Report on participation of the ICMR International Fellow (ICMR-IF) in Training/Research abroad

1. Name and designation of ICMR- IF: Dr. Akshaya Murugesan
2. Address : Assistant Professor, Department of Biotechnology, Lady Doak College, Madurai -625002, Tamilnadu
3. Frontline area of research in which training/research was carried out: In-vitro experiments on glioblastoma multiforme, Pharmacology.
4. Name & address of Professor and host institute:
Olli YLI-HARJA & Meenakshisundaram KANDAVELU
Faculty of Medicine and Health technology
Korkeakoulunkatu1, TE306,P.O.Box 553, 33101,
Tampere, Finland

5. Duration of fellowship with exact date: 01.01.2020 to 31.12.2020

6. Highlights of work conducted:

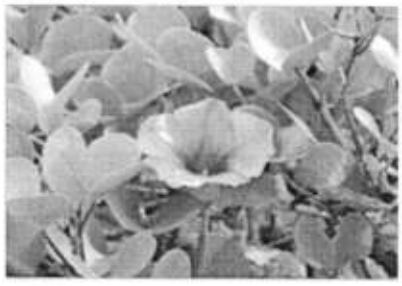
Task 1: Identification of novel molecular leads with potential anti-metastatic from the polyphenolic fractions of marine halophytes

Summary: The marine halophytes such as *Ipomoea-pes-caprae, Sonneratia apetala, Avicennia marina and Clerodendrum inerme* which was found to contain potent phenolic compound was selected for the study. The purified fractions were initially subjected for invitro cytotoxicity analysis for GBM cell lines such as LN229 and SNB19. Both LN229 and SNB19 exhibit mutated p53 proteins. LN229 was established from a patient with right frontal parieto-occipital glioblastoma with mutated p53 (TP53) and homozygous deletions in the p16 and p14ARF tumor suppressor genes. SNB19 was derived from a patient with the left parietooccipital glioblastoma tumor. The IC_{50} value was calculated. Both time dependent and concentration dependent analysis revealed that the *Avicennia marina and Ipomoea-pes-caprae* exhibited better cytotoxicity effect than the other two halophytes. Thus, the purified compounds from these plants were selected for further analysis. The IC50 value for both Avicennia and *Ipomoea was found to be 1 microgram.* Time dependents assay was performed for 24 hours and 48 hours whereas concentration dependent analysis was done for varying concentration like 0.1 µg, 1 µg, 2 µg, 5 µg, 8 µg and 10 µg. The IC_{50} value for both Avicennia and Ipomoea was found to be around 1 microgram, which was used for further analysis. whereas TMZ (positive control) was found to have higher IC_{50} of 87.76 ± 6.92 µM and 84.39± 2.60 µM for the respective cell lines.

Plants chosen for the study

Avicennia marina

Ipomea pes-caprae



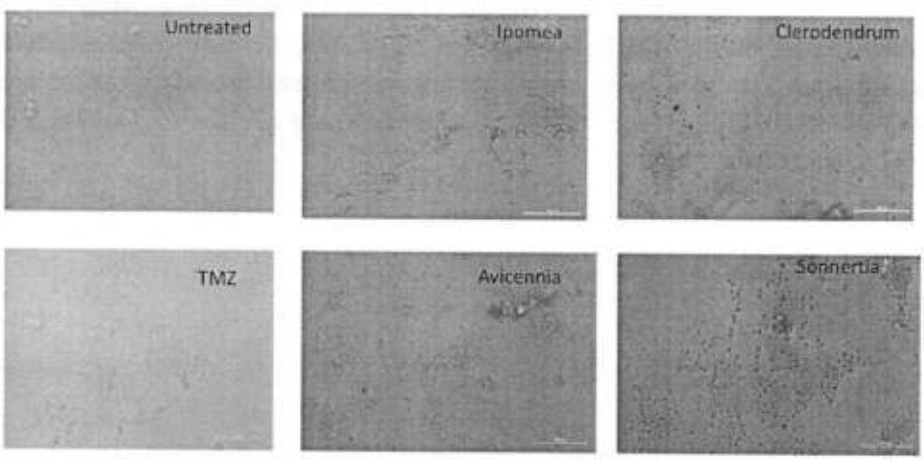
Sonneratia apetala

Clerodendrum inerme

Figure1: The halophytes such as *Avicennia marina, Ipomea pes-caprae, Sonneratia apetala and Clerodendrum inerme* collected from the coastal regions of Tamilnadu were processed, and the purified compounds were used for the further experimentation.

Cytotoxicity Assay

PHASE CONTRAST MICROSCOPIC IMAGES OF SNB19 TREATED WITH DRUGS



DOSE DEPENDENT ANALYSIS IN SNB19

		% of inhibition	S.D
TMZ	0.1	6.222041267	1.88926376
	1	5.588583878	1.67016536
	2	6.614616559	2.14460901
	4	-0.609140141	0.18034478
	8	-0.019003985	#DIV/0!
	10	28.34632775	5.86493233
Ipomea	0.1 micro	0.614996106	3.92622365
	1	40.57679315	5.14493257
	2	3.845568984	2.72557607
	4	10.80283343	2.36501658
	8	32.66302328	1.28035726
	10	56.09772809	6.86786308
Sonneratia	0.1 micro	-1.646391923	0.69422613
	1	11.40439197	10.1444828
	2	7.123516352	2.26538453
	4	7.91398862	2.3590256
	8	40.33040636	3.18447174
	10	86.98969837	9.65419102
Clerodendi	0.1 micro	0.636656073	0.52663521
	1	29.49463946	5.77970903
	2	9.323456514	3.29411919
	4	59.75257785	5.28813591
	8	83.97450149	3.58742092
	10	88.2640412	4.94824227
Avicennia	0.1 micro	2.651042586	2.73484838
	1	51.90510894	21.9090315
	2	28.51585492	1.16782971
	4	76.90315091	1.25834787
	8	93.03893478	2.28773269
	10	86.41071048	2.16504315

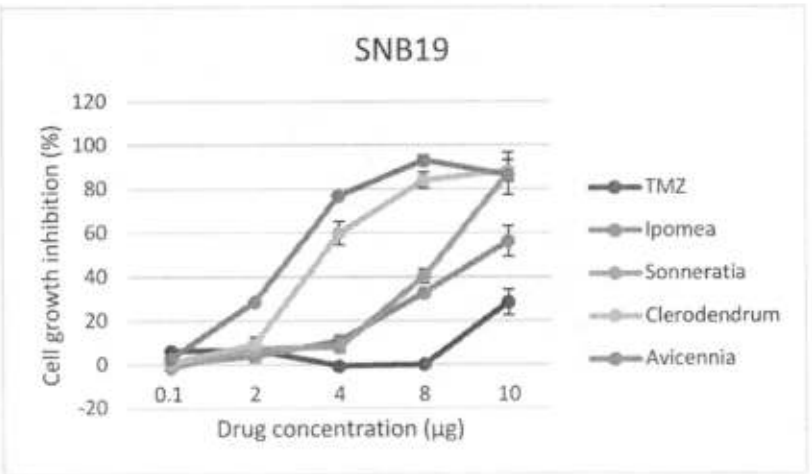
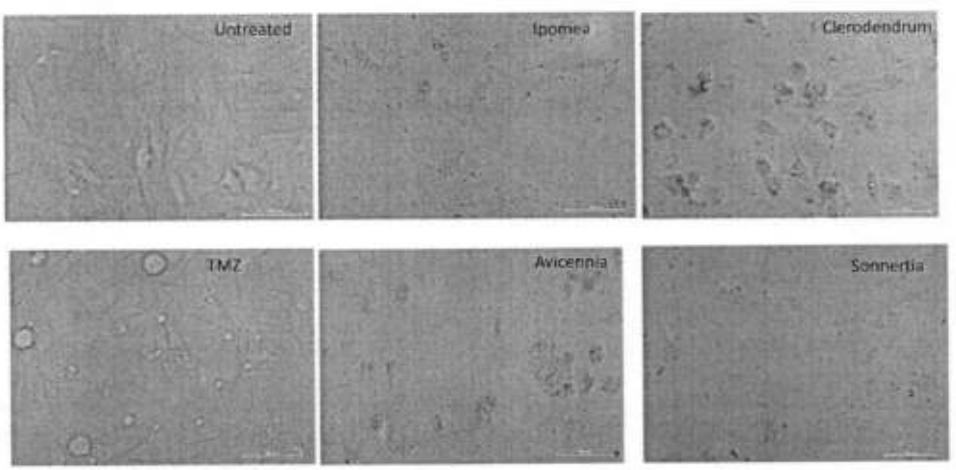


Figure 2: Cytotoxicity analysis for all the four plants in SNB19 cells: Effect of compounds on cell growth inhibition in multiple GBM cells. Cells treated with a series of concentrations (0.1µg, 1µg,4µg, 8µg and 10µg). The data were shown as means ±S.D with n = 5.

PHASE CONTRAST MICROSCOPIC IMAGES OF LN229 TREATED WITH DRUGS



DOSE DEPENDENT ANALYSIS IN LN229

		% of inhibition	S.D
TMZ	0.1	4.463498963	4.38776589
	1	6.907500245	3.44867766
	2	0.072968183	4.02705186
	4	5.563787804	1.05121578
	8	0.24872795	5.20763464
	10	17.6563542	6.18809941
Ipomea	0.1 micro	0.385524539	0.65062053
	1	6.770315248	7.74226021
	2	1.708946211	3.24832424
	4	8.306611518	1.13862829
	8	33.3628809	1.5945519
	10	83.51353157	4.66204685
Sonneratia	0.1 micro	-0.343558521	2.7652231
	1	33.17079782	9.3890422
	2	0.176184016	0.49335793
	4	39.5783753	2.29456226
	8	63.00376941	0.17807528
	10	89.69897013	4.59510472
Clerodendrui	0.1 micro	16.63404591	3.30517739
	1	59.24684044	6.7922942
	2	69.5731433	1.65589211
	4	54.41739723	2.38365197
	8	75.23276004	4.07584284
	10	99.60022199	0.79955601
Avicennia	0.1 micro	-0.878930664	0.52570572
	1	52.69545349	4.40338019
	2	57.93288492	0.95114447
	4	58.83321742	1.64356104
	8	73.09913352	3.24049889
	10	98.12290665	1.05139362

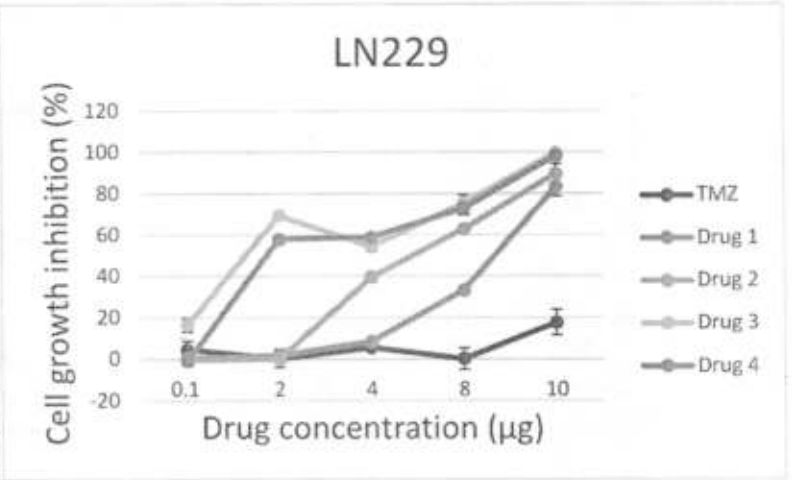
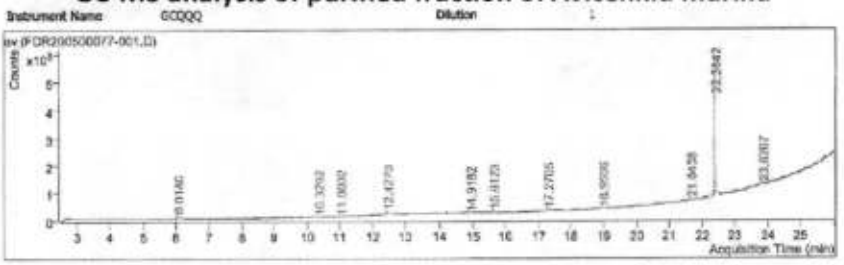


Figure 3: Cytotoxicity analysis for all the four plants in LN229 cells: Effect of compounds on cell growth inhibition in multiple GBM cells. Cells treated with a series of concentrations (0.1µg, 1µg,4µg, 8µg and 10µg). The data were shown as means ± S.D with n = 5.

Task 2: Elucidation of the structure of compounds and examined its potential bioactivity

Summary: The purified fraction were analysed for the presence of secondary metabolites and the structures were elucidated through the GC-MS analysis. The functional correlation with the potent secondary metabolites were identified from the PUBMED references. Avicennia contains 14 important secondary metabolites which are found to be involved in Anti-tumor activity, Antioxidant, cytotoxicity, insecticidal and nematicidal, antiviral, antifungal, phytotoxicity. Ipomea found to have 14 important secondary metabolites which possess Anti-inflammatory activity Anti-viral activity, Anti-malarial activity and Antioxidant activity.

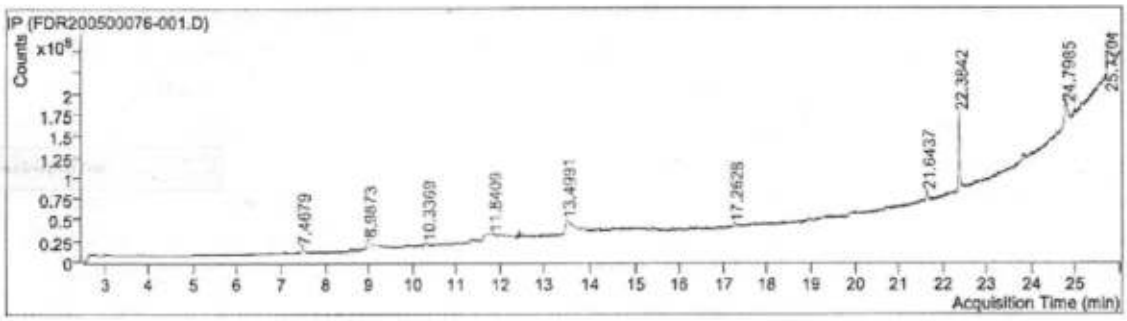
GC-MS analysis of purified fraction of *Avicennia marina*



Component RT	Compound Name	CAS#	Formula	Area	Match Score	Est. Conc.
6.0180	2-Pyrrolidinone, 1-methyl-	872-50-4	C5H9NO	78061844	75.7	
10.3292	3-Hydroxypropane-1,2-diyl diacetate	102-62-5	C7H12O5	9883431	63.8	
11.0002	Hibiscylhistidine	997530-35-4	C32H16N6O3	9450877	57.2	
12.3036	9-Hexadecenoic acid	2091-29-4	C16H30O2	10382760	59.2	
12.4270	2,4-Di-tert-butylphenol	96-76-4	C14H22O	21744415	61.1	
12.4733	Phenol, 2,6-bis(1,1-dimethylethyl)-	128-39-2	C14H22O	50194367	73.7	
14.9182	3,20-Dioxo-11-.rgfu(.-hydroxycoranine-1,4-diene	0-00-0	C21H27NO3	7133588	56.5	

Figure 4: ***GC-MS analysis on the profiling of secondary metabolites for Avicennia marina:*** *Avicenia* have 2-Pyrrolidinone, 1-methyl, 3-hydroxypropane-1,2 diyl diacetate, Hitidylhistitine, 9-hexadecenoic acid(Anti-tumor activity), 2,4-Di-tert-butyphenol,Phenol, 2,6-bis (1,1-dimethylethyl)(Antioxidant, cytotoxicity, insecticidal and nematicidal, antiviral, antifungal, phytotoxicity), 3,20-dioxo-11-,alpha hydroxyconanine-1,4-diene , 7 alpha-hydroxytestosterone, Hexadecanoiccaid, 2,3-dihydroxypropyl ester,9,12,15-octadecatrienoic acid, 2,3-dihydroxypropyl ester, Cyclohexane, 1,3,5-triphenyl,8,14-seco-3,19-epoxyandrostane-8,14-dione, 17-acetoxy-3 beta-methoxy, 4,4-dimethyl,Bis (2-ethylhexyl) phthalate (cytotoxicity),Normorphine.

Gc-Ms analysis for Ipomoea fraction



Component RT	Compound Name	CAS#	Formula	Area	Match Score	Est. Conc.
7.4679	4H-Pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl-	28564-83-2	C6H8O4	26100845	72.3	
8.9873	9-Octadecenoic acid, (2-phenyl-1,3-dioxolan-4-yl)methyl ester, trans-	56599-46-3	C28H44O4	91909851	61.0	
10.3369	2'-Deoxyuridine, 2TMS derivative	997772-86-0	C15H28N2O5Si2	8064221	56.7	
11.8409	Cinchonan-9-ol, (8.alpha.,9R)-	485-71-2	C19H22N2O	189664745	65.9	
13.4991	(E)-3-octadecenal	56554-99-5	C18H34O	196551121	66.5	
17.2628	Pyridine, 1,2,5,6-tetrahydro-1-methyl-4-[(5,6,7,8-tetrahydronapht-1-yl)aminomethyl]-	997405-49-5	C17H24N2	15903168	52.6	
21.6437	Cyclohexane, 1,3,5-triphenyl-	28336-57-4	C24H24	27235658	56.6	
22.3842	Mono(2-ethylhexyl) phthalate	4376-20-9	C16H22O4	166641343	81.1	
24.7985	3.beta.-Hydroxy-5-cholen-24-oic acid	5255-17-4	C24H38O3	104899906	62.4	
25.3704	Lupeol	545-47-1	C30H50O	261707626	77.3	

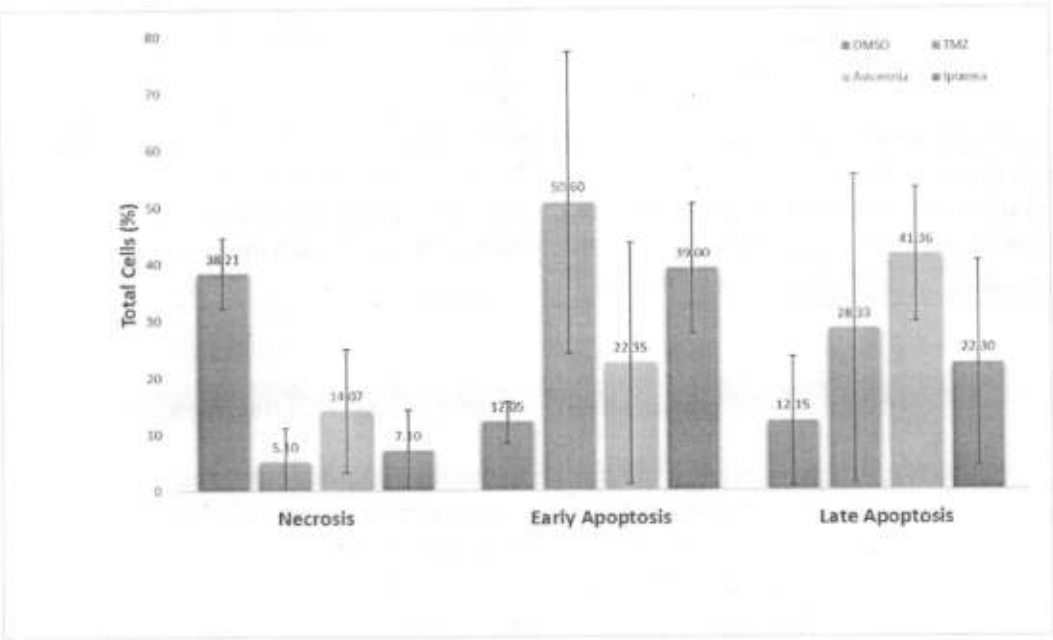
Figure 5: ***GC-MS analysis on the profiling of secondary metabolites for Ipome pes-carpe:*** *Ipomea* found to have the following compounds: 4H-Pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl, 9-octadecenoic acid, 2'-deoxyuridine,Cinchonan-9-ol,(E)-3-octadecenal, Pyridine, 1,2,5,6-tetrahydro-1-methyl,Cyclohexane, 1,3,5-triphenyl, Mono (2-ethylhexyl) phthalate,3, beta-Hydroxy-5-cholen-24-oic acid, and Lupeol.

Task 3: Validated the anti-metastatic property in GBM cell lines and patient's sample

Summary: Functional characterisation of the purified compounds were done such as analysis of apoptosis assay using Annexin V-FITC/PI Staining, estimation of caspase3/7 Activity, ROS, migration assay, and protein array. The changes in the cellular morphology such as reduction in nuclear size, chromatin condensation, and DNA fragmentation was observed under fluorescence microscope. In Avicennia treated LN229 and SNB19 cells, around 14% and 20% of cells were in necrotic, 22% and 35% in early apoptotic and 41% and 21% in late apoptotic stage, respectively. Similarly, Ipomea treated cells LN229 and SNB19 cells, around 7% and

20% of cells were in necrotic, 39% and 49% in early apoptotic and 22% and 20% in late apoptotic stage, respectively. The in-vitro scratch assay was done to evaluate the effect of the drugs on the migration rate of both glioblastoma cell lines. The images were captured at different time points such as 0h, 2h, 4h, 6h and 8h and the wound closure distance was calculated using Image J software. The calculated values were based on the scratch coverage rate up to 8hrs which is compared with DMSO (Control). The migratory percentage was significantly reduced as the time increased to 8 hours, in both the cell lines. In case of Ipomea, there observed grains during the experimentation period, which represents the death of GBM cell lines. Caspase, a cysteine proteases, mediates many biochemical and morphological changes associated with apoptotic cells. It is used as a biomarker for the detection of apoptosis process and hence the influence of both the drugs was assessed in glioblastoma cell lines. It was observed that Avicennia and ipomea treated cells showed 0.4 fold and 0.5 fold increase in the caspase 3/7 activity in SNB19 cells whereas LN229 cells showed 1.4 and 4.5 fold increase in the caspase activity. Likewise, ROS level was also estimated to understand the whether apoptosis was induced due to the ROS mediated cell death. Avicennia induced to about 2.5 and 2.6 fold increase in ROS in LN229 and SNB19 with 3.4 and 4.6 fold increase when treated with Ipomea. Thus, all the above experiments revealed that Ipomea was more effective in inducing the GBM cell death . All the data's was shown as means ± standard deviation of all biological and technical replicates. T-test was used to calculate the values and the results were considered statistically significant if the $p < 0.05$. Also, protein array specific for MAPkinase pathway was studied in GBM cells using RnD protein extraction kit for the blotting experiment. Avicennia treated cells found to show Down regulation of genes RIPK, CTNNB1 and PSAP with the upregulation of SPRY1, EZR, RASA3 was observed in both the cells. Ipomea treated cells showed downregulation of CTNNB1 and GADDs with upregulation of EZR, RASA3 and CAV1.

ESTIMATION OF APOPTOSIS IN LN229



ESTIMATION OF APOPTOSIS IN SNB19

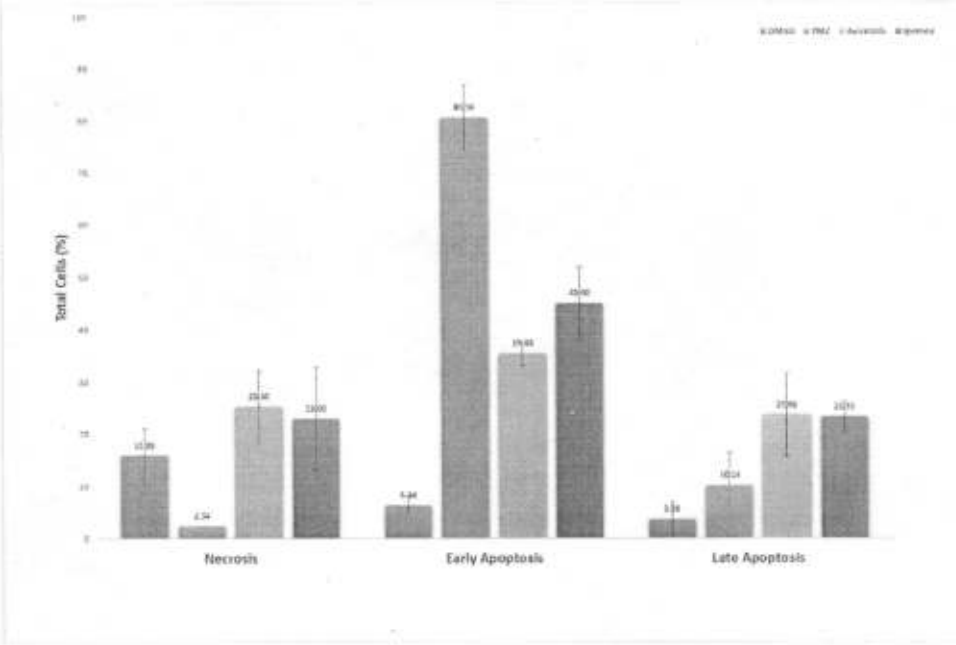


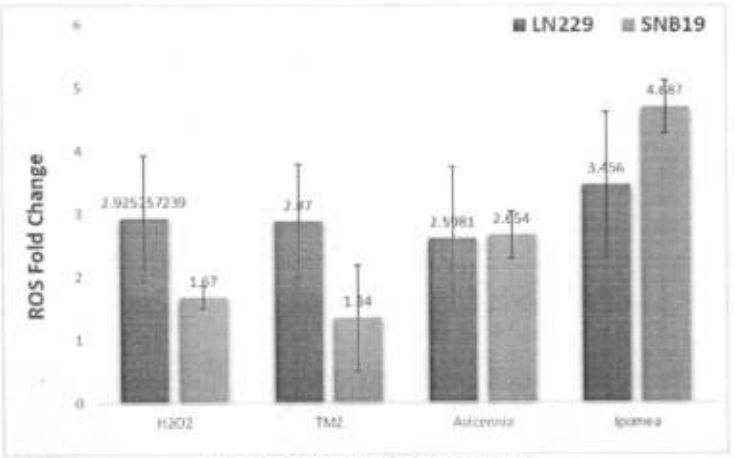
Figure 6: Effect of Avicennia and Ipomea on glioblastoma cell growth and apoptosis. Cell growth inhibition was determined with trypan blue solution for both the drugs and temozolomide (TMZ) against LN229 and SNB19 with IC50 concentrations at 24 hours post treatment.

Microscopic analysis of apoptosis in LN229

Microscopic analysis of apoptosis in SNB19

*Figure 7: Demonstrated images of untreated and treated cells using Dead Cell Apoptosis Kit with Annexin V-FITC and PI. Percentage of apoptosis, necrosis using Dead Cell Apoptosis Kit with Annexin V-FITC and PI in LN229 and SNB19 in treatment with DMSO, TMZ and the drugs at 24 hours post treatment. All experiments were performed with three biological repeats and two technical repeats. ** P < 0.001, * P < 0.05 compared to the TMZ.*

ESTIMATION OF REACTIVE OXYGEN SPECIES



ESTIMATION OF CASPASES

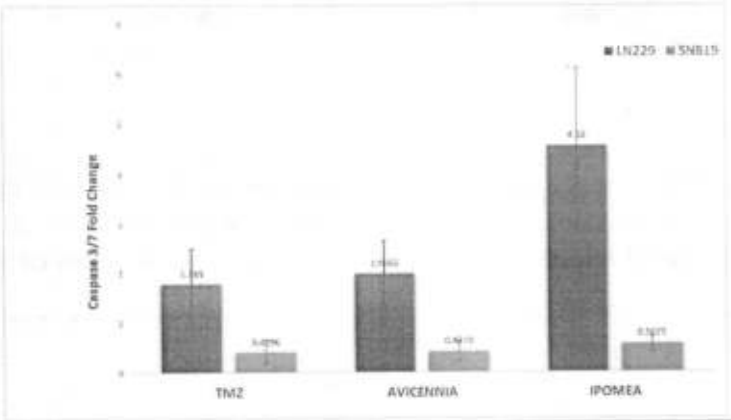
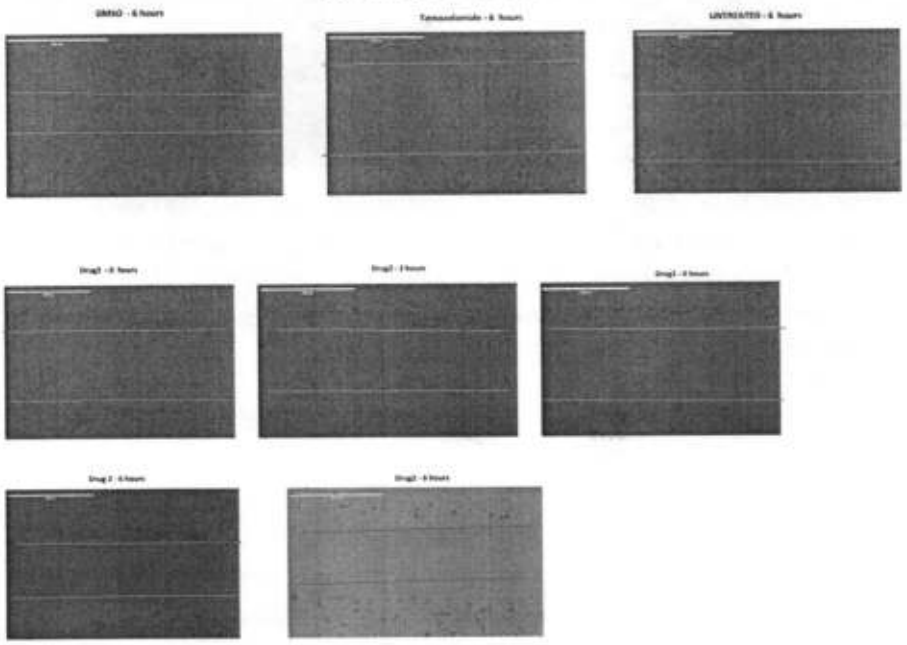


Figure 8: Estimation of Reactive oxygen species and the caspase3/7 for the induction of apoptosis upon treatment of Avicennia and Ipomea. Hydrogen peroxide was used as a positive control and TMZ used as a experimental control for ROS whereas TMZ was used as a control for caspase.

MIGRATION ASSAY - LN229



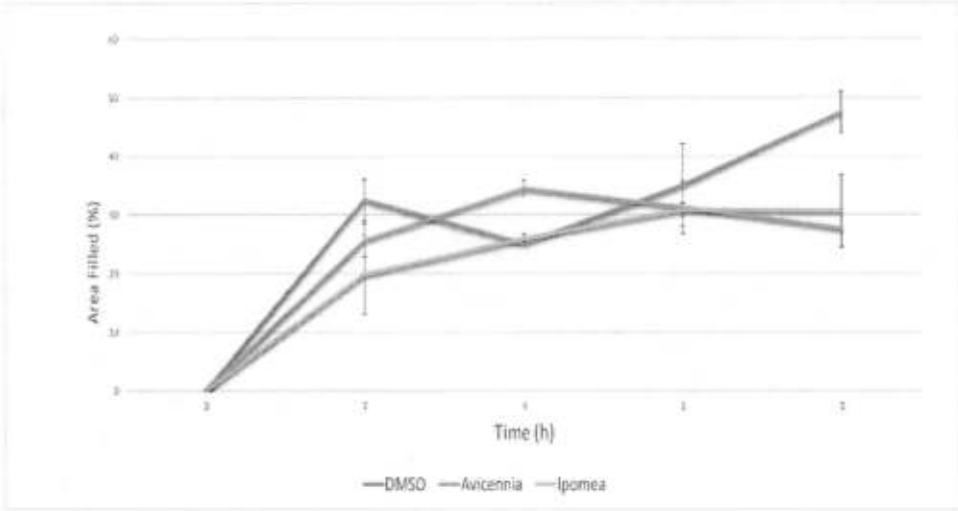
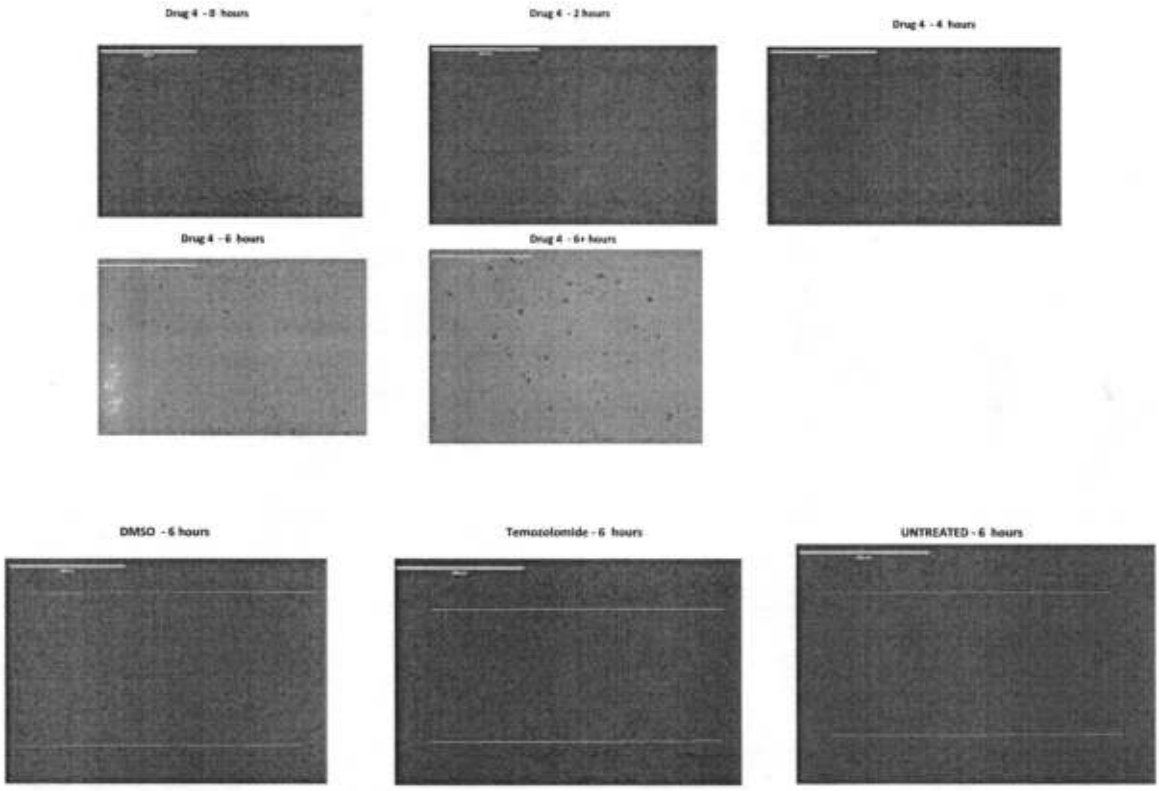


Figure 9: Analysis of cell migration and invasion assay in LN229: Effect of DMSO (control), Avicennia and Ipomea on cell migration (A), invasion (B) on GBM cells using transwell method. Microscopic images are the representation of the % of migration and invaded cells captured in 40X magnification. Data shown represent the mean ± s.e.m. of three independent experiments completed in triplicate with ** $p < 0.01$.

MIGRATION ASSAY -SNB19



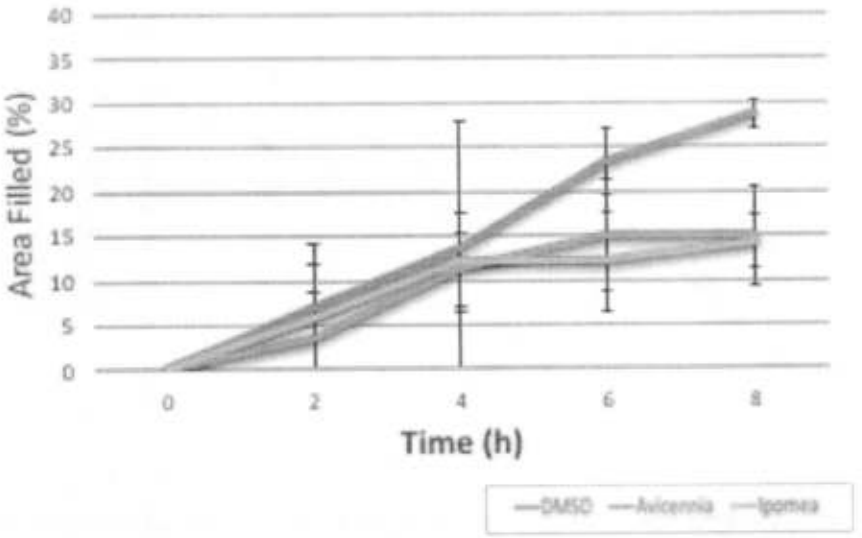


Figure 10: Analysis of cell migration and invasion assay in SNB19: Effect of DMSO (control), Avicennia and Ipomea on cell migration (A), invasion (B) on GBM cells using transwell method. Microscopic images are the representation of the % of migration and invaded cells captured in 40X magnification. Data shown represent the mean ± s.e.m. of three independent experiments completed in triplicate with ** $p < 0.01$.

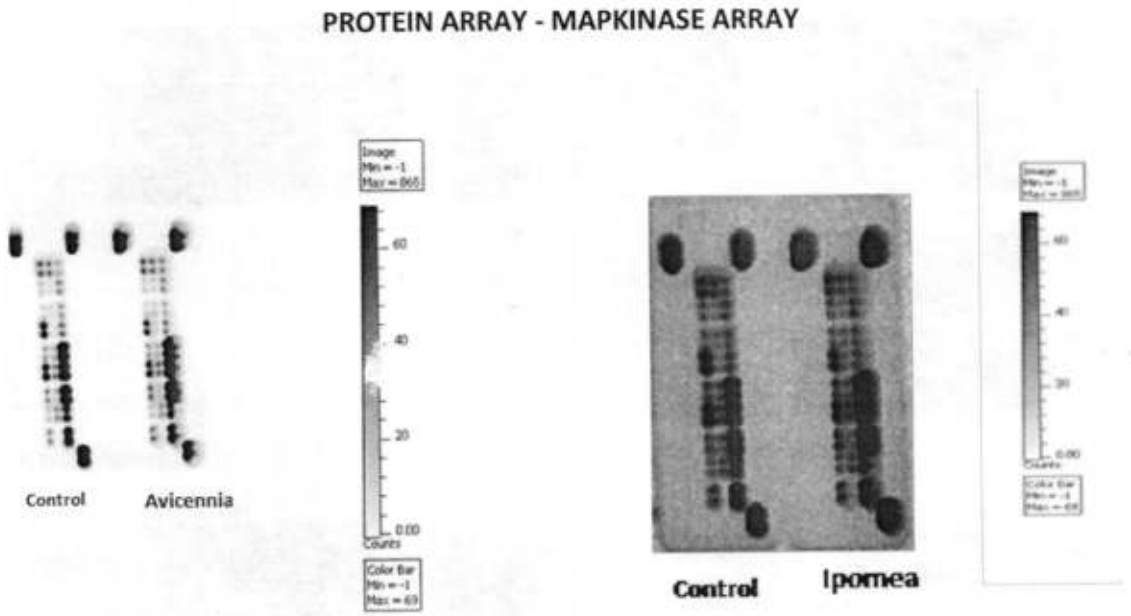


Figure 11: Proteome profiling of MAPkinase-associated proteins. (A) Array images showing the expression of total 35 apoptosis-associated proteins upon treatment with DMSO as control.

TECHNIQUE/EXPERTISE ACQUIRED:

Technical expertise gained: Invitro assays such as MTT assay, Apoptosis analysis, Migration invasion, ROS, caspase 3/7 assay, colony assay, cell cycle analysis using FACS, EMSA, protein array, Image processing using ImageJ software. Apart from the technical experience, other experience gained through this International training includes manuscript drafting, proposal writing.

RESEARCH RESULTS, INCLUDING ANY PAPERS, PREPARED/SUBMITTED FOR PUBLICATION

As an outcome of the research work carried out during the fellowship tenure, the following paper were published/submitted/in preparation.

Papers published:

1. Gnanavel, M.; **Murugesan, A.**; Konda Mani, S.; Yli-Harja, O.; Kandhavelu, M. Identifying the miRNA Signature Association with Aging-Related Senescence in Glioblastoma. Int. J. Mol. Sci. 2021, 22, 517.

Papers submitted:

2. Jeyalakshmi Kandhavelu, Ramesh Veeriah, Kumar Subramanian,Priyatharsini Rajendran, Olli Yli-Harja, Meenakshisundaram Kandhavelu*, **Murugesan, A*** "Vaccines, repurposed drugs and alternative biomedicines for the Management and prevention of nCOVID-19", Asian Pacific Journal of Tropical Medicine".

Manuscript in preparation:

3. Akshaya Murugesan, Phuong Doan, Olli Yli-Harja, Meenakshisundaram Kandhavelu*, "Identification of Novel molecular leads with potential anti-metastatic property from the polyphenolic fractions of marine halophytes".

PROPOSED UTILIZATION OF THE EXPERIENCE IN INDIA

Lady Doak College, Department of Biotechnology has the cell culture laboratory with basic facilities. The necessary Instruments like FACS, high-throughput microscopy with advanced softwares, Image processing software's will be proposed to procure. Also, the expertise I have acquired will be used to train the scholars, project fellows, master's candidates. New lab protocols will be proposed to be included in Bachelor's and Master's syllabus, so that young students will get an Idea of cell culture and cancer biology. Also, new course on Biomedical scientist will be proposed in the upcoming years, which will include all the techniques learnt during the training period.

AKSHAYA MURUGESAN

ICMR Sanction No:
INDO/FRC/452/Y-48/2019-20-IHD
dated 12th December 2019.

Ref-ID: 2019-00349 dated 6th June, 2019.