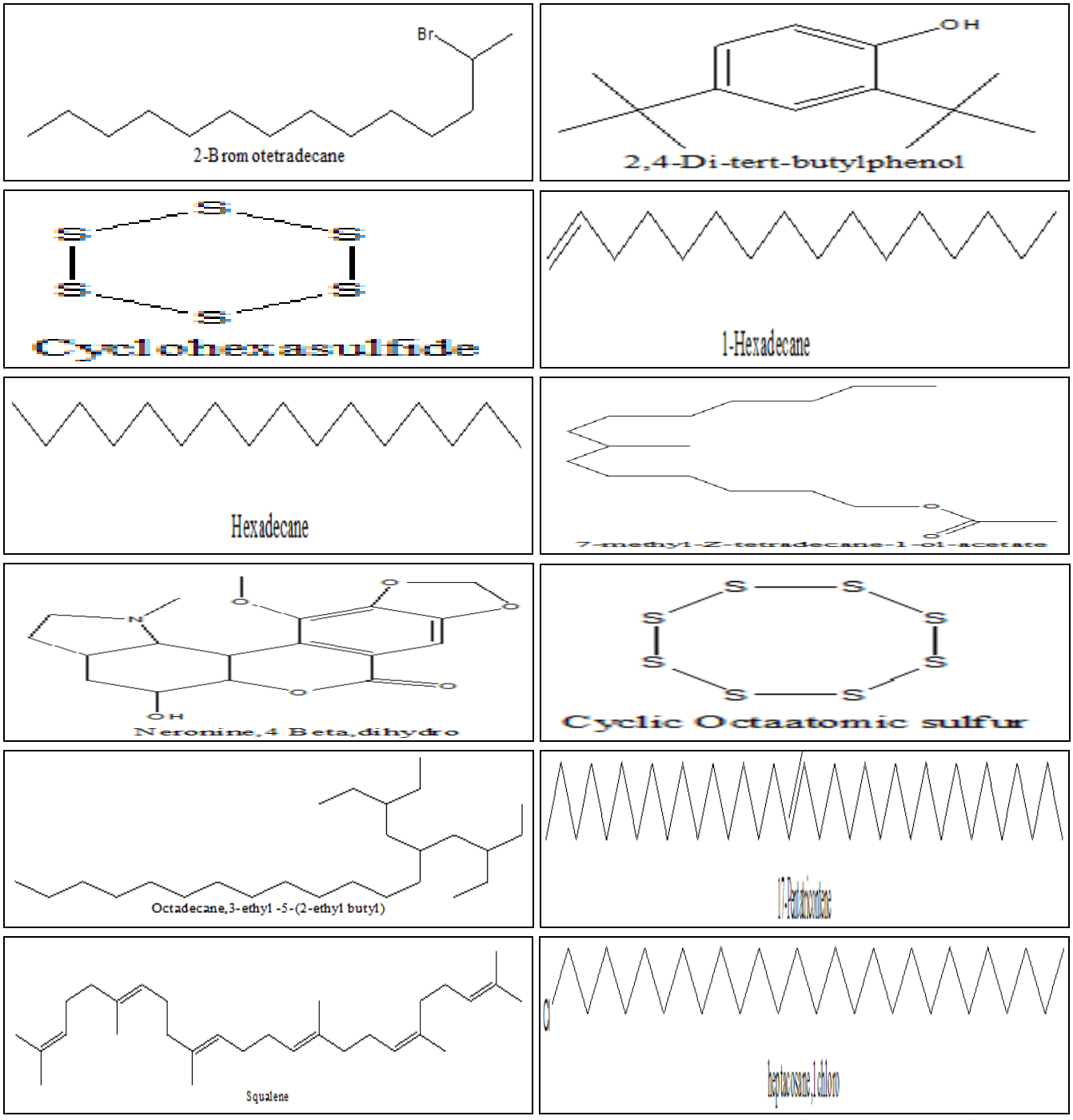


FIG. 6: PHYTOCHEMICAL COMPONENTS OF *TRIDAX PROCUMBENS* (TPDM EXTRACT)

Cytotoxicity Assay: The cytotoxic effect of *Dendrophthoe falcata* and *Tridax procumbens* plant extracts against HaCat (Skin cancer) cells with increasing concentrations of (10–80 µg/mL) for 24 h, which was confirmed by MTT and SRB assay.

The result shows that DFDM & TPDM extracts significantly induce cytotoxicity in a dose dependent manner **Fig. 7** and **8**. DFDM extracts shows cell inhibition 90.00 ± 0.09% & TPDM shows 91.29 ± 0.02% at 80 µg/ml. Both extracts shows strong cells inhabitation activity with IC_{50} values <20 µg/ml, 20 µg/ml respectively by MTT assay. The cell viability was gradually decreased, when treated with concentration of 10, 20, 40 & 80µg/ml. Simultaneously by SRB assay method DFDM extracts shows cell inhibition 86.96 ± 0.07% and TPDM shows 89.14 ± 0.27 with IC_{50} values < 20 µg/ml compare to IC_{50} values of std 5-fluorouracil <10µg/ml **Table 5**.

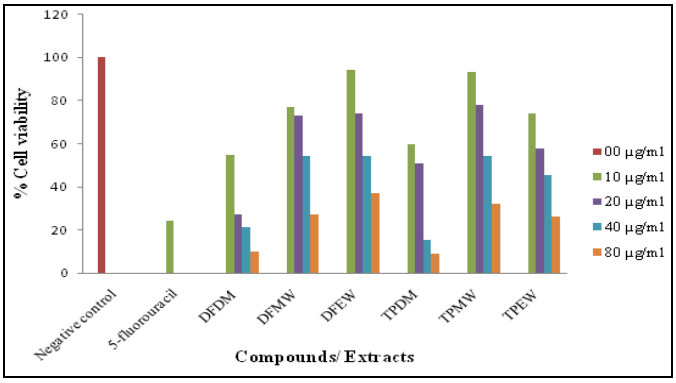


FIG. 7: CYTOTOXICITY OF EXTRACTS/ COMPOUND AGAINST HACAT (SKIN CANCER) CELLS LINE BY MTT ASSAY. Values are expressed in Mean ± s.e.m. from n=3 trials

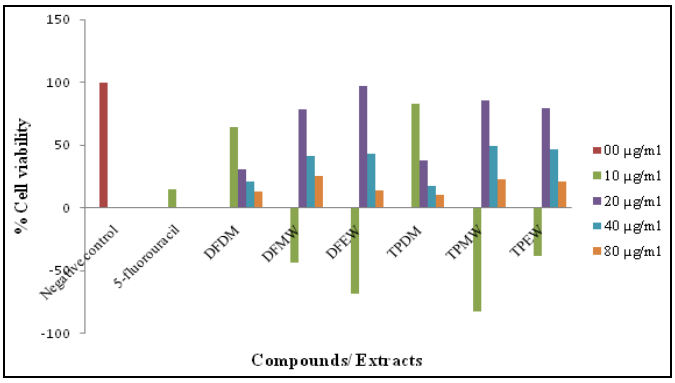


FIG. 8: CYTOTOXICITY OF EXTRACTS/ COMPOUND AGAINST HACAT (SKIN CANCER) CELLS LINE BY SRB ASSAY. Values are expressed in Mean ± s.e.m. from n=3 trials

TABLE 5: IC_{50} VALUES (µG/ML) OF *DENDROPHTHOE FALCATA* AND *TRIDAX PROCUMBENS* PLANT EXTRACTS AGAINST HACAT CELLS LINE BY MTT AND SRB ASSAY

S. no.	Compounds/Extracts	MTT Assay	SRB Assay
		IC_{50} Values (µg/ml)	
1	Negative control	00	00
2	5-fluorouracil(Std.)	< 10	< 10
3	DFDM	< 20	< 20
4	DFMW	> 40	< 40
5	DFEW	> 40	< 40
6	TPDM	20	< 20
7	TPMW	> 40	40
8	TPEW	< 40	< 40

CONCLUSION: The present study demonstrated that DFDM and TPDM extract exhibits strong cell inhibition effects on HaCat (Skin cancer) cells line by MTT and SRB assay. HPTLC and GC-MS study shows the presence of phytochemicals such as hexadecanoic acid butyl ester, Tetradecene, Squalene, Cyclic Octaatomic sulfur that may inhibit cells growths by increased intracellular reactive oxygen species (ROS) generation and decreased mitochondrial membrane potential also apoptosis of cells by the oxidative DNA damage. Reviews of research explore that DFDM and TPDM extracts have potent anticancer properties against the HaCat cells line. Present bioactive compounds were responsible for anticancer activity.

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CONFLICTS OF INTEREST: Authors declares no conflicts of interest

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