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Chemical Profiling of *Kalingathi Kadugu*, A Herbomineral Siddha Formulation Through Gas Chromatography-Mass Spectrometry (GC-MS) Analysis

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ABSTRACT

Kalingathi Kadugu **(KK)** is a herbomineral Siddha formulation with its reference from the classical Siddha text ***"Agathiyar vallathi 600"***. Among the nine indications, ***Karupai Kaluthuputru*** **(Cervical cancer)** has been specially mentioned in the text. Cervical Carcinoma, the fourth most common cancerous disease diagnosed in women worldwide, is caused by several factors such as human papillomavirus (HPV) etc. Compared to other treatment methods, chemotherapy is the principal and most feasible method. The higher dosage accompanies many post-treatment clinical consequences along with side effects. Nowadays discovering molecules from classical traditional systems of medicine such as Siddha become imperative as the system has many promising formulations like kalingathi kadugu for cancer therapy.

The study intends to analyze the presence of the active compounds within the formulation KK obtained from the classical Siddha literature *"Agathiyar vallathi 600"*.

KK was prepared from the Classical Siddha literature *"Agathiyar vallathi 600"* as per SOP. The raw drugs were authenticated by the Chief consultant of Walter Siddha Research Centre, Tirunelveli and GC-MS analysis was performed in SAIF- IIT Madras as per standard guidelines. GC-MS analysis was performed for KK. GC-MS screening of the drug KK unveiled the presence of multiple compounds such as Lanosterol, 9,19-cyclolanostan-3-ol,24 methylene-(3β), Tetradecane etc., exhibiting diverse reported biological activities including potentially beneficial anti-tumor activity against tested carcinoma cells, therefore it deserves furthermore clinical research in the prospective.

Keywords: *Agathiyar vallathi* 600, Anti-angiogenesis, Cervical cancer, *Kalingathi kadugu*.

INTRODUCTION

In the current scenario, lifestyle changes may lead to the development of carcinoma in the cervix. Despite many technological developments, Cancer has emerged as a prevalent and significant health concern, leading to substantial human suffering and mortality. According to WHO, Cervical carcinoma stands as the sixth most frequently diagnosed cancer in women and 99% of cervical cancer is due to human papillomavirus (HPV) which is easily spread through skin-to-skin contact (WHO, 2024). In 2020, globally 604000 new cases of cervical cancer were diagnosed, among these 342000 deaths occurred. The curable rate is high if cervical cancer is diagnosed early.

While chemotherapy remains the primary and viable treatment approach for cancer compared to other therapeutic modalities the higher dose of this chemotherapy treatment accompanies many post-treatment clinical consequences along with side effects. While numerous drugs have been identified as cancer chemotherapeutic agents, no single compound has been reported to have null toxicity. Cisplatin, the standard treatment for cervical cancer, is associated with post-treatment toxicity. Nowadays discovering molecules from classical traditional systems of medicine such as Siddha, Ayurveda, etc., has emerged in cancer drug discovery research. Natural compounds serve as an invaluable resource for the development of potent therapeutics. In the current scenario, the leading structure for new drug discoveries is from the natural resources that have Biologically derived substances with high structural diversity. The natural components in the drugs show high effectiveness by focusing on targeting structures of utmost importance. (Faruck, 2016).

Numerous formulations were present in the Siddha system of medicine for cancer treatment. Our group primarily focuses on discovering natural product-derived medications for the treatment of cancer from the Siddha system of medicine. In this study, we explored the anticancer activity of KK formulation from the classical *"Agathiyar vallathi 600"* literature. In this formulation, the major ingredient is *Citrullus colocynthis* (*kalingathi*). *Citrullus colocynthis* seems a potential anticancer herbal medicine via various efficient compounds and is reported to trigger apoptosis in colorectal cancer cells also. (Abdulridha et al., 2020; Mohammed Al-Zharani et al., 2022). Therefore to find out the compounds responsible for anticancer properties and active principles we have performed GC-MS analysis in the formulation KK.

MATERIALS AND METHODS

(a)Preparation:

KK has been prepared as per the Siddha text "***Agathiyar Vallathi 600"*** (Uthamarayan et al., 1980). after following proper purification methods for its ingredients as per the Siddha textbook *"**Saraku Suthi Seimuraikal"*** (Anaivaari Ananthan, 2008). as shown in figure 1. The raw drugs were authenticated by the Chief consultant of Walter Siddha Research Centre, Tirunelveli.

(b) Gas chromatography- Mass spectrometry (GC-MS) Standard operating procedures:

Gas chromatography-mass spectrometry (GC-MS) is a diagnostic tool utilized for detecting the presence of active compounds in the formulations. **The acquisition method** of GC- MS of scan type is followed and the methods are mentioned in figure 2.

RESULT

GC–MS profile of the KK extract:

The formulation KK showed greater efficacy in cytotoxic activity against cervical cancer cell lines with all the advantages of micro-particle size. Consequently, the extracted portion underwent methylation to enhance volatility, and both fractions were subsequently analyzed using GC/MS.

The compounds recognized in the KK extract are presented in Table 1. The compounds identified as hits within the herbal formula are Lanosterol (63.24%), 9,19-cyclolanostan-3-ol,24

methylene -(3β) (60.97%), 3,3-Diethoxy-1-propanol, propyl ether (50.26%), Dodecane (28.49%), Tetradecane (27.72%), W-18(20.61%), methyl-3,3-dimethyl cyclopropane-1, trans-2-dicarboxylate (19.66%), 2-propanol,1 (1-methylethoxy) (12.24%) and Butanoic acid,2-ethyl-3 hydroxy-ethyl esteror 3-BH (6.75%) as shown in figure 3.

DISCUSSION

Secondary metabolites derived from plants often play a crucial role in treating a spectrum of conditions (Eng Soon Teoh, 2015). Gas chromatography-mass spectrometry (GC-MS) is an analytical method that integrates gas chromatography with mass spectrometry for the identification and quantification of organic substances in classical drug formulations. GC–MS analysis of KK unveiled the existence of multiple bioactive compounds, including Lanosterol(63.24%), 9,19-cyclolanostan-3-ol,24 methylene -(3β)(60.97%), 3,3-Diethoxy-1-propanol, propyl ether (50.26%), Dodecane(28.49%), Tetradecane(27.72%), W-18(20.61%),methyl-3,3-dimethyl cyclopropane-1,trans-2-dicarboxylate (19.66%), 2-propanol,1 (1-methylethoxy) (12.24%) and Butanoic acid,2-ethyl-3 hydroxy-ethyl ester(6.75%) with several known biological activities as shown in figure 5,6,7.

The GC-MS analysis of herbomineral formulation KK has three major hits namely Lanosterol (63.24%), 9,19-cyclolanostan-3-ol,24 methylene -(3β) (60.97%) and 3,3-Diethoxy-1-propanol, propyl ether (50.26%) as shown in figure 4.

(a) Lanosterol:

Lanosterol has a score of 743 in KK and has Anti-angiogenesis, Antitumor and Antiviral activities (Nourhan Hisham Shady et al., 2021). as shown in figure 6.

Claudia Stäubert Et al Found the potential of lanosterol in controlling function in maintaining cholesterol homeostasis which may be critical for **drug-resistant leukaemia cancer cells** and observed cancer drug resistance. Further, they revealed the novel connection between drug resistance and increased flux of lanosterol (Claudia Stäubert et al., 2016).

Lanosterol synthase (LSS), a crucial rate-limiting enzyme in cholesterol biosynthesis, may have a notable impact on oxidative stress. Antioxidants play a vital role in mitigating the toxic effects of free radicals in various diseases, including cancer. (Hui Hua et al. 2019).

Pengjuan Ma found that LSS protection plays an antifibrotic role in maintaining lens transparency. They also suggested that regulating lanosterol and sterol biosynthesis could be promising plans for averting and treating fibrotic cataracts (Pengjuan Ma et al., 2023).

Further, it was found that 3β-Hydroxylanosta-8,24-dien-21-al which is a lanosterol-type triterpene can inhibit tumour promotion and reduce the percentage of mice bearing papillomas (medchemexpress).

(b) 9, 19-cyclolanostan-3-ol,24 methylene -(3β):

9,19-cyclolanostan-3-ol,24 methylene -(3β) or 24-methylene cycloartenol (24-MCA) is derived mainly from Euphorbia species that have Anti-tumor and Anti-inflammatory activities (24-methylene cycloartenol, PUBCHEM). In our analysis, this compound is present in 60.97%

The two phytosterols 24-methylene cycloartenol (24-MCA) and cycloartenol (CA), found in Ficus krishnae exert antidiabetic activity by promoting an increase in the population of beta cells and restoring pancreatic beta cells to their natural insulin secretion function. (Ajikumaran Nair Sadasivan Nair et al., 2020**;** medchemexpress)

The use of 24-methylene-9,19-cyclolanostan -3-ol in drugs, food or drink improves pancreatic functions (Tanaka Miyuki, 2006).

(c) 3,3-Diethoxy-1-propanol, propyl ether:

3,3-Diethoxy-1-propanol, propyl ether present in 50.26% has anti-tumour, antimicrobial, excellent humectant, low toxicity, antioxidant, anti-inflammatory and anti-ulcer properties (Lan-Xiang Liu et al., 2015; Nastaran Hashemzadeh et al., 2022; Dinesh Shantilal Patel et al., 2017).

Further, the compound Butanoic acid,2-ethyl-3 hydroxy-ethyl ester shows anti-tumour activity through various mechanisms viz., promotion of TCA cycle, promotion of protein synthesis, reduction in inflammation & enhancement of antioxidant capacity, improvement of metabolic homeostasis and attenuation of proteolysis as shown in figure 10. Other compounds present in the formulation KK such as Butanoic acid,2-ethyl-3 hydroxy-ethyl ester (Ethyl-3 hydroxybutyrate), 2-propanol, and 1(1-methyl ethoxy) show antitumor activity and apoptosis action (Kurita-Ochiai et al., 2008; Siqi Feng et al., 2019). The secondary metabolites namely Tetradecane present in the formulation show antimicrobial activity (Zeinab Nasr, 2022). whereas Butanoic acid,2-ethyl-3 hydroxy-ethyl ester shows anti-cachexia activity (Zhou Y et al., 2023). Studies documented that these compounds induce programmed cell death in various cancer cells, indicating their potential as anticancer agents as shown in Figure 9. Many chemotherapeutic drugs including cisplatin, doxorubicin, fluorouracil, and vincristine exert their anticancer effects by inducing apoptosis in tumor cells, making them valuable for

oncology therapy (Gavamukulya et al., 2014; Milner et al., 2002). Further characterization and assessment are needed for the tentatively identified compounds to elucidate the structures present in formulation KK.

Similarly, the presence of a secondary metabolite, Dodecane, in the fungal extract, at a concentration of 28.49%, exhibited significant anti-tumor activity, particularly against HPV18+ human cervical cancer HeLa cells. This activity was confirmed through GC-MS analysis, highlighting its promising potential in cancer treatment. (Kumari et al., 2018; Serban Moldoveanu, 2019).

This assay shows that KK formulation is a source of anti-tumor and antioxidants that might impede the advancement of various conditions induced by free radicals, and proliferation such as cancers. However, the constituents that are accountable for the antioxidative capacity are also present in the formulation KK. The correlation between the chemical structures of the identified compounds and their known pharmacological activities indicates a prevalence of anti-inflammatory, antioxidant, and anticancer properties among the compounds.

Non-polar compounds such as Lanosterol,9,19-cyclolanostan-3-ol,24 methylene -(3β), etc. have a cytotoxic effect that is soluble in the lipid bilayer, so they can easily cross the cell membrane (Nicole Peiris, chem. libretexts). There may be certain restrictions within this study. First, no investigation was carried out for incursion, displacement and colonization of the cells when treated with KK formulation. It's crucial because the majority of cancer-related fatalities are ascribed to metastasis. The second restriction is the scarcity of toxicological investigation of KK formulation using in vivo animal studies.

The result of GC-MS verified the existence of selective compounds that were noted to stimulate programmed cell death. Therefore, it can be deduced that the anticancer potential, especially for cervical cancer, observed in the KK could be credited to the existence of these compounds.

CONCLUSION

To conclude, the data unveiled that KK formulation has secondary metabolites namely Lanosterol(63.24%), 9,19-cyclolanostan-3-ol,24 methylene -(3β)(60.97%), 3,3-Diethoxy-1-propanol, propyl ether (50.26%), Dodecane(28.49%), Tetradecane(27.72%), W-18(20.61%),methyl-3,3-dimethyl cyclopropane-1,trans-2-dicarboxylate (19.66%), 2-propanol,1 (1-methylethoxy) (12.24%) and Butanoic acid,2-ethyl-3 hydroxy-ethyl ester(6.75%). Further the top three hits namely Lanosterol, 9,19-cyclolanostan-3-ol,24

methylene -(3β) and 3,3-Diethoxy-1-propanol, propyl ether were derived from GC-MS analysis showed Antitumor activity especially cervical cancer via apoptosis and anti-angiogenesis as shown in table 2 and figure 8,9. This may be a promising formulation since the KK formulation contains natural Compounds effective even in apoptosis-resistant cells.

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CONFLICT OF INTEREST

The authors have declared that no competing interests exist.

DECLARATION OF COMPETING INTERESTS

The authors affirm that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

ABBREVIATIONS

1.	**HPV**	Human papilloma virus
2.	**KK**	*Kalingathi kadugu*
3.	**GC-MS**	Gas chromatography-mass spectrometry
4.	**WHO**	World Health Organization
5.	**IC50**	Half-maximal inhibitory concentration
6.	**CAS**	Chemical Abstracts Service
7.	**LSS**	Lanosterol Synthase
8.	**TCA**	Tricarboxylic Acid Cycle
9.	**3HB**	Ethyl-3 hydroxybutyrate

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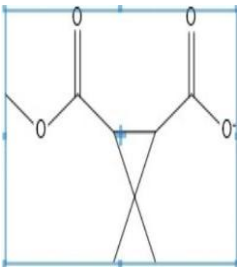
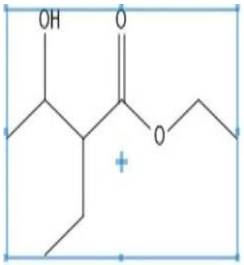
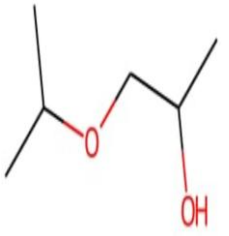
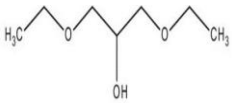
TABLES

Table 1: Phytoconstituents identified in the *Kalingathi Kadugu* extract via gas chromatography-mass spectrometry.

S. NO.	Retention time	Compound name	Molecular formula& molecular weight	Chemical structure	Score	Probability (%)	CAS#
1	3.999	(-)-methyl-3,3-dimethyl cyclopropane-1,trans-2-dicarboxylate	C8H11O4 Molecular weight: 171.17g/mol (chemdraw)		672	19.66	98628

2	4.216	Butanoic acid,2-ethyl-3 hydroxy-ethyl ester	C8H16O3 Molecular weight: 160.21g/mol(chemdraw)		625	6.75	45719
3	4.842	2-propanol,1 (1-methylethoxy)	C6H14O2 Molecular weight: 118.17g/mol (chemeo - high quality chemical properties)		648	12.24	18333
4	5.810	3,3-Diethoxy-1-propanol, propyl ether	C10H22O3 (pubchem) Molecular weight: 190.28g/mol (Atman Chemicals)		746	50.26	83574
5	7.898	Dodecane	C12H26 Molecular weight:170.34g/mol (Dodecane, 2021)		883	28.49	26119
6	12.709	Tetradecane	C14H30 Molecular weight: 198.39g/mol (Tetradecane, 2021)		857	27.72	26185

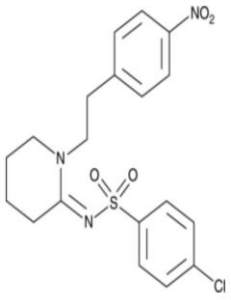
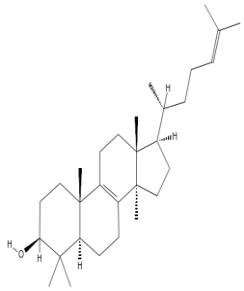
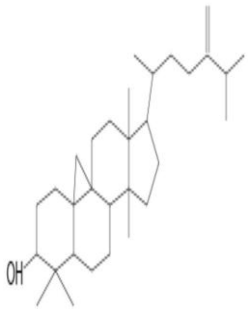
7	43.287	W-18	C19H20CIN3O4S Molecular weight: 421.90g/mol. (cayman chemical)		552	20.61	207754
8	47.533	Lanosterol	C30H50O (NIST chemistry webbook) Molecular weight: 426.71g/mol (lanosterol,2021)		743	63.24	262184
9	49.806	9,19-Cyclolanostan-3-ol, 24-methylene-3β	C31H52O (spectrabase) Molecular weight: 440.74g/mol		711	60.97	22724

Table 2: Significance of compounds present in Kalingathi kadugu

S.NO.	Compound name	Significance
1	Lanosterol	Anti-angiogenesis, Anti-tumor and Antiviral activity
2	9,19-cyclolanostan-3-ol,24 methylene -(3β)	Anti-tumor and Anti-inflammatory activity
3	3,3-Diethoxy-1-propanol, propyl ether	Anti-tumor activity

FIGURES

Figure.1: Ingredients of *Kalingathi kadugu* namely *Piper longum*. Sodium chloridum impura, magnetite, *Croton tiglium*, cinnabar (mercuric sulphide), *Euphorbia nivulia*, asafoetida, *Citrullus colocynthis* and *dry* ginger *(Zingiber officinale)*

Tune File:	atune	**Ion Source:**	EI	**Source Temperature:**	230 °C
Quad Temperature:	150 °C	**Fixed Electron Energy:**	70 eV	**Acquisition Type:**	Scan
Stop Time:	53.5 min	**Solvent Delay:**	3 min	**Trace Ion Detection:**	Off
Gain Factor:	1	**EM Saver:**	Off	**EM Saver Limit:**	N/A

Figure 2: Acquisition method of GC-MS analysis

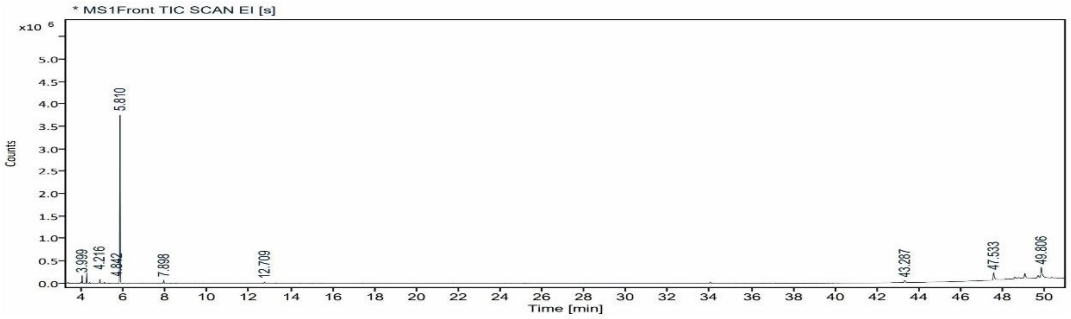


Figure 3: Chromatogram of KK extract using Gas Chromatography-Mass Spectrometry

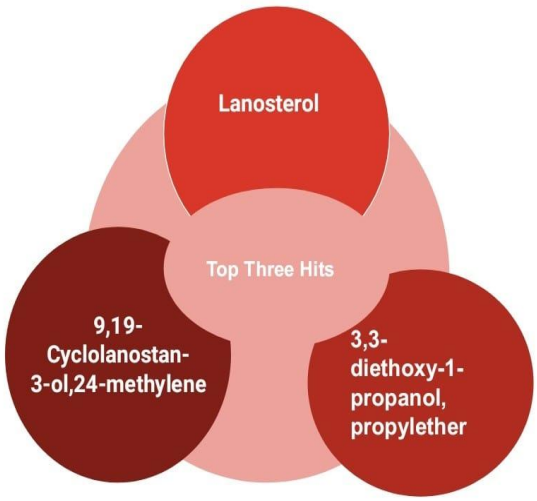


Figure 4: Represents the top three compounds with Antitumor activity

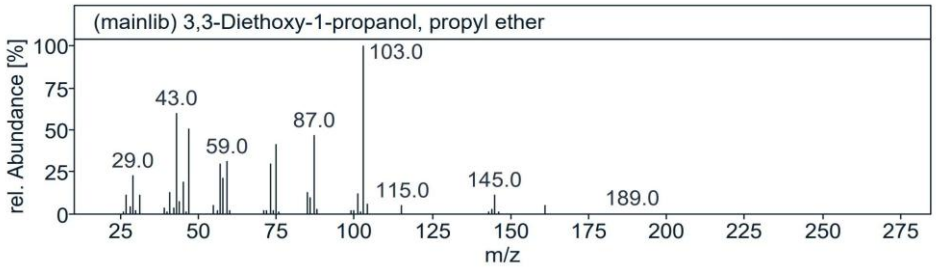


Figure 5: Graph representing retention time of 3,3-Diethoxy-1-propanol, propyl ether

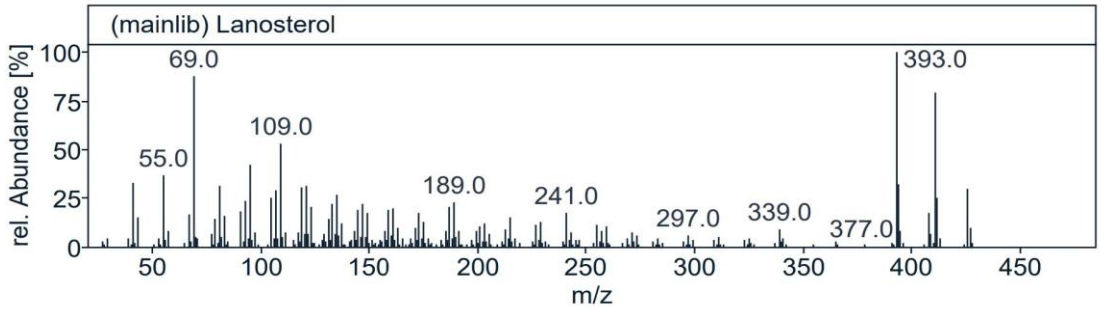


Figure 6: Graph representing retention time of Lanosterol

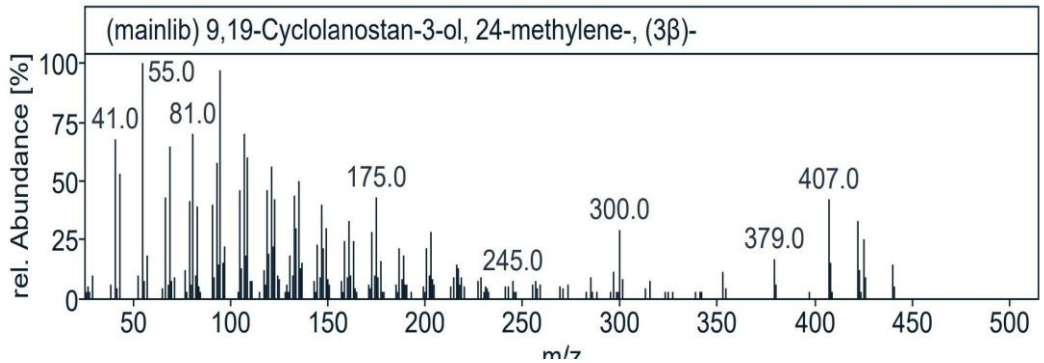


Figure 7: Graph representing retention time of 9,19-Cyclolanostan-3-ol,24-methylene-(3β

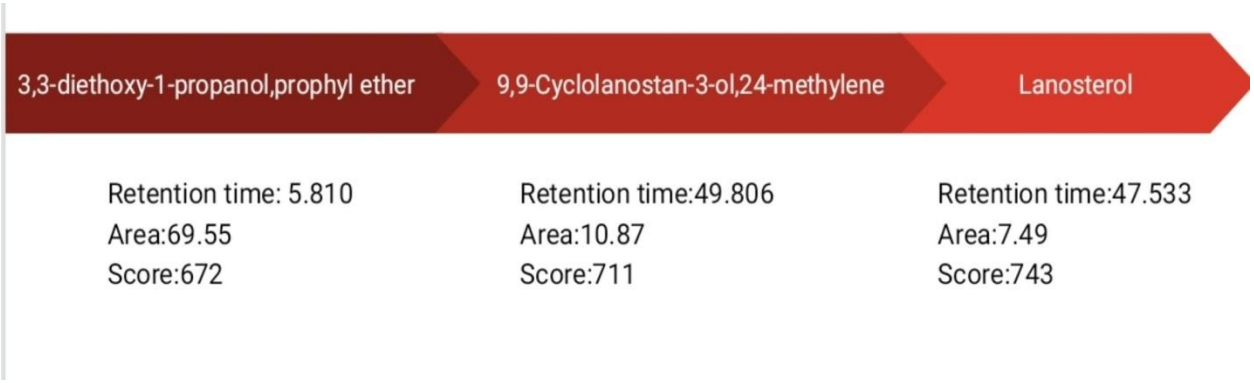


Figure 8: Top three Hits obtained from GCMS analysis

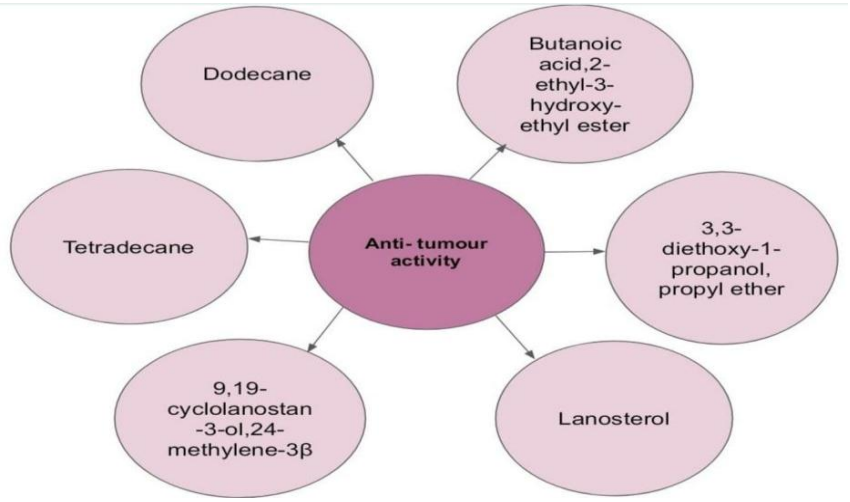


Figure 9: Compounds having anti-tumour activity obtained from GC-MS analysis

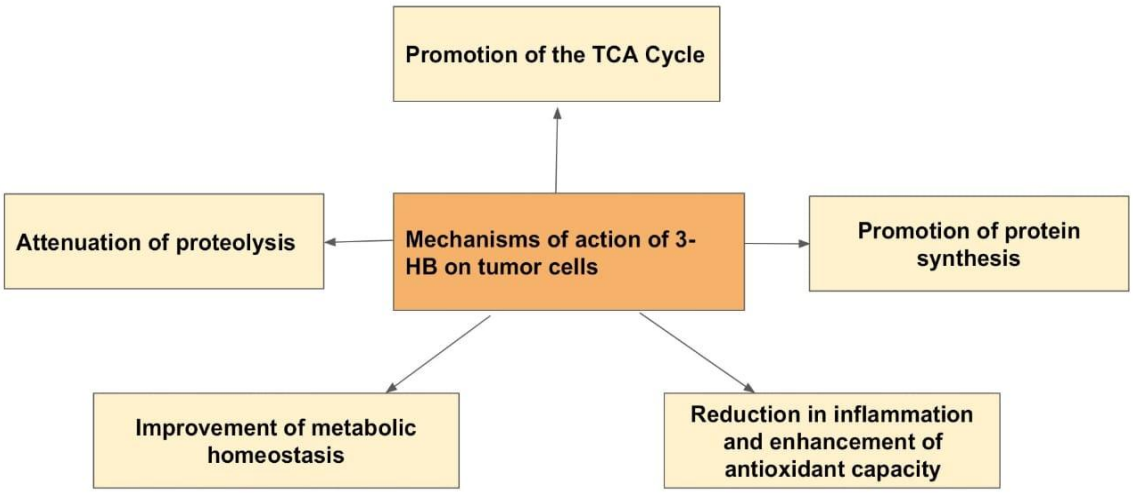


Figure 10: The mechanism of action of 3-HB (Butanoic acid,2-ethyl-3 hydroxy-ethyl ester) on tumour cells.