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Comparative evaluation of anti-cancer properties from different Indian plants and seaweed using in vitro and silico technique

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ABSTRACT

Background/aim: *Non-small cell lung cancer (NSCLC) remains one of the main contributors to the global health burden. Researchers have been exploring alternative treatments as chemotherapy has shown a tremendous amount of adverse side effects on patient's health.*

Materials and methods: *This study focuses on comparing the anti-neoplastic effects of Syzygium aromaticum (clove), Myristica fragrans (mace), Sargassum wightii (a marine brown algae), and Trigonella foenum-graecum (fenugreek) using in vitro and in silico techniques. This study employs MTT assay using A549 cell lines to study the potential of anti-proliferative as well as DPPH assay to study the anti-oxidative properties of the samples. To understand the molecular mechanism by which these phytochemicals work, the study uses ligand-protein docking with cancer-critical proteins like AKT1, EGFR and KRAS.*

Results: *The study reveals that Myristica fragrans (mace) has the lowest LD50 value indicating being most cytotoxic towards A549 lung cancer cells while Trigonella foenum-graecum (fenugreek) seeds have the highest anti-oxidative properties amongst the samples. Docking studies also indicate efficient binding of some of the bioactive compounds like Piperitol and Sarsasapogenin with cancer-critical proteins.*

Conclusion: *In conclusion, these plant and seaweed samples show potential for future studies in drug development of alternative therapies for cancer.*

Keywords: *Non-small lung cancer,alternative therapies, antioxidant properties, anti-proliferative properties, Myristica fragrans, Trigonella foenum-graecum*

Introduction

It has been decades since lung cancer has been a global health burden for cancer-related mortalities across the globe, mainly due to its diagnosis at a very late stage and lack of effective treatment strategies (1). The complexity of the molecular pathways that cause tumorigenesis and lead to resistance to therapy has posed a major obstacle in its treatment. Oncogenes such as KRAS (Kristen Rat Sarcoma Viral oncogene homolog), AKT1 (Protein Kinase B), and EGFR (Epidermal Growth Factor Receptor) play a crucial role in such molecular pathways, promoting lung cancer progression––(24). Cancer treatments have evolved a lot in recent years such as chemotherapy, radiation, and immunotherapy though, the effectiveness of such treatments in advanced stages has been low, along with its adverse effects(5). Hence, there has been growing interest among researchers to explore alternative treatments, especially those originating from natural sources to enhance conventional treatment strategies. Many bioactive compounds from different medicinal plants found across the globe have shown promising results in preventing cell proliferation, apoptosis and oxidative stress in cancer cells(6).

This study mainly focuses on the potential anticancer effects of bioactive compounds derived from clove, mace, seaweed, and Fenugreek. There have been many studies reporting the antibacterial, anti-inflammatory, and antioxidant properties of these plants––(710).

The current study intends to use molecular docking to examine the binding interactions between bioactive compounds derived from *Syzygium aromaticum* (clove), *Myristica fragrans* (mace), *Sargassum wightii* (a marine brown algae) and *Trigonella foenum-graecum* (fenugreek) with cancer critical proteins: KRAS, AKT1 and EGFR. This approach will help to identify the phytochemicals that are key for preventing the action of these cancer-critical proteins in the progression of lung cancer, which could lead to the development of targeted therapies for lung cancer by identifying inhibitors for KRAS, AKT1 and EGFR(11).

There have been extensive records of therapeutic uses of these traditional medicines but their effects specific to lung cancer cells remain unexplored. This provides researchers with an opportunity to investigate the potential anticancer properties of these plants and its phytochemicals. This study also aims to assess the cytotoxic and antioxidant effects of these phytochemicals on the lung cancer cells using in vitro assays. The MTT assay is used to determine the cytotoxic effects of the phytochemicals on A549 cell lin(12) while the DPPH assay is used to assess the antioxidant capacity of the plant extracts(13). This study seeks to identify potential bioactive compounds responsible for its anticancer effects, which can be further developed as drug candidates for therapeutic uses for alternative treatment of cancer.

Materials and Methods

Preparation of crude extracts

The plant samples were obtained from the authenticated vendor in Ahmedabad, Gujarat. The plant materials were identified by the Botany Department of St. Xavier's College (Autonomous), Ahmedabad. Phytochemical extraction from *Syzygium aromaticum* (clove), *Myristica fragrans* (mace), *Sargassum wightii* (marine brown algae), and *Trigonella foenum-graecum* (fenugreek) was performed using methanol as solvent. This method was chosen because methanol is a polar solvent that effectively extracts a wide range of bioactive compounds, including alkaloids, flavonoids, phenolic acids, and other polyphenols, which are known for their anticancer and antioxidant properties'(14). The extraction was carried out using maceration. Plant and algal materials were shade dried and powdered finely, 10 g each were weighed accurately and placed in separate glass flasks. The powdered materials were then immersed in methanol (100 mL) at room temperature. The mixture was kept under constant agitation (usingan orbital shaker) to enhance the solubility of phytochemicals. After 48 hours of maceration, the mixture was filtered through a Whatman No. 1 filter paper to separate the solid plant and algal residue from the liquid extract. The solvent was evaporated at 40°Cto concentrate the extracts and stored at 4°C till further use.

Phytochemical Screening

The crude methanolic extracts were analysed using O-HRLCMS (Q-Exactive Plus Biopharma, Thermo Scientific) at SAIF, IIT Bombay, India. The column used for chromatographic separation was Syncronics C18: 100 x 2.1 mm, 1.7 microns (Thermo Scientific). Gradient elution was performed using 0.1% formic Acid in Milli-Q water and Acetonitrile. The compounds were identified by Compound Discoverer 3.2 SP1 (Thermo Scientific).

Maintenance of A549 cell line

In the present study, A549 cell line was grown in HAM F-10 media with 10% FBS (Foetal Bovine Serum). The pH of the media was 7.4. The culture conditions were kept constant by keeping the T-25 culture flasks in the CO_2 Incubator (ESCO CellMate CO_2 Incubator) at 37°C and 5.0% CO2 concentration. Nutrient mixture F-10 HAM and Foetal Bovine Serum (FBS) was obtained from HiMedia. Hydrochloric acid (HCl), Sodium Hydroxide (NaOH) and Sodium Bicarbonate ($NaHCO_3$) powder were obtained from SRL Private Limited.

In silico Studies

Molecular docking is one of the most used computational tools of Computer Aided Drug Design (CADD). It involves ligands and proteins. Proteins are the target on which the potential binding of ligand molecules is tested with different conformations. It predicts the possible conformations assessed through scoring functions(15).

Molecular Docking and Visualization of molecular interactions

Autodock4 and Discovery Studio Visualizer (BIOVIA) have been used in this study to examine the types and affinities of interactions between the phytochemicals found in plant extracts and some of the critical proteins involved in cancer metabolism. Positive interactions between these molecules could point to a potential mechanism of action for how these phytochemicals may prevent cancer.

Autodock4 was used to obtain the docking scores while the interactions of the protein-ligand complex were studiedusing BIOVIA Discovery Studio Visualizer software.A genetic algorithm (GA) was used to find the best conformation for the docking. Lamarckian output was used to enhance localized optimization(16). Structures of macromolecules AKT1 (PDB ID: 3O96), KRAS (PDB ID:4OBE), and EGFR (PDB ID: 7A2A) were retrieved from Protein Data Bank while structures of all the ligands were retrieved from PubChem.

Antioxidant Activity Assay

This method assesses antioxidant capacity using free radicals to measure hydrogen production or radical scavenging ability. DPPH becomes purple colored upon reaction with an odd electron, with absorbance at 517 nm(17). Ascorbic acid and methanol were obtained from SDFCL, and DPPH from HiMedia.

MTT Cell Cytotoxicity Assay

The MTT reagent is a mono tetrazolium salt that can pass through the cell membranes and the mitochondrial inner membrane of viable cells. It is reduced by metabolically active cells to form a violet-blue water-insoluble molecule called formazan. This reaction produces a cooler that can be used to measure intracellular formazan production, which forms the basis of the MTT assay. The assay is commonly used to measure cell metabolic activity, but it has also been used to infer other cellular processes, such as viability, which may not be well-supported(18). The 96 well plate was seeded by trypsinizing 80% confluent T-25 culture flask. After 24 hours, fresh media along with varying concentrations of sample and standard drug (doxorubicin) is added to the wells. Controls were also added. Doxorubicin was used as positive control. The 96-well plate was incubated in the Incubator (ESCO CellMate CO_2 Incubator) at 37 °C and 5.0% CO_2 concentration for 24 hours. Media is aspirated from all wells and 20µl of MTT solution is added to the 96 well plates and incubated for 3 hours in the Incubator at 37°C and 5.0% CO_2 concentration. 150µl of DMSO was then added to the wells. The plate was read at 490 nm using Microplate Reader (Thermo Fisher Scientific).

RESULTS

Phytochemical screening

OHRLCMS was performed for crude extracts of all the samples. As shown in the Figure 1, peaks were obtained for all the samples: *Syzygium aromaticum, Sargassum wightii, Myristica fragrans* and *Trigonella foenum-graecum*, both in positive as well as negative mode indicating presence of bioactive compounds.

In silico studies anddocking analysis

Some of the significant bioactive compounds (Figure 2) from *Syzygium aromaticum*, *Sargassum wightii*, *Myristica fragrans* and *Trigonella foenum-graecum* were used as ligands in protein-ligand interaction studies. The docking score for the phytochemicals is represented in Table 1.

Molecular docking for AKT1

The docking analysis of AKT1 with the different phytochemicals from the plant samples under study showed potent binding efficiency with the cancer-critical protein. 1,4,7-Cycloundecatriene, 1,5,9,9-tetramethyl showed best docking score of -7.39 kcal/mol from *Syzygium aromaticum,* similarly Fucosterol (-9.42 kcal/mol) from *Sargassum wightii*, Piperitol

(-6.26 kcal/mol) from *Myristica fragrans* and Sarsasapogenin (-9.3 kcal/mol) from *Trigonella foenum-graecum* as shown in the Table 1.

Molecular docking for KRAS

Numerous phytochemicals from the plant samples gave effective binding interactions with KRAS protein. Copaene had highest binding affinity of -7.39 kcal/mol from *Syzygium aromaticum*, while Fucosterol (-7.67 kcal/mol) from *Sargassum wightii,*Piperitol (-4.75 kcal/mol) from *Myristica fragrans* and Diosgenin (-8.75 kcal/mol) from *Trigonella foenum-graecum* as shown in the Table 1.

Molecular docking for EGFR

Protein-ligand studies with EGFR also gave significant results. Copaene had the best binding affinity of -8.04 kcal/mol from *Syzygium aromaticum*, while Fucosterol (-6.58 kcal/mol) from *Sargassum wightii*, Verrucosin (-6.08 kcal/mol) from *Myristica fragrans*, and Sasapogenin (-9.36 kcal/mol) from Trigonella foenum-graecum, as shown inTable 1.

Protein-Ligand Interactions

There wasnumber of significant protein-ligand interactions observed between the bioactive compounds from the four plant species under the study and the cancer critical proteins. Amongst those, a few interactions are shown in the Figure 3. Copaene from Syzygium aromaticum shows Alkyl interactions with Ile740, Pro741, Leu792, Pro794 and Lys864 of KRAS protein. Fucosterol from Sargassum wightii forms Hydrogen bond with Asp119 in KRAS and Alkyl interactions with Phe28, Lys117, Lys147 and Leu120. Piperitol from Myristica fragrans interacts with AKT1 protein by the formation of a Hydrogen bond with Ser205 residue of AKT1, pi-pi stacked interaction with Trp80, Alkyl interactions with Leu210, Leu264, Lys268, and Val270. Sarsasapogenin from Trigonella foenum-graecum shows strong interaction with EGRF protein by the formation of Hydrogen bond with Pro794, and Alkyl interactions with Leu718, Leu792, Val726, Lys745, Met790, Lys745, Leu788 and Ala743.

MTT Cell Toxicity Assay

The findings of the cell cytotoxicity experiment against A549 are illustrated in the Figure 4. Figure 4(a) illustrates a dose-dependent cytotoxicity in all samples, with Doxorubicin serving as the positive control. LD_{50} was determined by nonlinear regression analysis using GraphPad Prism 10.1.0. Figure 4(b) illustrates that *Syzygiumaromaticum*exhibited the highest LD_{50} at 457.3 µg/ml, followed by *Sargassum wightii*at 264.1 µg/ml, *Trigonella foenum-graecum*at 176.9 µg/ml, and *Myristica fragrans* at 150.1 µg/ml, which had the lowest LD_{50} among the samples. The LD_{50} for the control Doxorubicin was 109.2 µg/ml. The findings demonstrate that *Myristica fragrans* has the highest cytotoxicity against A549 lung cancer cells.

DPPH Antioxidant Assay

The antioxidant activity of all the samples was measured using DPPH (2,2-diphenyl-1-picrylhydrazyl) assay. Figure 5(a) represents the standard curve obtained using Ascorbic Acid as a standard. Figure 5(b) shows the comparison between IC_{50}values of different samples with *Trigonella foenum-graecum* having the lowest IC_{50} value of 35.41 µg/ml indicating highest antioxidant potential amongst the sample, while *Syzygium aromaticum* had IC_{50} of 56.6 µg/ml, *Sargassum wightii* 67.43 µg/ml and *Myristica fragrans* of 102.6 µg/ml.

Discussion

Researchers across the world have been trying to find a cure for cancer for decades, though the treatments seem harsh and leave adverse effects on patients' health in the long term, not to mention the financial burden that it causes. Recent studies have shownsome promises as seen in CAR-T cell therapy for cancer treatment, though it comes with its own limitations (19). Many researchers are attempting to identify drugs that can be repurposed as effective therapies for Alzheimer's disease (AD). Several recent studies have highlighted epidermal growth factor receptor (EGFR) inhibitors approved for use as anti-cancer drugs as potential candidates for repurposing as AD therapeutics. In cancer, EGFR inhibitors target cell proliferation and angiogenesis, and studies in AD mouse models have shown that EGFR inhibitors can attenuate amyloid-beta (Aβ) pathology and improve cognitive function. In this review, we discuss the different functions of EGFR in cancer and AD and the potential of EGFR as a dual molecular target for AD diseases. In addition, we describe the effects of anti-cancer EGFR tyrosine kinase inhibitors (TKIs) on AD pathology and their prospects as therapeutic interventions for AD. By summarizing the physiological functions of EGFR in cancer and AD, this review emphasizes the significance of EGFR as an important molecular target for these diseases(Choi et al., 2023). The requirement for sophisticated medical infrastructure for such treatments would limit its availability to thecommon man. Millions across the globe suffer from cancer and only a small percent would be able to access and afford such treatments(20). This scenario calls for developing readily available drugs that show promising results. Hence, researchers are trying to explore plant-based metabolites that can be potent in preventing and treating cancer. Current studies have focused on four such plants and algal species found in India, which haveshown promising results for cancer treatment.

The cytotoxicity of the plants was measured in the present study using MTT assay. Studies have reported LD50 for Syzygium aromaticum in range of 142.5 µg/ml to 2879.8 µg/ml(21), 2.11 µg/ml to 78.15 µg/ml for Myristica fragrans (22), 200 µg/ml to 250 µg/ml for Sargassum wightii (23) and 1055 µg/ml for Trigonella foenum-graecum (24). The current study has shown similar results as shown in the figure though some variations in LD50 wereobserved, all the plant and algal samples had shown potential against A549 cancer cells. ROS (Reactive Oxygen Species) can be generated during chemotherapy. These radicals can interact with cellular components like lipids, proteins, sugars as well as DNA. Though DNA is well protected, accumulation of ROS can still lead to mutations and damage DNA repair mechanisms(25). Cellular membrane is also affected by ROS due to the presence of lipids as a primary constituent. This can affect cellular integrity ultimately leading to necrosis and cell death. In the current study, antioxidant properties of *Syzygium aromaticum, Sargassum wightii, Myristica fragrans* and *Trigonella foenum-graecum* have been studied using DPPH assay. Many phytochemicals from plants, especially flavonoids are known to quench the free radicals generated in cells, enabling them to act as antioxidants –(26). All the plants and algal species studied here has shown antioxidant properties, though *Trigonella foenum-graecum* had the highest among the four. KRAS plays a crucial role in tumorigenesis and metastasis of cancer cells. KRAS is found to be the most mutated form of the RAS family. Hence, KRAS has been targeted for cancer therapy in recent years(27).

Diosgenin, a compound derived from *Trigonella foenum-graecum* gave the best docking score with KRAS, which could be indicative of potential inhibition of the cancer-critical molecule. Similarly, AKT1 is usually activated in cancer cells which leads to tumorigenesis as well as resistance towards chemotherapy. Hence, researchers have identified AKT1 as a potential target for treatment of cancer (28). Fucosterol from Sargassum wightii had the highest binding affinity with AKT1, which suggests that it could act as a potential inhibitor for AKT1. Lastly, EGFR was also studied in current research. EGFR plays an important role in proliferation of cells. Overexpression of EGFR is often associated with lung cancer, breast cancer and glioblastoma. EGFR is involved in modulation of several metabolic processes related to cell proliferation. Therefore, it has been an attractive target for researchers to prevent cancer''(29). In the current study Sarsasapogenin showed the strongest interaction with EGFR. Hence, it can be further examined for its potential to act as an inhibitor for EGFR in cancer therapy. Current study shows immense potential of *Syzygium aromaticum, Sargassum wightii, Myristica fragrans* and *Trigonella foenum-graecum* in the treatment of cancer and developing targeted therapies.

Conclusion

The current study shows tremendous potential for plant-based therapeutics against lung cancer. The bioactive compounds assessed in this study have shown promising results in developing targeted alternative therapies for cancer treatment.

Acknowledgment and/or disclaimers

We would like to acknowledge OHRLCMS facility at SAIF, IIT Bombay for the analysis of phytochemical profile using Q-Exactive Plus Biopharma-High Resolution Orbitrap system.

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Conflicts of Interest: The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

Table 1: The docking score for the phytochemicals for the selected plants and sea-weed

Macromolecule:		AKT1	KRAS	EGFR
Sr. no.	Ligand	Docking Scorekcal/mol	Docking Score kcal/mol	Docking Score kcal/mol
Syzygium aromaticum				
1	3-Allyl-6-methoxyphenol	-4.91	-5.37	-5.18
2	alphaFarnesene	-5.77	-5.81	-6.18
3	Caryophyllene	-7.27	-7.77	-7.92
4	Caryophylleneoxide	-4.91	-7.21	-7.35
5	Copaene	-6.8	-8.11	-8.04
6	1,4,7-Cycloundecatriene, 1,5,9,9-tetramethyl	-7.39	-5.48	-7.52
7	Eugenolacetate	-4.96	-5.39	-5.25
8	2-Pentanone, 4-hydroxy-4-methyl	-6.49	-7.36	-7.44
9	2-Pentanone-3-methylene	-4.2	-4.35	-4.32
10	2,4,4,6-Tetramethyl-4,5-dihydro-1,3-oxazine	-5.47	-5.66	-4.95
Sargassum wightii				
1	Fucosterol	-9.42	-7.67	-6.58
2	AromandreneOxide	-7.43	-7.44	-6.17
3	GuaiaDiene	-7.16	-7.16	-5.95
4	Fucoxanthin	-5.94	-6.86	-4.85
5	Phytane	-4.82	-4.95	-4.31
6	pseudocumene	-4.69	-4.92	-4.2
7	MethylIndole	-4.45	-4.81	-4.18
8	EthylBenzene	-4.4	-4.78	-4.14
9	Methylisoeugenol	-4.09	-4.7	-4.06
10	Toluene	-4.03	-4.57	-3.68
Myristica fragrans				
1	Gamma terpinene	-4.52	-1.66	-4.53
2	Verrucosin	-5.19	-3.91	-6.08
3	Vanillin	-3.07	-3.48	-3.96
4	Terpinen-4-ol	-4.98	-4.72	-5.29
5	Safrole	-3.92	-3.98	-4.35
6	Sabinene	-4.96	-4.42	-5.36
7	Piperitol	-6.26	-4.75	-5.97
8	P cymene	-4.7	-4.36	-4.8
9	Palmitic acid	-2.66	-2.65	-2.77
10	Oleic acid	-3.21	-2.13	-3.17

Trigonella foenum-graecum				
1	Diosgenin	-8.8	-8.75	-8.46
2	Gentianine	-4.46	-4.53	-5.62
3	Gitogenin	-8.6	-8.04	-7.73
4	Isovitexin	-3.99	-3.27	-2.54
5	Neotigogenin	-8.57	-7.72	-8.58
6	Neurine	-1.28	-2.62	-1.18
7	Quercetin	-3.8	-2.83	-3.9
8	Choline	-1.57	-3.11	-1.38
9	Yuccagenin	-8	-8.08	-9.16
10	Sarsasapogenin	-9.3	-7.25	-9.36

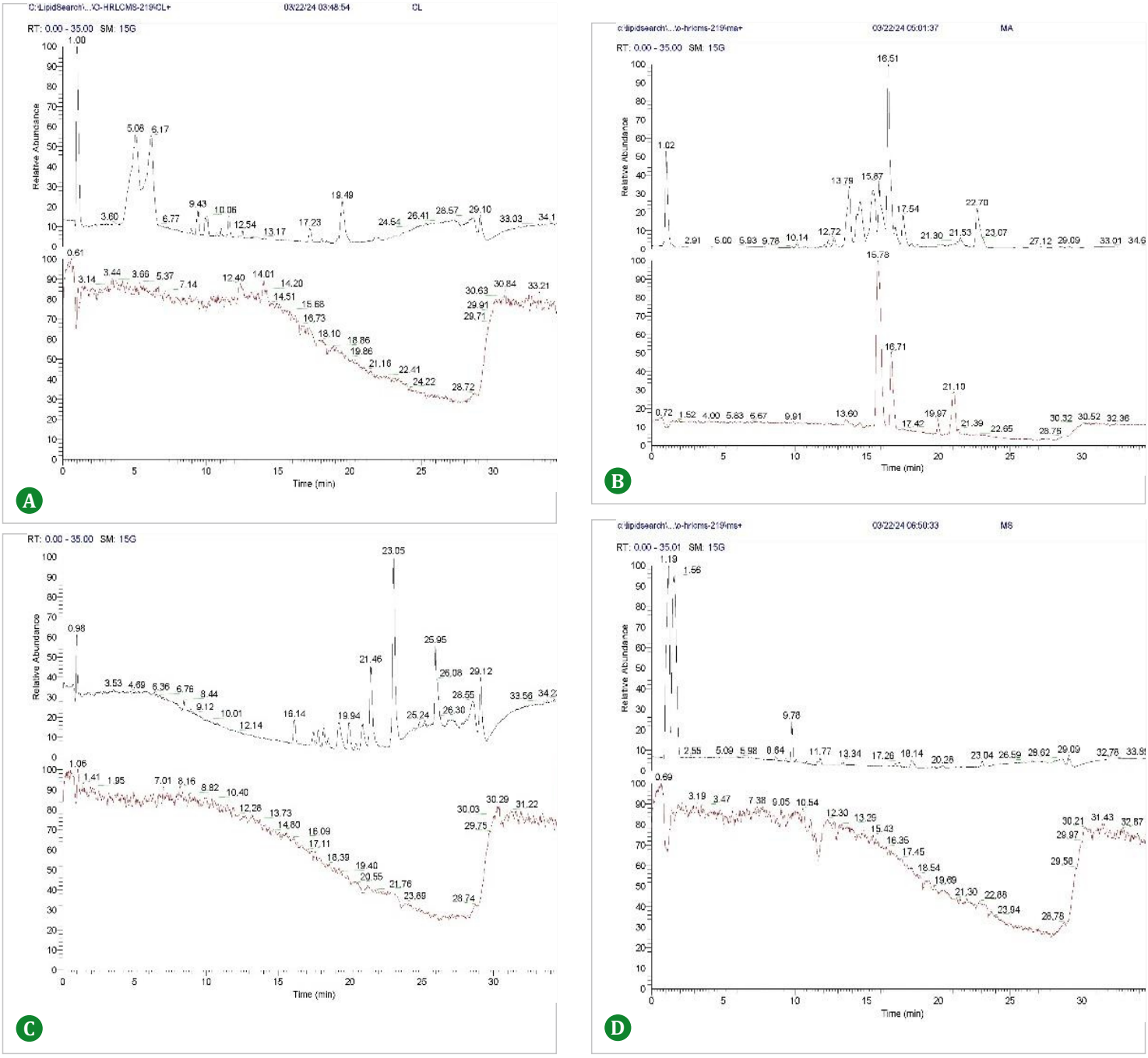
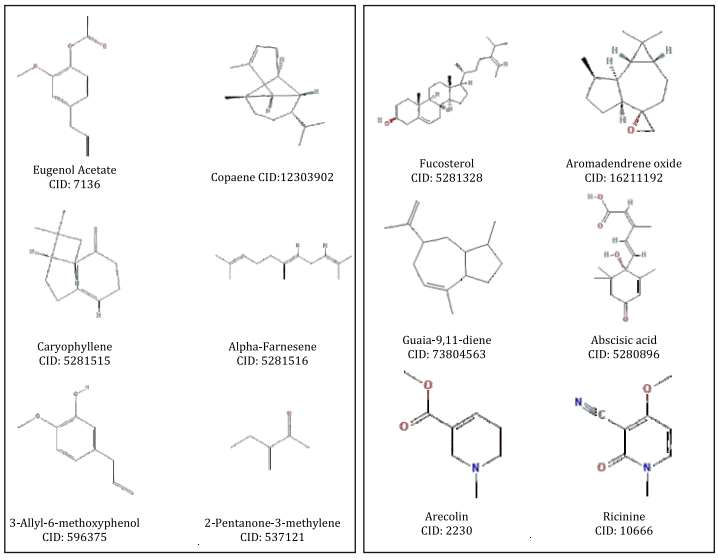
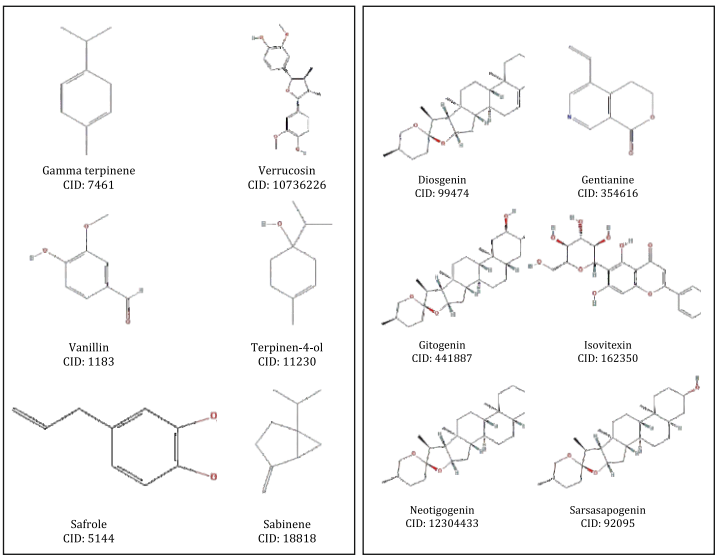


Figure 1. HRLCMS Chromatogram for both positive mode (top) and negative mode (bottom) obtained from the extracts of the samples, indicating retention time on X axis and relative abundance on Y axis. Chromatogram for samples (a) Syzygium aromaticum; (b) Sargassum wightii; (c) Myristica fragrans; (d) Trigonella foenum-graecum

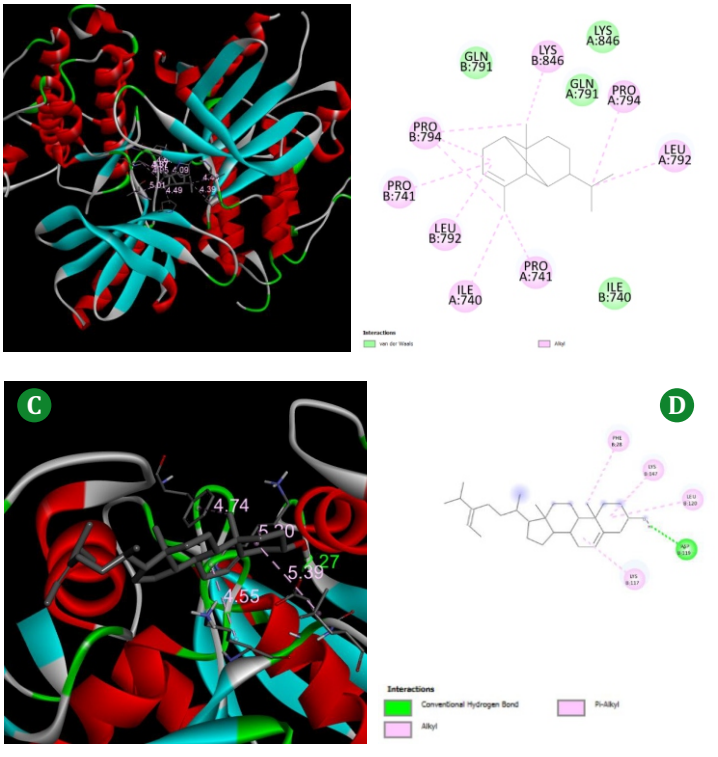


(a) Syzygium aromaticum *(b) Sargassum wightii*



(c) Myristica fragrans *(d) Trigonella foenum-graecum*

Figure 2: Some of the bioactive compounds found in (a) Syzygium aromaticum; (b) Sargassum wighti; (c) Myristica fragrans; (d) Trigonella foenum-graecum



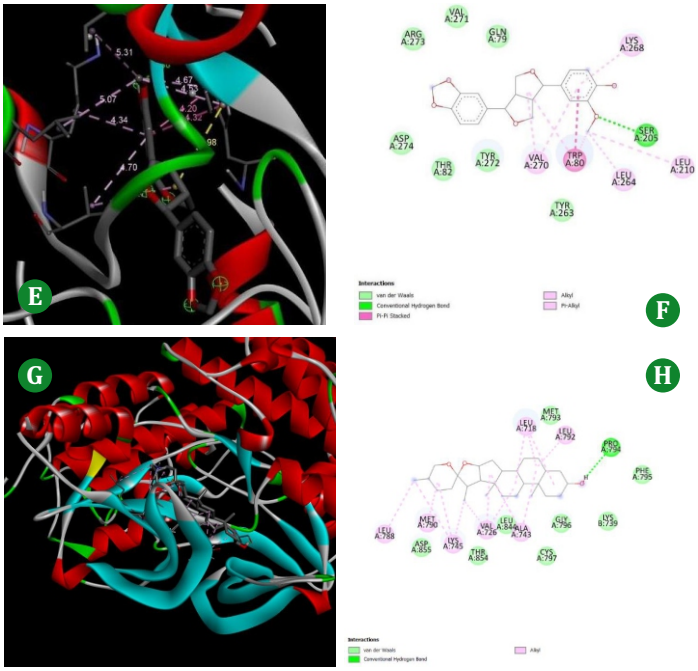


Figure 3: (a) 3-D interaction between Copaene and KRAS (b) 2-D interaction between Copaene and KRAS (c) 3-D interaction between Fucosterol and KRAS (d) 2-D interaction between Fucosterol and KRAS (e) 3-D interaction between Piperitol and AKT1 (f) 2-D interaction between Piperitol and AKT1 (g) 3-D interaction between Sarsasapogenin and EGFR (h) 2-D interaction between Sarsasapogenin and EGFR

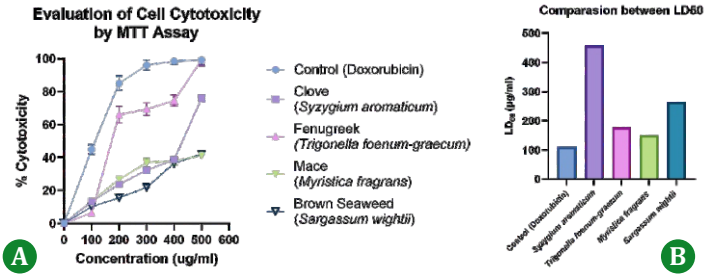


Figure 4: (a) Graphical representation of dose dependent relationship between concentration of samples and cell cytotoxicity using MTT assay (b) Comparison between LD50 values of different samples

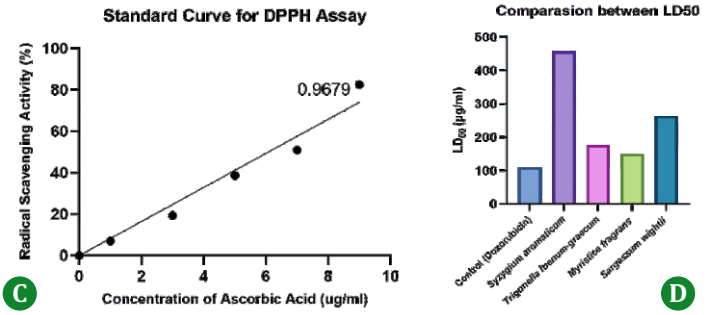


Figure 5: (a) Standard curve for DPPH assay using Ascorbic Acid as standard (b) Comparison between IC50 values obtained in DPPH Assay for different samples

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