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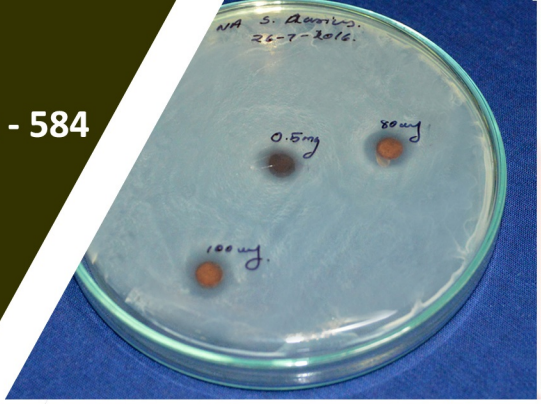

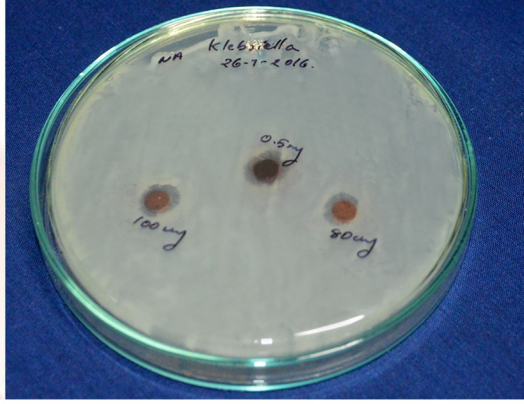

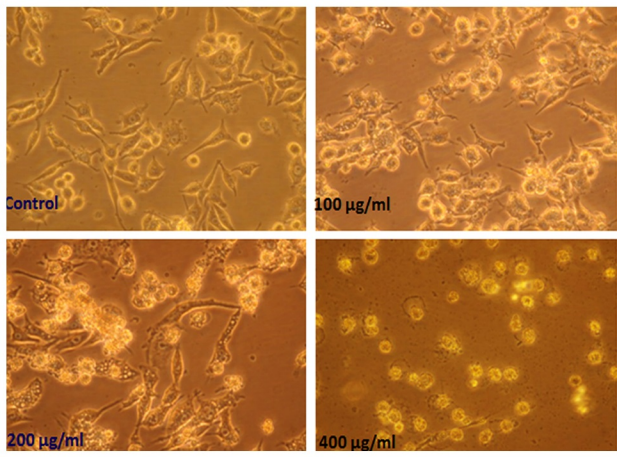

Bioactivity guided fractionation and mechanistic elucidation of biomolecules from Cocculus laurifolius DC. of Southern Western Ghats

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Bioactivity guided fractionation and mechanistic elucidation of biomolecules from *Cocculus laurifolius* DC. of Southern Western Ghats

Project Technical Report

[Project # KFRI RP 710/2015]

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December 2021

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An Institution of Kerala State Council for Science, Technology and Environment (KSCSTE)
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PROJECT DETAILS

Title of the Project	Bioactivity guided fractionation and mechanistic elucidation of biomolecules from *Cocculus laurifolius* DC. of Southern Western Ghats
Objectives	To identify the biologically active molecules from *C. laurifolius* and understand the mechanistic aspects of their bioactivity **A**. Analysis of pharmacological and biological activities of *C. laurifolius* extracts and bioactivity guided fractionation. **B**. Isolation and characterization of phytochemical constituents of bark and leaves of *C. laurifolius.* **C**. Mechanistic studies on biological properties of the isolated molecules.
Funding agency	Kerala Biotechnology Commission, Government of Kerala
Investigators	Dr. R. Jayaraj Principal Investigator Senior Scientist Forest Ecology & Biodiversity Conservation Division KSCSTE - KFRI Dr. P. Sujanapal Co-Investigator Senior Scientist Sustainable Forest Management Division KSCSTE - KFRI
Project Staff	Mr. Roshan Lal
Duration	04 Years 09 months

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INVESTIGATORS

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ABSTRACT

Exploration, extraction and screening biological diversity and indigenous knowledge for commercially valuable genetic and biochemical resources is a continuous ongoing process. One such major activity is the identification of novel bioactive molecules for drug development research. In addition to the drug development, these plant products have diverse applications including fragrance extraction, pest control, extraction of essential oils, cosmetic applications and in food industry. The current project was formulated with the objectives to identify the biologically active molecules from *Cocculus laurifolius,* study the bioactivities and understand the mechanistic aspects of their bioactivity. The bark and leaves of *C. laurifolius* were extracted using different solvents and chloroform extract is found to contain maximum phytochemicals including alkaloids, anthocyanin, isoflavones, flavonoids, tannins, saponins, lignins, phenols and coumarins. The extracts were fractioned based on bioassays and the most active fraction is found to be cytotoxic to human cervical cancer cells and murine macrophage cells with an IC50 of 35 µg/ml and 200 µg/ml respectively. The cells showed typical apoptotic morphology including cell shrinkage, blebbing and destruction of actin filaments and cellular structure. The bark chloroform extract had shown potential antimicrobial activity against *Staphylococcus aureus* and *Klebsiella pneumoniae*. The methanolic extract of both leaf and bark of *C. laurifolius* showed antifeedant, repellent activities and contact toxicity at varying degrees. The present study had brought out the cytotoxic, anti-microbial and pesticidal properties of *C. laurifolius*. Among this, the cytotoxic and pesticidal properties were reported for the first time. Exploring the pesticidal properties of crude extract and developing appropriate formulations may help in developing cost-effective and eco-friendly technologies for pest control in agriculture and related fields.

INTRODUCTION AND OBJECTIVES

1. INTRODUCTION AND OBJECTIVES

Plants and their bioactive properties attain importance because of their involvement in indigenous knowledge on health support systems, contribution to biodiversity and generation of income through local trading as well as global business. Indigenous knowledge plays a crucial role in exploring the possibilities of utilizing the medicinal plants for diverse applications. The indigenous knowledge is defined as "the totality of all knowledge and practices established on past experiences and observations that is held and used by people" (Masango, 2010). During the past few decades, the interest to study the medicinal plants and their traditional uses has been increasing steadily. Use of traditional knowledge for medicinal and other uses either by tribal people or by other indigenous communities in India has increased over recent years (Savithramma et al., 2007; Pattanaik et al., 2008; Namsa et al., 2009; Upadhyay et al., 2010). Investigation of medicinal plants for identification of novel bioactive molecules either for therapeutic applications or for other commercial applications is a continuous ongoing process. The bioassay and toxicity studies for ensuring the efficacy and safety of ethnomedicinal plants are essential in ensuring utility and safe use of biomolecules.

The worldwide market value of traditional medicinal products is estimated to be around US $ 80-90 billions, which is at par with modern medicine industry (Karki, 2000). World Health Organization (WHO) emphasizes that medicinal plants have crucial role in the health care of 80 % population in developing countries, among that traditional medicines play major role. The herbal medicines constitute the most prominent part in the traditional medicinal systems (Farnsworth et al., 1985). Medicinal plants are defined as "*Plants that are recognized by people to have reliable and effective medicinal values, are commonly used in treating and preventing specific ailments and diseases, and play an essential role in health care*".

India is one of the leading countries in exploring and utilizing its traditional knowledge system and practice. Along with conventional practice, the folklore medicines have been an inspiring basis for modern medicines too. The rich plant resources from nature are always the back bone of Indian systems of medicines -

Ayurveda, Unani and Siddha. In addition to the systemic use, these plant resources have a rich heritage of ethno-botanical usage by tribal communities. Active ingredient separation and characterization for therapeutic use based on traditional knowledge system has been in practice for many decades. Isolation and identification of biologically active molecules and modified derivatives with enhanced activity and or reduced toxicity is one of the success paths in utilization of plants as resource material.

The number of medicinal plants in India, both indigenous and introduced has been estimated to be between 8,000 to 8,500 species of higher plants. The number of plants having clinically useful chemical compounds or confirmed therapeutic properties are around 700 species. Only a small portion of the plants were successfully analysed and so far, yielding about 120 therapeutic agents of identified structure from about 90 species of plants. The Western Ghats is considered as one of the biodiversity hotspots of the world (Myers et al., 2000) and harbours about 5000 plant species (Nair and Daniel, 1996). The Western Ghats is very rich in its medicinal plant wealth. The forests and hills of this region is a treasure house of medicinal plants. The medicinal plant species of the region represent a variety of life forms ranging from lichen, algae, herbs, shrubs, climber and trees, which are annuals to perennials. Out of which some are used for traditional and folk medicinal practices. Many are exploited commercially for their active enzymes and their commercial value.

Some of the plant derived drugs includes vinblastine, taxol, podophyllotoxin, camptothecin, digitoxigenin, gitoxigenin, digoxigenin, tubocurarine, morphine, codeine, atropine, aspirin, pilocarpine, capsaicin, allicin and curcumin among others. Identification of active principles and elucidation of their mechanism were successful in all these cases and led to the introduction of either active principle or its derivatives to the modern system of medicine. Eventhough, the plant extract or crude materials show activity in most cases, the major obstacles in introducing and establishing the use of herbal medicines to the modern system is the inadequate scientific validation and documentation. Attempts are made by researchers across

the world to scientifically establish the structure and mechanism of active principles in herbal extracts and preparations so that their use can be augmented and time frames of drug discovery can be redefined.

Though the plants are mainly explored for their medicinal properties, the other uses include fragrance extraction, pest control, extraction of essential oils, cosmetic applications and food industry. These plant products are considered to be safe in comparison with synthetic drugs, that are regarded as unsafe to environment and human beings. One of the major applications of plant products in addition to medicinal properties, is in the pest management, both for domestic and farming applications. Plant extracts and essential oils are safe, eco-friendly and more compatible with environmental components compared to synthetic pesticides. These botanical pesticides have better options for pest control, if they can be categorized based on their differential efficacy against different pests (Emden, 1989). The advantages of botanical pesticides are their rapid degradation, lack of persistence and less or nil bioaccumulation in the environment, which are the major problems in synthetic pesticide use.

Through the present work, the investigators explored the biologically active molecules from *Cocculus laurifolius* DC. of Menispermaceae, which is commonly known as "Aadukolli". The name "Aadukolli" emerged from the local knowledge that probably the leaves of this plant are fatal to goat. This observation essentially indicates the presence of some highly biologically active molecules in the plant. The first isolated alkaloid "morphine" had references back to Greek literature, where leaves from the poppy plants were used as pain reducers. Based on these previous success stories, the present study covered a complete bio-spectrum analysis including isolation, characterization and bioactivity studies of *C. laurifolius*.

The following objectives were covered under the study;

A. Analysis of pharmacological and biological activities of C. *laurifolius* extracts and bioactivity guided fractionation.

B. Isolation and characterization of phytochemical constituents of bark, and leaves of *C. laurifolius*.

C. Mechanistic studies on biological properties of the isolated molecules.

REVIEW OF LITERATURE

2. REVIEW OF LITERATURE

2.1. Importance of medicinal plants and its bioprospecting

Plants play crucial role in existence of human life; as food, medicines, support systems, providing essential nutrients among others. Plants have been used for medicinal purposes long before prehistoric period. The plants and its products are the backbone of many traditional medicinal systems including, Greek, Chinese, Egyptian, Unani and Ayurveda. The use of herbs for over 4000 years as medicine by Unani Hakims, Indian Vaidyas and European and Mediterranean cultures was well documented. Well-developed traditional medical systems such as Unani, Ayurveda and Chinese Medicines uses herbal therapies systematically, while indigenous cultures such as Rome, Egypt, Iran, Africa and America used herbs in their healing rituals. Medicinal plants are considered as a rich resource of ingredients which can be used in drug development either pharmacopeial, non-pharmacopeial or synthetic drugs. In addition, various other uses of the plants and its products have been identified. Apart from the medicinal uses, herbs are also used in natural dye, pest control, food, perfumes, cosmetics are some among others.

Majority of the world population depends mainly on plants and plant extracts for health care and maintenance. Among the entire plant species, more than 30 %, were in use for medicinal purposes at one time or other. The plant derived drugs in the world market accounts for about Rs. 2 lakh crores and Indian contribution is around Rs.2000 crores. The annual production of medicinal plant raw material costs approximately Rs. 200 crores. Among the 2,50,000 higher plant species on earth, more than 80,000 are considered to have medicinal properties. India is one of the world's 12 biodiversity hotspots with the presence of over 45000 different plant species across the region. Among India's biodiversity centres, the Western Ghats occupies the primary position.

In India, herbs and herbal preparations have been used in traditional medicinal systems such as Siddha, Unani and Ayurveda since ancient times. The Unani system uses about 700 species, Ayurveda about 700 species, Siddha about 600 and modern medicine around 30 species. The drugs are prepared either from the whole

plant or from different plant parts like stem, leaves, root, bark, flower, seed, etc. Some medicines are prepared from excretory plant product such as gum, resins and latex. Even the allopathic system of medicine has adopted a number of plant-derived drugs. There are several reasons for the wide use of traditional medicinal systems, which include inadequate supply of drugs, demand from population, prohibitive cost of treatments, side effects of several allopathic drugs, allopathic drug resistance are some among them**.** The scientific evaluation of herbs used in traditional medicines and derivation of drugs through bioprospecting are some of the widely adopted strategies in drug development in the modern era. In this scenario, based on the scientific evaluation and requirement, systematic conservation of the concerned medicinal plants is of great importance.

A major problem in traditional medicinal systems is the absence of drug standardisation, information and quality control. Most of the traditional medicines are in the form of crude extract of a single plant or a mixture of several plant ingredients and a synergistic effect is expected. When the individual active principles are isolated, it fails to give anticipated activity. The active principles are present in different parts of the plant like stem, root, heartwood, bark, flower, fruit, leaf, or plant exudates. The separation of these active principles can be achieved by different processes, mainly solvent extraction. Solvent extraction is the separation of the required constituents from plant materials using a solvent based on the polarity. The active principles are extracted from raw materials either as pure form or as precursors for new chemical entities.

As of today, in the modern drug industry, there are more than 120 distinct chemical entities extracted from plants that are considered to be drugs. Many other drugs presently available in market are synthetic alterations of naturally occurring chemical substances. Around 25% of the world's pharmaceutical products has its origin from the indigenous communities/knowledge, which occupies more than 2000 billion dollars share in global market (Jones and Jones, 2002).

Bioprospecting has played crucial role in the discovery of new drugs since the starting of modern drug development. More than 8000 species of medicinal plants are in use across the world for health care requirements. The pharmaceutical industry has many useful drugs (Table -2.1) which were derived from medicinal plants (Taylor, 2000).

Table – 2.1: List of some common drugs derived from plants

S. No	**Drug**	**Action/ Clinical use**	**Plant Source**
1.	Aspirin	Analgesic, anti-inflammatory and antipyretic	*Salix alba*
2.	Atropine	Anticholinergic	*Atropa belladonna*
3.	Artemisinin	Anti-malaria	*Artemisia annua*
4.	Codeine	Analgesic, anti-tussive	*Papaver somniferum*
5.	Colchicine	Anti-tumour	*Colchium autumnale*
6.	Cynarin	Cholerectic	*Cynara scolymus*
7.	Digitoxin/Digoxin	Cardiotonic	*Digitalis purpurea*
8.	Epihedrine	Sympathomimetic	*Ephedra vulgaris*
9.	Hyoscyamine	Anticholinergic	*Hyoscyamus niger*
10.	Methyl salicylate	Rubefacient	*Gaultheria procumbens*
11.	Morphine	Analgesic	*Papaver somniferum*
12.	Nicotine	Insecticide	*Nicotiana tabacum*
13.	Physostigmine	Cholinestrase inhibitor	*Physostigma venenosum*
14.	Paclitaxel	Anti-cancer	*Taxus brevifolia*
15.	Podophyllotoxin	Anti-neoplastic	*Podophyllum peltatum*
16.	Quinidine/ Quinine	Anti-malarial	*Cinchona ledgeriana*
17.	Silymarin	For hepatic disorders	*Silybum marianum*
18.	Theophylline	Diuretic, vasodilator	*Theobroma cacao*
19.	Tubocurarine	Skeletal muscle relaxant	*Chondodendron tomentosum*
20.	Vincristine, vinblastine	Anti-neoplastic	*Catharanthus roseus*
21.	Yohimbine	Alpha-2 adenoreceptor blocker	*Pausinystalia yohimbe*

The economic value of plants or living organisms for pharmaceutical purposes is enormous and it benefits all sectors, including pharmaceutical companies, host country and indigenous community. This was ensured by United Nations Convention on Biological Diversity (CBD) in 1992. The Convention establishes the control and sovereignty of local agency over the biological resources and its diversity (Kumar and Tarui, 2004). Bioprospecting from the local medicinal plants provides an opportunity for preservation of the traditional knowledge, revenue

generation for indigenous communities through raw material collection, employment avenues etc (Martin, 2001).

There are certain limitations associated with the bioprospecting, such as over exploitation of natural resources such as forest, fields and waters, destruction of biological diversity, patenting of local information leading to restricted use, resistance in sharing of monetary benefits and imbalance in ecosystem (Moran, 1992; RAFI, 1994; Zakrzewski, 2002)

2.2. Western Ghats and its medicinal plant resources

The Western Ghats mountains is extending from Kanyakumari to Maharashtra and runs approximately 1,600 km through the State's Tamil Nadu, Kerala, Karnataka, Goa, Maharashtra and Gujarat. Western Ghats is one of the thirty-three recognized ecological sensitive zones in the world and one of the four such sensitive zones in India. There are about 5800 plant species were identified from Western Ghats and 35% of them are endemic to the region. The levels of endemism are very high in this area with 2000 species of higher plants, 87 species of amphibians, 84 species of fishes, 15 species of birds, 89 species of reptiles and 12 species of mammals (Daniel, 1997).

More than 900 species from the Western Ghats are used in various systems of medicine (KFRI, 2012; SMPB, 2012). Among them, 140 are exploited on a commercial scale. An analysis showed that roots and tubers of about 50 species are used in large scale for commercial purpose. The other major useful parts of medicinal plants are fruits and seeds (35 species), bark (20 species), wood (5 species), leaves (15 species) and exudations (5 species). About 28 species are collected as such and whole plant is used for medicinal preparations. The Ayurvedic medicine industry is still meeting majority of their raw drugs requirements from the forests. Among the forest types of Kerala, more than 40 per cent of raw drugs are coming from moist deciduous forest (Basha, 1990).

Some of the major medicinal plants of the Western Ghats are *Aegle marmelos, Asparagus racemosus, Bacopa monnieri, Baliospermum montanum, Celastrus paniculatus, Coscinium fenestratum, Crateva magna, Embelia ribes, Hemidesmus indicus, Holarrhena*

pubescens, Holostemm ada-kodien, Kaempferia rotunda, Limonia acidissima, Nervilia aragoana, Oroxylum indicum, Plumbago indica, Rotula aquatica, Rubia cordifolia, Saraca asoca and *Trichosanthes lobate* (Warrier et al., 2001). Many among this as well as some other plants are reported to be threatened and listed in IUCN Red List. The medicinal plants of the Western Ghats have played crucial roles in the traditional knowledge and indigenous medical systems in the region. This diversity and availability of medicinal plants has contributed well to the growth of Ayurveda in Kerala.

2.3. Bioactive molecules from plants

Since the development of medical systems, molecules from natural origin have played crucial roles in traditional medicine in different cultures and in present times, medicinal plants occupy the most vital position as the source of new chemical molecules as drugs (Zyad et al., 2018). Plants and their chemical constituents have been essential in curing diverse forms of diseases, including cancer, bacterial infection and inflammation (Merina et al., 2012; Tilaoui., 2015). Among the treatments available for many of these diseases, many are associated with severe side effects, disturbance of natural metabolic functions, change of immune system or hormonal balance, etc (Latosinska and Latosinska, 2013). Hence, there is a need to search from nature for alternative and safe drugs and/or methods for the treatment of many of the ailments. The plants can provide variety of chemical entities towards this. The secondary metabolism in plants naturally evolves in response to the varied environmental conditions, leading to production of complex functional molecules with diverse chemical and biological properties (Verdine, 1996). The Indian subcontinent is known as the region with high diversity of plants with novel biomolecules and also called as "the botanical garden of the world" (Garg et al., 2007; Umadevi et al., 2013).

One of the major activities well observed or evaluated in phytochemicals is cytotoxicity. Many plants extracts or isolated biomolecules were evaluated for their toxicity to cells and mechanism also has been studied. Many studies have

established that cytotoxic phytochemicals either induce apoptosis and necrosis or obstruct variety of cell-signaling pathways, thereby, leading to cell death or cell cycle arrest. A detailed list of such studies is given below (Table – 2.2).

Table – 2.2 : Cytotoxic properties of bioactive compounds

No	Plant species	Bioactive Compounds	Mechanism of Cytotoxicity	References
1.	*Adenanthera pavonina*	-	-	Kumar et al., 2013
2.	*Aegiceras corniculatum*	Aegicoroside A, sakurasosaponin, fusarine, fusamine etc.	-	Akter et al., 2013; Vinh et al., 2017; Ding et al., 2012
3.	*Aloe vera*	-	Increased *p53* and reduced *Bcl-2* gene expression	Shalabi et al., 2015
4.	*Annona muricata*	-	-	Gavamukulya et al., 2014
5.	*Annona squamosa*	Eupafolin, apigenin, rhamnetin etc.	-	Sumithra et al., 2014
6.	*Aquilaria malaccensis*	Benzaldehyde, pinene, octanol, germacrene, hexadecanal, etc.	-	Hegde et al., 2018; Ibrahim et al., 2011; Adam et al., 2018.
7.	*Aristolochia ringens*	-	-	Akindele et al., 2014
8.	*Aristolochia longa*	-	-	Daoudi et al., 2013
9.	*Artemisia annua*	Dihydro-artemisinin	-	Jirangkul et al., 2014; Hosoya et al., 2008; Efferth et al., 2011; Isani et al., 2019
10.	*Arbutus andrachne*	-	-	Abu-Rish; 2016
11.	*Artocarpus heterophyllus*	Noratocarpin, cudraflavone, artocarpin, brosimone, kuwanon, albanin, etc.	Inhibition of melanin biosynthesis	Patel and Patel, 2011; Arung et al., 2010; Arung et al., 2010; Arung et al., 2007
12.	*Asplenium nidus*	Gliricidin-7-*O*-hexoside	-	Jarial et al., 2018
13.	*Averrhoa bilimbi*	Nonanal, tricosane, squalene, malonic acid, etc.	-	Nair et al., 2016; Ahmed et al., 2016
14.	*Azadirachta indica*	Nimonol, 3′-(3-hydroxy-3-methyl-butyl)naringenin,	-	Amer et al., 2010; Jafari et al., 2013; Takagi et al., 2014; Akihisa, 2014;

No	Plant species	Bioactive Compounds	Mechanism of Cytotoxicity	References
		4′-*O*-methyl-lespedezaflavanone C, triterpenoids, limonoids etc.		Kikuchi et al., 2011; Kitdamrongtham et al., 2014; Chen et al., 2011
15.	*Barleria grandiflora*	-	Upsurge in ascitic fluid	Manglani et al., 2014; Kumar et al., 2013
16.	*Berberis aristata*	-	-	Serasanambati et al., 2015 Gaidhani et al., 2013
17.	*Bidens pilosa*	-	-	Sundararajan et al., 2006
18.	*Caesalpinia sappan*	Brazilin A	Reduction of *Bcl-2* apoptotic inhibitor	Bukke et al., 2018
19.	*Calligonum comosum*	Catechin, kaempferol, mequilianin, etc.	Increased *p53* and reduced *Bcl-2* gene expression	Shalabi et al., 2015; Badria et al., 2007
20.	*Cedrus deodara*	-	Interaction with caspase 3,8 and 9 proteins	Gaidhani et al., 2013; Shashi et al., 2006
21.	*Cenchrus ciliaris*	-	-	Alothman et al., 2018; Awaad et al., 2019
22.	*Centaurea antiochia*	-	-	Artun et al., 2016
23.	*Centaurea nerimaniae*	-	-	Artun et al., 2016; Saranya et al., 2019
24.	*Chrysanthemum coronarium*	Campesterol	Inhibition of fibroblast growth factor-mediated cell proliferation	Abu-Rish et al., 2016 Choi et al., 2007
25.	*Clerodendrum viscosum*	-	Apoptosis	Akter et al., 2013
26.	*Clerodendron infortunatum*	-	Apoptosis through interaction with *Bax*, *Bcl-2*, caspases 8 and 10 proteins	Chacko et al., 2015
27.	*Cotinus coggygria*	-	-	Artun et al., 2016
28.	*Cordia dichotoma*	-	apoptosis, nuclear condensation and ROS production	Rahman et al., 2016
29.	*Croton caudatus*	-	-	Rosangkima and Jagetia, 2015
30.	*Cocculus hirsutus*	Coclaurine, haiderine, lirioresinol etc.	Interactions with Aurora kinase, c-Kit, FGF, *NF-kB*, *Bcl-xL* and VEGF	Thakkar et al., 2014; Thavamani et al., 2016
31.	*Crataegus microphylla*	-	-	Artun et al., 2016 Bura et al., 2016

No	Plant species	Bioactive Compounds	Mechanism of Cytotoxicity	References
32.	*Curcuma longa*	Curcumin, β-sesquiphellandrene	Apoptosis	Srivastava and Srivastava, 2015; Ramsewak et al., 2000; Kuttan et al., 1985; Tyagi et al., 2015; Atsumi et al., 2005
33.	*Delphinium staphisagria*	Astragalin, paeonoside, petiolaroside, etc.	-	Daoudi et al., 2013; Ramírez-Macías et al., 2012
34.	*Dillenia pentagyna*	-	Reduction in glutathione level	Rosangkima et al., 2015; Prasad et al., 2008
35.	*Dillenia indica*	-	-	Akter et al., 2013
36.	*Diospyros peregrina*	-	-	Akter et al., 2013; Alex et al., 2012
37.	*Euphorbia tirucalli*	-	-	Munro., 2015
38.	*Ficus beecheyana*	*p*-Coumaric acid, chlorogenic acid, caffeic acid, gallic acid, rutin etc.	Interaction with Fas, Fas-L, *p53*, *Bcl-2* and caspases (3,8,9) proteins	Yen et al., 2018
39.	*Ficus carica*	-	-	Jawad etal., 2014
40.	*Ficus racemosa*	-	-	Khan et al., 2016 Sukhramani et al., 2013; Gavhane et al., 2016
41.	*Gnaphalium luteoalbum*	Apigenin, luteolin, jaceosidin, gnaphalin, etc.	-	Akter et al., 2013; Al-Snafi et al., 2019
42.	*Helicteres isora*	Cucurbitacin B and isocucurbitacin B	-	Shaikh et al., 2014; Bean et al., 1985
43.	*Hibiscus calyphyllus*	Ursolic acid, β-sitosterol and lupeol	Interaction with caspases 3 and 9, topoisomerase I, *Bcl-2*, *Bax*, DNA polymerase and poly (ADP-ribose)-polymerase	Alam et al., 2018; Kim et al., 2000; Choi et al., 2003; Gallo and Sarachine, 2009
44.	*Hibiscus deflersii*	Ursolic acid, β-sitosterol and lupeol	Interaction with caspases 3 and 9, topoisomerase I, *Bcl-2*, *Bax*, DNA polymerase and poly (ADP-ribose)-polymerase	Alam et al., 2018; Kim et al., 2000; Choi et al., 2003; Gallo and Sarachine, 2009
45.	*Hibiscus micranthus*	Ursolic acid, β-sitosterol and lupeol	Interaction with caspases 3 and 9, topoisomerase I, *Bcl-2*, *Bax*, DNA polymerase	Alam et al., 2018; Kim et al., 2000; Choi et al., 2003;

No	Plant species	Bioactive Compounds	Mechanism of Cytotoxicity	References
			and poly (ADP-ribose)-polymerase	Gallo and Sarachine, 2009
46.	*Hypericum kotschyanum*	-	-	Talib and Mahasneh, 2010
47.	*Hymenodictyon excelsum*	-	DNA fragmentation and apoptosis	Akter et al., 2013; Khairunnisa and Karthik, 2014
48.	*Inula viscosa*	-	-	Talib et al., 2010
49.	*Jasminum sambac*	-	-	Talib and Mahasneh, 2010; Akter et al., 2013
50.	*Lavandula angustifolia*	-	-	Talib and Mahasneh, 2010
51.	*Lannea coromandelica*	2-Palmitoylglycerol, myricadiol, pyrogallol, isovanillin	Apoptosis and DNA fragmentation	Akter et al., 2013; Weerapreeyakul et al., 2016, Yun et al., 2014
52.	*Leea indica*	-	-	Ghagane et al., 2017
53.	*Limonium densiflorum*	Myricetin, isorhamnetin and *trans*- 3-hydroxy-cinnamic acid	-	Medini et al., 2015
54.	*Luffa cylindrica*	Kaempferide, eriodictyol 7-*O*-glucoside and apigenin 7-*O*-glucouronide	Interaction with caspases 3 and 8	Talib et al., 2010; Abdel-Salam et al., 2019; Abdel-Salam et al., 2016
55.	*Manilkara zapota*	Lupanes, oleananes, ursanes, manilkoraside, etc.	DNA fragmentation	Sumithra et al., 2014; Awasare et al., 2012
56.	*Mirabilis jalapa*	Rotenoids and mirabilis antiviral protein (MAP)	-	Talib and Mahasneh, 2010; Kale and Mukundan, 2015; Xu et al., 2010
57.	*Morus nigra*	-	-	Qadir et al., 2014; Souza et al., 2017
58.	*Myristica fragrans*	38 Essential oils	Apoptosis through *Bcl-2* inhibition	Piaru et al., 2012; Duan., 2009
59.	*Narcissus tazetta*	-	-	Talib and Mahasneh, 2010
60.	*Nepeta italica*	-	-	Artun et al., 2016
61.	*Ocimum sanctum*	-	Regulation of humoral immunity and	Karthikeyan et al., 1999; Godhwani et al.,

No	Plant species	Bioactive Compounds	Mechanism of Cytotoxicity	References
			stimulation of cell mediated immunity	1988; Mediratta et al., 1988; Mandal and Chatterjee, 1994
62.	*Olea europaea*		Interaction with *Bcl-2*, *Bax*, and *p53* proteins	Artun et al., 2016; Jawad et al., 2014; Fares et al., 2011
63.	*Oldenlandia corymbosa*	-	Apoptosis	Pandey et al., 2012
64.	*Ononis hirta*	-	-	Talib and Mahasneh, 2010
65.	*Ononis sicula*	-	-	Talib and Mahasneh, 2010
66.	*Origanum sipyleum*	-	-	Artun et al., 2016
67.	*Parthenium hysterophorus*	-	-	Saranya et al., 2019; Kumar et al., 2013
68.	*Phagnalon rupestre*	-	-	Talib et al., 2010
69.	*Phyllanthus emblica*	Trihydroxy-sitosterol	-	Sumalatha, 2013; Qi., 2013; Desouky et al., 2008
70.	*Picrorhiza kurroa*	Curcubitacin	Apoptosis	Rajkumar., 2011; Masood, 2015
71.	*Piper longum*	Piperine	Apoptosis	Gaidhani et al., 2013; Sunila E., Kuttan , 2004
72.	*Piper regnellii*	Eupomatenoid-5	-	Longato, 2011
73.	*Plectranthus stocksii*	-	-	Muniyandi et al., 2017
74.	*Populus alba*	-	-	Talib and Mahasneh, 2010; Abdel-Hameed et al., 2016
75.	*Pterocephalus pulverulentus*	-	-	Artun et al., 2016
76.	*Rosa damascena*	Nerol, geraniol, β-citronellol, linalool, nonadecane and phenylethyl alcohol	-	Talib and Mahasneh, 2010; Artun et al., 2016 Abdel-Hameed et al., 2016
77.	*Salvia pinardi*	-	-	Talib and Mahasneh, 2010
78.	*Salvia officinalis*	α-Humulene and *trans*-caryophyllene	-	Jawad et al., 2014

No	Plant species	Bioactive Compounds	Mechanism of Cytotoxicity	References
79.	*Salvia hypargeia*	-	-	Artun et al., 2016
80.	*Saraca asoca*	-	-	Akter et al., 2016
81.	*Saururus chinensis*	Aristolactram, dihydroguaiatric acid, sauchinone, saucerneol D, manassantin A and B, saucerneol F etc.	Inhibition of DNA topoisomerase I and II	Alaklabi et al., 2017 Lee., 2009
82.	*Saurauja roxburghii*	-	Interaction with caspases 3 and 9 topoisomerase I and $p21^{WAF1}$ cell-cycle regulator	Mazumder et al., 2011; Mazumder et al., 2011
83.	*Scorzonera tomentosa*	-	-	Artun et al., 2016
84.	*Senecio scandens*	-	-	Rosangkima et al., 2015
85.	*Solanum khasianum*	-	-	Rosangkima et al., 2015
86.	*Solanum nigrum*	-	-	Shokrzadeh et al., 2010; Patel., 2009
87.	*Syzygium cumini*	Pelargonidin-3-*O*-glucoside, cyanidin-3-*O*-malonyl glucoside, delphenidin-3-*O*-glucoside, ellagitannins etc.	-	Yadav et al., 2011; Nazif, 2007 Banerjee and Narendhirakannan, 2011
88.	*Syringa vulgaris*	-	-	Talib and Mahasneh, 2010
89.	*Tabernaemontana divaricata*	Ervachinine, cononitarine, conofoline, conophylline and cisplatin	-	Dantu et al., 2012; Bao et al., 2013
90.	*Tecoma stans*	Rutin, luteolin, diosmetin, skytanthine, etc.	-	Jayachandran et al., 2017; Zhu et al., 2008; Marzouk et al., 2007
91.	*Teucrium polium*	-	-	Talib and Mahasneh, 2010; Abu-Rish et al., 2016
92.	*Teucrium sandrasicum*	-	-	Artun et al., 2016
93.	*Tillandsia recurvata*	1,3-di-*O*-Cinnamoyl-	-	Lowe et al., 2013

No	Plant species	Bioactive Compounds	Mechanism of Cytotoxicity	References
		glycerol and (*E*)-3-(cinnamoyloxy)-2-hydroxypropyl 3-(3,4-dimethoxyphenyl) acrylate		
94.	*Verbascum sinaiticum*	Sinaiticin, hydrocarpin and flavonolignans	-	Talib and Mahasneh, 2010; Afifi et al., 1993
95.	*Vitis vinifera*	Quercetin 3-*O*-β-D-4C_1 galactoside and quercetin 3-*O*-β-D-4C_1 glucuronide	Apoptosis	Handoussa et al., 2013; Nirmala et al., 2017; Jawad et al., 2014
96.	*Withania coagulans*	Myricetin, quercetin, gallic acid, etc.	-	Maqsood et al., 2018
97.	*Withania somnifera*	-	-	Gaidhani et al., 2013
98.	*Zea mays*	Maysin	-	Balasubramanian and Padma, 2013; Kim et al., 2003
99.	*Zingiber officinale*	Gingerol		

All these studies have clearly indicated the involvement of phytochemicals in cellular mechanisms and its modulation.

Insecticidal activities are one of the major uses explored in many plant species. Natural products from plants have fascinated researchers in recent years as potential sources of new pesticides. One of the primary plants recorded to have pesticidal properties was tobacco (*Nicotiana tabacum*). The use of tobacco leaf infusion to kill aphids led to the isolation of the alkaloid, nicotine (Rosaline Marion Bliss, 2005). The pesticides include insecticides insect repellents, molluscicides, antifeedants, fungicides and herbicides.

Pyrethrum has attained commercial attention due to its high effectiveness and broad-spectrum insecticidal activity (David, 2005). Another plant which has got attention is *Azadirachta indica,* which is effectively used to control insect pests. It's

activity is due to the presence of the compound azadirachtin (Rajapake and Ratnaseka, 2008). Investigation showed that extracts of three plants, *Plearostylia opposita* (Wall) Alston (Celastraceae), *Aegle marmelos Correa* (Rutaceae) and *Excoecaria agallocha* (Euphorbiaceae) were insecticidal (Samarasekera Javaneththi, 1997; Paul, 2011).

Asimina tribola (Annonaceae) used by traditional communities in Africa and Americas, has found to possess pesticidal, antitumor, and anti-feedant properties. The bioactive component "asimicin" is active against many insects including blowfly larvae, *Calliphora vicina* Meig, the spotted spider mite, the melon aphid, and *Aedes aegypti* larvae (Ratnayake et al., 1993). The genus *Piper* (family Piperaceae) is probably one of the most studied species. Extractives from *P. guineense, P. longum*, and *P. retrofractum* which are known to be active against *Callosobruchus maculatus*, the garden insect, *Zonocerus variegatus* L, and mosquito larvae (Ivbijaro and Agbaje, 1986; Dyer et al., 2004). Some of the successful plants and phytochemicals which were commercialised are given in Table – 2.3

Table -2.3. List of some commercial botanical pesticides

No	**Plant Species**	**Product/Trade name**	**Mode of action**	**Targets**
1	*Lonchocarpus* spp *Derris eliptica*	Rotenone	Insecticidal	Aphids, bean leaf beetle, cucumber beetles, leafhopper, red spider mite
2	*Chrysanthemum cinerariaefolium*	Pyrethrum/Pyrethrins	Insecticidal	Crawling and flying insects such as cockroaches, ants, mosquitoes, termites
3	*Nicotiana tabaccum*	Nicotine	Insecticidal antifungal	Aphids, thrips, mites, bugs, fungus gnat, leafhoppers
4	*Azadirachta indica [Dogonyaro* (Nigeria)]	Azadirachtin/Neem oil Neem cake Neem powder Bionimbecidine(GreenGold)	Repellent Antifeedant Nematocide sterilant Anti-fungal	Dandruffs(shampoos) eczema, nematodes, sucking and chewing insects(caterpillars, aphids, thrips, maize weevils)

5	Citrus trees	d-Limonene, Linalool	Contact poison	Fleas, aphids, mites, paper wasp, house cricket, dips for pets
6	*Shoenocaulon officinale*	Sabadilla dust	Insecticidal	Bugs, blister beetles flies, caterpillars, potato leafhopper
7	*Ryania speciosa*	Ryania	Insecticidal	Caterpillars, thrips, beetles, bugs, aphids
8	*Adenium obesum(Heliotis sp)*	Chacals Baobab (Senegal)	Insecticidal	Cotton pests, particularly the larvae of ballworm

The pesticidal and phytochemical screenings of many higher plants based on traditional knowledge clearly indicate that plants are endowed with pesticidal properties and can be utilized for use in agriculture and related fields. The requirement to use plant-based products arises from the fact that the synthetic pesticides are harmful to the entire ecosystem due to high persistence and toxicity.

2.4. Phytochemical potential of *C. laurifolius*

C. laurifolius DC. is shrub or considered as small sized tree up to 1-2 m high with striate, glabrous branches and branchlets (Ajaib and Khan, 2012). It is reported from shrublands and open forests of Taiwan, Indonesia, Japan, Laos, Malaysia, Myanmar, Thailand, Nepal, India & South East Asia (Ji, 2008; Kottaimuthu et al., 2008). The roots of this plant, is used in traditional Chinese medicine for the treatment of hernia, headache and injuries from falls. There are few alkaloids – dihydroerysotrine, erythroculine, dihydroerysodine- isolated from the plant earlier (Prelog et al., 1956; Ripperger et al., 1983; Millington et al., 1974; Juichi et al., 1977). Neuromuscular blocking action of isocorydine methochloride, a new quaternary alkaloid from *C. laurifolius* was reported (Mukherjee et al., 1984). The ethanolic extract of *C. laurifolius* leaves possesses potential anticonvulsant and neuroprotective effect against strychnine induced convulsions (Maqbool et al., 2019). Bark and leaf of *C. laurifolius* was reported to possess antimicrobial and antioxidant activities (Ajaib et al., 2017). No other bioactivity studies were reported on this plant. However, the studies cited above indicate the potential of this plant in processing highly active biomolecules.

MATERIALS AND METHODS

3. MATERIALS AND METHODS

3.1. Plant materials

The leaves and bark of the plant, *C. laurifolius* were collected from Mattupetty, Idukki, foot hills of southern Western Ghats during pre – monsoon and monsoon periods. Herbarium specimens were prepared and accessed to Kerala Forest Research Institute Herbarium (KFRI) with accession number 18026. Thoroughly washed bark and leaf samples of all the test plants were individually shade dried and then powdered with the help of a blender. The plant powder (approx. 10 g) was used for the extraction. The samples were stored at deep freezer (-20^{0}C) until future use.

3.2. Solvent extraction using Soxhlet apparatus

In this method, the finely ground crude plant material is placed in a porous bag or "thimble" made of strong filter paper (A), which is placed in upper extractor chamber (B) of the Soxhlet apparatus (Figure - 3.1). The extracting solvent (`300 ml) in solvent flask (C) is heated, and its vapours condense in condenser (D). The condensed extract drips into the thimble containing the crude drug, and extracts it by contact. When the level of liquid in extractor chamber (B) rises to the top of siphon tube, the liquid contents of extractor chamber (B) siphon into solvent flask (C).

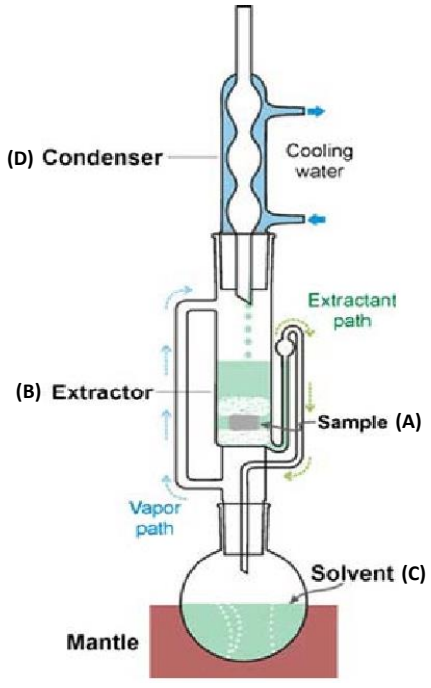


Figure -3.1 Soxhlet apparatus

This continuous process was carried out until a drop of solvent from the siphon tube does not leave residue when evaporated. The advantage of this method is that large amounts of compounds can be extracted with a much smaller quantity of solvent. The process is repeated 6-7 times, until the extraction is complete.

The extraction was carried out using hexane, petroleum ether, chloroform, ethyl acetate, methanol and water. After the extraction, the extracted solvent was concentrated using rotatory evaporator. The extracted solvent was condensed to `50 ml and stored at -20^{0}C until further use. Further fractionation, qualitative phytochemical analysis and bioassays were carried out using these extracts.

3.2. Fractionation of extracts

Fractionation of the extracts (wherever required) was achieved using column chromatography. The column chromatography was performed in 600 mm column with bore size 30 mm. Silica (100 - 200 mesh size, 115 g) was used to pack the column (`300 mm in length). The extracts mixed with silica were loaded in to the top of the column. Different solvents in different combinations were used for the elution (details given in the results section). Thus, obtained fractions were concentrated using rotary evaporator and stored in glass vials at -20^0 C until further use.

3.3. Qualitative phytochemical analysis

The condensed bark and leaf extracts were used for preliminary qualitative screening of phytochemicals. The analysis was carried out for leaf and bark extracts. Tests for alkaloids, flavanoids, saponins, glycosides, tannins, steroids, fatty acids were standardized for leaf samples as well as bark and young stem.

Test for Alkaloids:

Hager's Test – Plant extract was treated with few drops of Hager's reagent (saturated picric acid solution). Formation of yellow precipitate would show a positive result for the presence of alkaloids.

Test for Flavonoids:

Alkaline reagent Test – Crude extract of the plant when treated with sodium hydroxide solution, shows increase in the intensity of yellow color which would become colorless on addition of few drops of dilute Hydrochloric acid, indicates the presence of flavonoids.

Test for Saponins

Frothing test –The presence of saponins in the crude extract was determined by vigorously shaking with distilled water and allowed to stand for 10 minutes for saponin content as follows: no froth indicate absence of saponins and stable froth more than 1.5 cm indicates the presence of saponins.

Test for Glycosides

Keller Killiani Test – Plant extract was treated with few drops of glacial acetic acid and ferric chloride solution and mixed. Concentrated sulphuric acid was added, and observed for the formation of two layers. Lower reddish-brown layer and upper acetic acid layer which turns bluish green would indicate a positive test for glycosides.

Tests for Sterols and Triterpenoids

Salkowski's test–Leaf and bark extracts were treated in chloroform separately with few drops of concentrated sulphuric acid, shaken well and allowed to stand for some time. Appearance of red colour in the lower layer indicates the presence of sterols while formation of yellow coloured lower layer indicates the presence of triterpenoids.

Test for Fattyacids

One ml of extract was mixed with 10 ml of ether. These extracts were then allowed for evaporation on filter paper and dried. The appearance of transparence on filter paper indicates the presence of fatty acids.

Test for Steroids

The extract (1 ml) was dissolved in 10 ml of chloroform and equal volume of concentrated sulphuric acid was added by sides of the test tube. The upper layer

turns red and sulphuric acid layer showed yellow with green fluorescence. This indicated the presence of steroids.

Test for Tannins

The presence of tannins was confirmed by adding 2 ml of extract to few drops of 1 % lead acetate. Formation of a yellowish precipitate indicates the presence of tannins (Savithramma et al., 2011).

Test for Quinones

Formation of blue green or red coloration while mixing diluted NaOH with 1 ml of crude extract indicates the presence of quinones (Sony and Sosa, 2013).

Test for Coumarins

The coumarins were tested by adding 10% of NaOH to 2 ml of extract and chloroform. The resultant mixture developed a yellow colour indicating the presence of coumarins (Sony and Sosa, 2013).

3.4. Thin Layer Chromatography (TLC)

For the standardization of TLC procedures, the pre-coated plates were used to analyse the samples. Mixture of mobile phases were used to enhance the maximum separation between compounds while doing TLC. Different mixtures of mobile phases, at different concentrations were also tried. Three dilutions of samples were prepared. The mobile phases taken were;

a) Chloroform: Methanol: Water (80:20:2)
b) Ether: Acetone: Diethyl amine (80:20:5)
c) Chloroform: Methanol (90:10)

The samples were spotted on the bottom of the plates and allowed to run using different mobile phases as given above. After sufficient run time the plates were allowed to dry. The plates were observed under UV light at 254 nm and images were captured. The plates were further treated using Dragendorff's reagent for confirming the presence of alkaloids.

3.5. Bioactivity studies

3.5.1. Cytotoxicity studies - Cell viability assay

The cell lines, human cervical cancer (HeLa) cells and murine macrophage (U-937) cells were obtained from National Centre for Cell Science (NCCS), Pune. The HeLa cells were grown in minimum essential medium (Eagle) without tryptose phosphate broth and supplemented with 2 nM L-glutamine. Earle's BSS adjusted to contain 1.5 g/l sodium bicarbonate, 0.1mM non-essential amino acids and 1.0mM sodium pyruvate 90%, fetal calf serum 10%, and gentamycin (80 µg/ml). Cells were maintained at 37^0C in a humidified atmosphere of 95 % air and 5 % CO_2 in an incubator.

U-937 cells were grown in RPMI- 1640 medium with L-glutamine supplemented with 7.5%w/v of sodium bicarbonate, 10% fetal bovine serum albumin and gentamycin (80 µg/ml). Cells were maintained at 37^0 C in a humidified atmosphere of 95 % air and 5 % CO_2 in an incubator. The assay in U-937 cells were carried out at cell culture facility at DRDO-BU Centre for Life Sciences, Coimbatore.

In order to access the biological activity of the extracts, cell viability assay was performed. The MTT cell proliferation assay measures the cell proliferation rate and when metabolic events lead to apoptosis or necrosis, the reduction in cell viability is observed. The reduction of tetrazolium salt is a reliable way to examine cell viability/proliferation. The yellow tetrazolium MTT (3-(4, 5-dimethylthiazolyl-2) - 2, 5-diphenyltetrazolium bromide) is reduced by metabolically active cells, in part by the action of dehydrogenase enzymes, to generate reducing equivalents such as NADH and NADPH. The resulting intracellular purple formazan can be solubilized and quantified by spectrophotometric means. Since reduction of MTT can only occur in metabolically active cells the level of activity is a measure of the viability of the cells.

3.5.2. Anti-oxidant evaluation - DPPH Radical Scavenging assay

The free radical scavenging activity was measured as described by Blois (1958). DPPH was dissolved in methanol to a concentration of 0.025 g/L. The plant extract at various concentrations was diluted with dimethyl sulfoxide (DMSO) to get

sample solution. The sample solution (50 µL) was taken and 1950 µL DPPH working solution was added to sample. After a 20 min reaction at room temperature, the absorbance of the solution was measured at 515 nm. DPPH working solution was taken as blank. The free radical scavenging activity of each fraction was determined by comparing its absorbance with that of a blank solution (no sample). The ability to scavenge the DPPH radical was expressed as percentage inhibition and calculated using the following equation:

The results are calculated as

DPPH scavenging activity (%) = $(A_0-A_1)/A_0 \times 100$

where A_0 is the absorbance of the control and A1 is the absorbance of the sample.

3.5.3. Anti-microbial studies

Antibacterial activity of *C. laurifolius* extracts was tested against the following microorganisms by disc diffusion method: *Streptococcus aureus*, *Salmonella typhi* and *Klebsiella pneumoniae*. The samples were procured from MTCC (Microbial Type Culture Collection) and were sub-cultured on nutrient agar, an isolated single colony was inoculated in 20 ml nutrient broth. The overnight culture was used to prepare bacterial lawns. Approximately 20 ml of molten and cooled nutrient agar media was poured in sterilized petri dishes. The plates were left overnight at room temperature to check for sterility. The samples were swabbed onto the plates. As a preliminary qualitative assay was conducted with 5 mm diameter sterile disc solvent control, paper control, positive control and 5 ml sample (respective solvent extracts) were loaded and plates were incubated at 37^0C for 24 hours and examined the zone of inhibition which appear as a clear area around the wells. Organism with zone of inhibition was further taken for dosage analysis. Discs with three different concentrations (80 µg, 100 µg and 500 µg) of the chloroform extracts were prepared and placed on swabbed plates. Then the plates were incubated at 37^0C for 24 h. The diameter of the clearing zones was measured in mm using the antibiotic zone scale. The experiment was done in triplicate for each pathogenic bacterium and compared with the standard antibiotic sensitivity chart.

3.5.4. Insecticidal activity studies

3.5.4.1. Anti-feedant activity

Anti-feedant activities of plant extracts were studied using leaf disc no-choice bioassay method (Ranjith et al., 1997). Fresh castor leaf disc (4.5 cm dia) were dipped in 0.5, 1.0, 2.5 and 5.0% concentrations of crude aqueous methanolic extracts against *Spodoptera litura* stored at individual boxes. The leaf disc treated with water was used as negative control. In each dish, wet cotton was placed to avoid early drying of the leaf discs and single fourth instar larva was introduced in to each dish. Progressive consumption of treated or control leaf area by the larvae after 24 hours was recorded using graph paper. Leaf area, eaten by larvae in treatment was corrected from the negative control and shrinkage percentage. Four replications were maintained for each treatment. The percent of feeding and anti-feedant activity in the no choice method was calculated based on following formula (Balikai et al., 1999).

$$Per\ cent\ feeding = \frac{(Initial\ area\ for\ feeding - area\ left\ over\ after\ feeding)}{Initial\ area\ given\ for\ feeding} \times 100$$

$$Antifeedant\ activity(\%) = \frac{(\%\ protection\ in\ treatment - \%\ protection\ in\ control)}{(100 - \%\ protection\ in\ control)} \times 100$$

3.5.4.2. Repellent activity

The repellency of insect was tested with choice bioassay (Chattopadhyay et al., 2019). The fresh castor leaf disc of 4.5 cm dia. was treated with plant extracts (100 mg/ml) on one piece and with water on another. Then the leaves were exposed to ten, third instar larvae of *S. litura* by placing them in the middle of each box. After three hour, the number of larvae present at treated or control were counted. Repellent index (RI) was calculated as

$$Repellent\ Index = \frac{(C - T)}{(C + T)} \times 100$$

C = Number of larvae in control diet

T = Number of larvae in treated diet

If RI > 50, the extract is repellent and RI < 50, the extract is non repellent (Isman, 2006). All the experiments were repeated four times.

3.5.4.3. Contact toxicity

This experiment tested the hypothesis that topically applied plant extracts exhibit contact toxicity to *S. litura* larvae. Contact toxicity of the plant extracts were evaluated using first instar larvae. For each replicate, 10 larvae were transferred to a Whatman No. 1 filter paper disc in a 90 mm disposable plastic dish. Three replicate Petri-dishes of 10 larvae were treated with each plant extract. Each larva was treated topically with aqueous-methanol plant extract using a 50 µl micropipette. In the control treatment, larvae were treated with methanol in water. After application of treatments, the larvae were allowed to dry for 10 min at 25 ± 2°C and subsequently transferred individually into castor leaves containing plastic containers. Following treatment application, larvae were maintained at 25 ± 2°C and mortality was assessed after 24 Hr. The experiment was repeated four times.

3.6. GC-MS studies

The chemical composition of the plant extract was analyzed using GC–MS. The extracts were filtered through 0.22 µm syringe filter before the analysis. One microlitre of the filtered sample was analyzed using GC–MS (QP-2010-S Shimadzu) equipped with Rxi-5Sil MS column of 30 m in length, 0.25 mm in diameter, and 0.25 µm thickness. The GC-MS was employed with helium as the carrier gas at a constant flow of 1 mL/min. The oven temperature started at 80 °C and remained at this temperature for 4 minutes increasing to 280 °C at 5° C/min ramp rate. Injection port was adjusted at 260 °C and splitless injection mode was used. EI mode was at 70 eV, while a mass spectrum was recorded in the 50–500 amu range and ion source

temperature was maintained at 200 °C. The components of the extracts were identified by comparing the retention times of chromatographic peaks using quadrapole detector with NIST and Wiley library.

3.7. Statistical analysis

Data were expressed as mean ± SE from four replicates per each treatment. Data of anti-feedant and feeding activity were analysed by one-way analysis of variance followed by Dunnet's test for comparison between respective control and treatment groups. For all other the experiments, data were analysed by one-way analysis of variance followed by Student- Newman-Keul's multiple mean comparison test. The level of significance was set at $p \leq 0.05$. Data of all the results in this study were obtained from at least three independent experiments with similar pattern.

RESULTS

4. RESULTS

4.1. Qualitative phytochemical analysis and bioactivity studies of crude extracts

4.1.1. Qualitative phytochemical studies of bark and leaf crude extracts

The phytochemical studies of the plant were started with the extraction using soxhlet extraction methods. The bark and leaf of the plants were collected as detailed in the materials and method. Initially a sequential extraction method was adopted using different solvents such as hexane, petroleum ether, chloroform, ethyl acetate, methanol and water. The preliminary qualitative phytochemical studies showed maximum phytochemicals in chloroform extracts. The qualitative phytochemical analysis of the bark extract (Table – 4.1) showed the presence of number of biomolecules in varying degrees. The alkaloids were present in all the extracts, maximum was noted in chloroform followed by hexane and ethyl acetate. The tannins were present only in aqueous extract, however only minimal quantities were observed. Saponins were significantly present only in chloroform extract. Glycosides, flavonoids, coumarins and fatty acids were sparingly present in all the extracts. Terpenoids were present in all the extracts except in aqueous extract. Quinones were significantly present in chloroform extract; however, traces were noted in other extracts too.

The results of the leaf extracts indicated that the phytochemicals were present in varying degrees in all the extracts. The alkaloids and terpenoids were present in almost all the extracts significantly except in aqueous extract. Tannins, saponins, steroids, glycosides, flavonoids, coumarins, fatty acids and quinones were present moderate to mild levels in almost all the extracts. Certain phytochemicals were absent in some extracts. The results are given in Table - 4.2.

From these results it was observed that many of the phytochemicals were present significantly in chloroform extracts of both *C. laurifolius* bark and leaf. However, all the extracts were subjected to further bioactivity studies to ascertain their activity potential.

Table - 4.1: Qualitative phytochemical analysis of bark extracts

Phytochemicals	Solvents					
	Hexane	Pet. ether	Chloroform	Ethyl acetate	Methanol	Water
Alkaloids	+++	+	++++	++	++	-
Tannins	-	-	-	-	-	+
Saponins	-	+	+++	-	+	-
Steroids	++	+	+	++	++	-
Glycosides	+	+	+	++	-	-
Terpenoids	+++	+++	++++	++	++++	+
Flavonoids	+	-	+	-	-	+
Coumarins	-	-	-	-	+	+
Fattyacids	+	+	-	++	+	+
Quinones	-	-	+++	+	+	++

Table - 4.2: Qualitative phytochemical analysis of leaf extracts

Phytochemicals	Solvents					
	Hexane	Pet. ether	Chloroform	Ethyl acetate	Methanol	Water
Alkaloids	++	+	++++	+	+	-
Tannins	-	++	+	+	-	+
Saponins	-	+	++	-	++	-
Steroids	++	-	+	+	++	+
Glycosides	+	-	+	+	-	-
Terpenoids	+++	++	+++	+	+++	+
Flavonoids	+	-	+	-	+	+
Coumarins	+	-	-	++	-	+
Fattyacids	+	+	+	+	+	+
Quinones	-	-	++	+	-	+

4.1.2. Bioactivity studies of bark and leaf crude extracts

4.1.2.1. Antioxidant activity (DPPH) assay

The anti-oxidant activity of hexane, petroleum ether, chloroform, ethyl acetate, methanol and water extracts of both bark and leaf of *C. laurifolius* were analysed using DPPH assay. The free radical scavenging activity of each fraction was determined by comparing its absorbance with that of a blank solution (no sample). The ability to scavenge the DPPH radical was expressed as percentage inhibition. All the extracts were assayed at a concentration of 30 µg/ml.

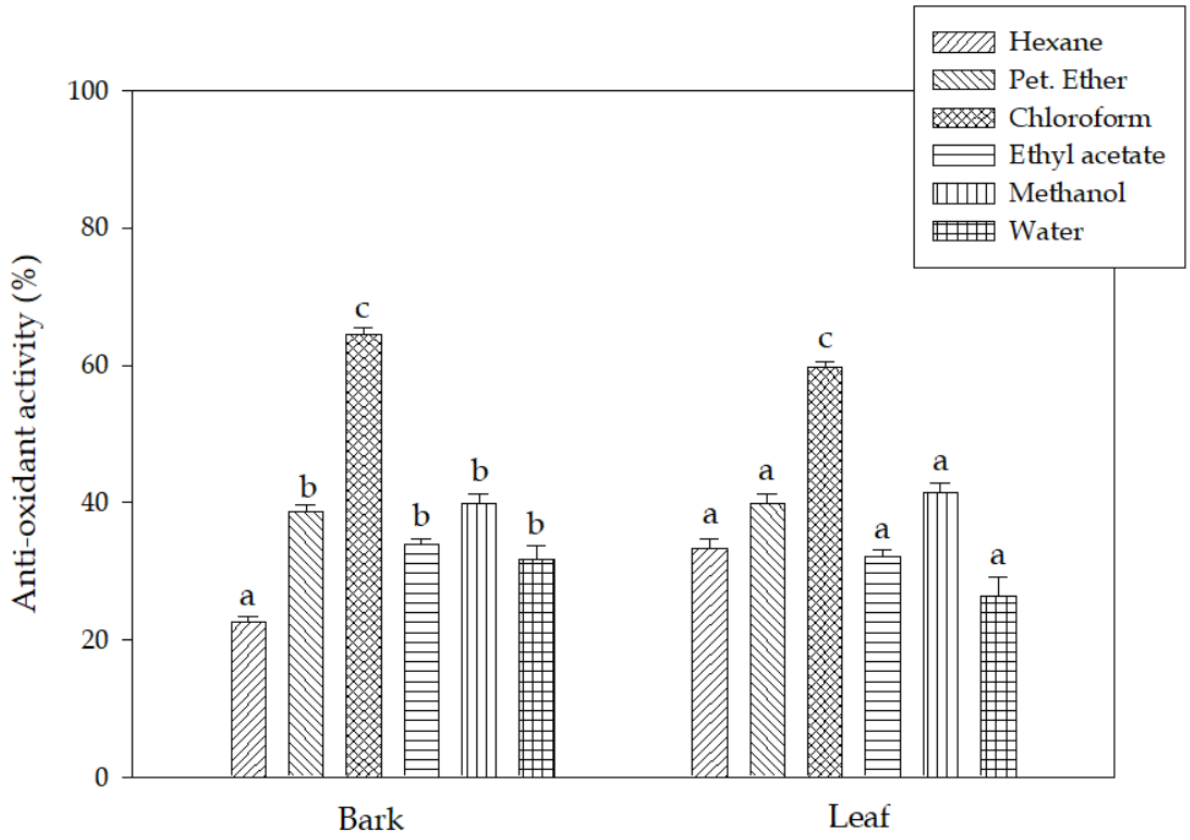


Figure – 4.1: Anti-oxidant activity of bark and leaf extracts of *C. laurifolius* in different solvents. The values are expressed as mean ± SE of four replicates per experiment. In each group mean followed by different alphabets are significantly different at $p \leq 0.05$ by Student-Newman-Keul's multiple comparison test.

The DPPH method revealed the scavenging of free radicals as 22.58, 38.7, 64.51, 33.87, 39.78 and 31.72 percentage respectively for hexane, petroleum ether, chloroform, ethyl acetate, methanol and water extracts of *C. laurifolius* bark. In leaf extracts of *C. laurifolius* it was 33.33, 39.78, 59.67, 32.25, 41.39 and 26.34 percentage

respectively for hexane, pet. ether, chloroform, ethyl acetate, methanol and water extracts (Figure - 4.1)

4.1.2.2. *In vitro* Cytotoxicity (MTT) assay

The cytotoxic potential of different solvent extracts was assayed using mitochondrial dehydrogenase (MTT) assay (Figure – 4.2). Cell viability was examined by the ability of the cells to cleave the tetrazolium salt, MTT [3-(4,5-dimethylthiazol-2-yl)-2,5- diphenyl tetrazolium bromide by the mitochondrial enzyme succinate dehydrogenase. The cytotoxicity of all the six extracts were assayed in human cervical cancer (HeLa) cells. The cytotoxicity of all the extracts were assayed at a concentration of 30 µg/ml. The optical density at 570 nm was measured as a measure of cell viability.

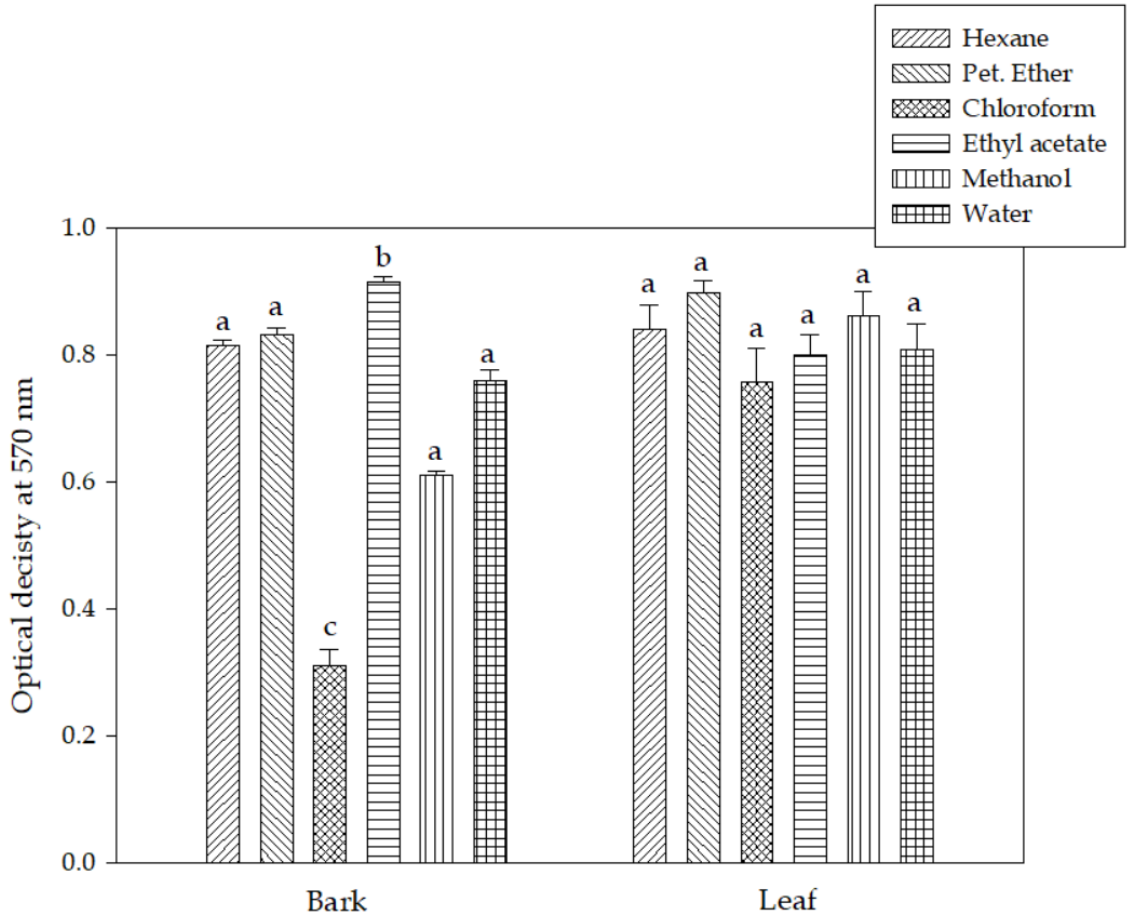


Figure – 4.2: Cytotoxicity activity of bark and leaf extracts of *C. laurifolius* in different solvents. The values are expressed as mean ± SE of four replicates per experiment. In each group mean followed by different alphabets are significantly different at $p \leq 0.05$ by Student-Newman-Keul's multiple comparison test.

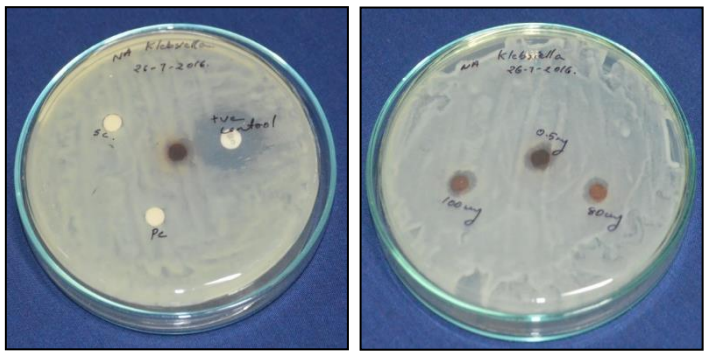
The cell viability assay showed that bark chloroform extract is significantly toxic compared to all other extracts. The increased optical density indicates the presence of more viable cells. The optical density shown by bark chloroform extract is 0.310, which is very much lower compared to extracts in other solvents. The bark methanol extract also showed a lowered optical density, however which is much higher than the chloroform extract. The study indicated that the bark chloroform extract of *C. laurifolius* contains molecules with cytotoxic potential. Further purification is required to isolate biologically active molecules.

4.1.2.3. Antimicrobial activity assay

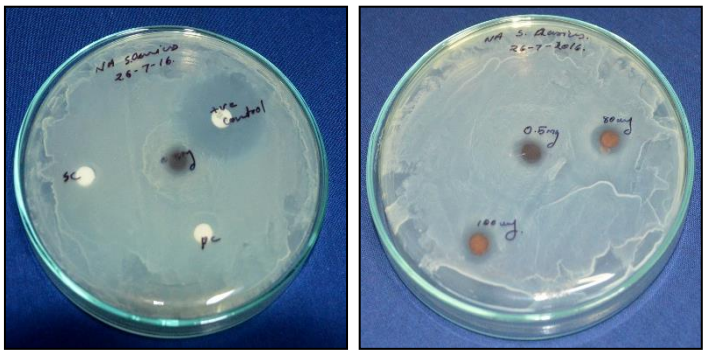
The anti-microbial activity of hexane, petroleum ether, chloroform, ethyl acetate, methanol and water extracts of both bark and leaf were attempted against *Streptococcus aureus*, *Salmonella typhi* and *Klebsiella pneumoniae*. Three concentrations of extracts 80, 100 and 500 µg/ml were attempted. The result indicated that the bark chloroform extract exhibited antimicrobial properties against these organisms. The chloroform extract of *C. laurifolius* showed potential inhibition against *Streptococcus aureus* (Zone of inhibition (ZOI) 13 mm, 14 mm, 17 mm), *Salmonella typhi* (ZOI - 10 mm, 13 mm, 18 mm) and *Klebsiella pneumoniae* (ZOI - 08 mm, 10 mm, 13 mm) for 80, 100 and 500 µg/ml respectively. No other extracts were shown any significant inhibition of these organisms (Figure – 4.3).

The studies clearly indicated that the bark chloroform extract of *C. laurifolius* possess significant bioactivity in terms of anti-oxidant, cytotoxic and anti-microbial activities. Further studies were taken up with the bark chloroform extract of *C. laurifolius*.

(A) *Klebsiella pneumoniae*



(B) *Staphylococcus aureus*



(C) *Salmonella typhi*

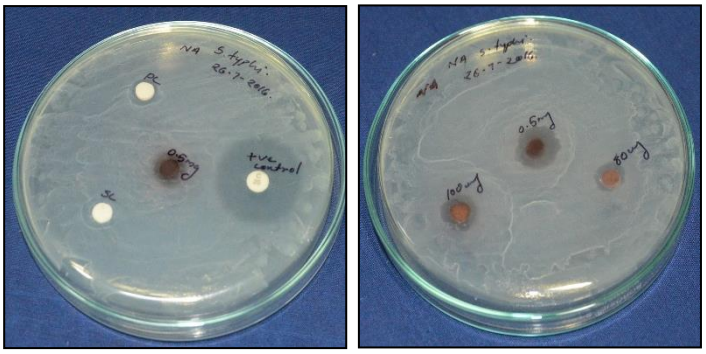


Figure – 4.3: Antimicrobial effect of *C. laurifolius* bark chloroform extract on different microorganisms (A) *Klebsiella pneumoniae*, (B) *Streptococcus aureus* and (C) *Salmonella typhi*.

4.1.2.4. Insecticidal activity studies

The insecticidal activities of the extracts were evaluated using feeding and antifeedant activity, repellent activity and contact toxicity methods. During the preliminary screening, only methanolic extracts showed some activity hence further dosimetry studies were carried out using methanolic extract.

4.1.2.4.1. Feeding and antifeedant activity

Antifeedant activities of leaf and bark crude extracts of *C. laurifolius* was studied using leaf disc no-choice bioassay. The efficacy assessment was carried out by comparing the leaf area consumed in the treated leaves that of control by the third instar larvae of *Spodoptera litura* for their antifeedant activity. Four doses (0.5, 1.0, 2.5 and 5 %), of both leaf and bark methanolic extracts were tested for its antifeedant activity (Figure- 4.4). The leaf extract showed significant antifeedant activity compared to control at different concentrations. *C. laurifolius* extracts showed

significant antifeedant activity from 1 % exposure onwards and maximum activity was observed at 5 % exposure (86.11%). In bark extracts, maximum activity was noted with 41.29 % antifeedant activity on exposure of 0.5% extract followed by 47.29 % in 1 % exposure, 74.46 % in 2.5 % exposure and maximum of 92.17 % in 5 % exposure. Similar to antifeedant activity assay, feeding activity was also recorded on the exposure of leaf and bark extracts. Extracts at 5% extract exposure, showed 13.87 % inhibition.

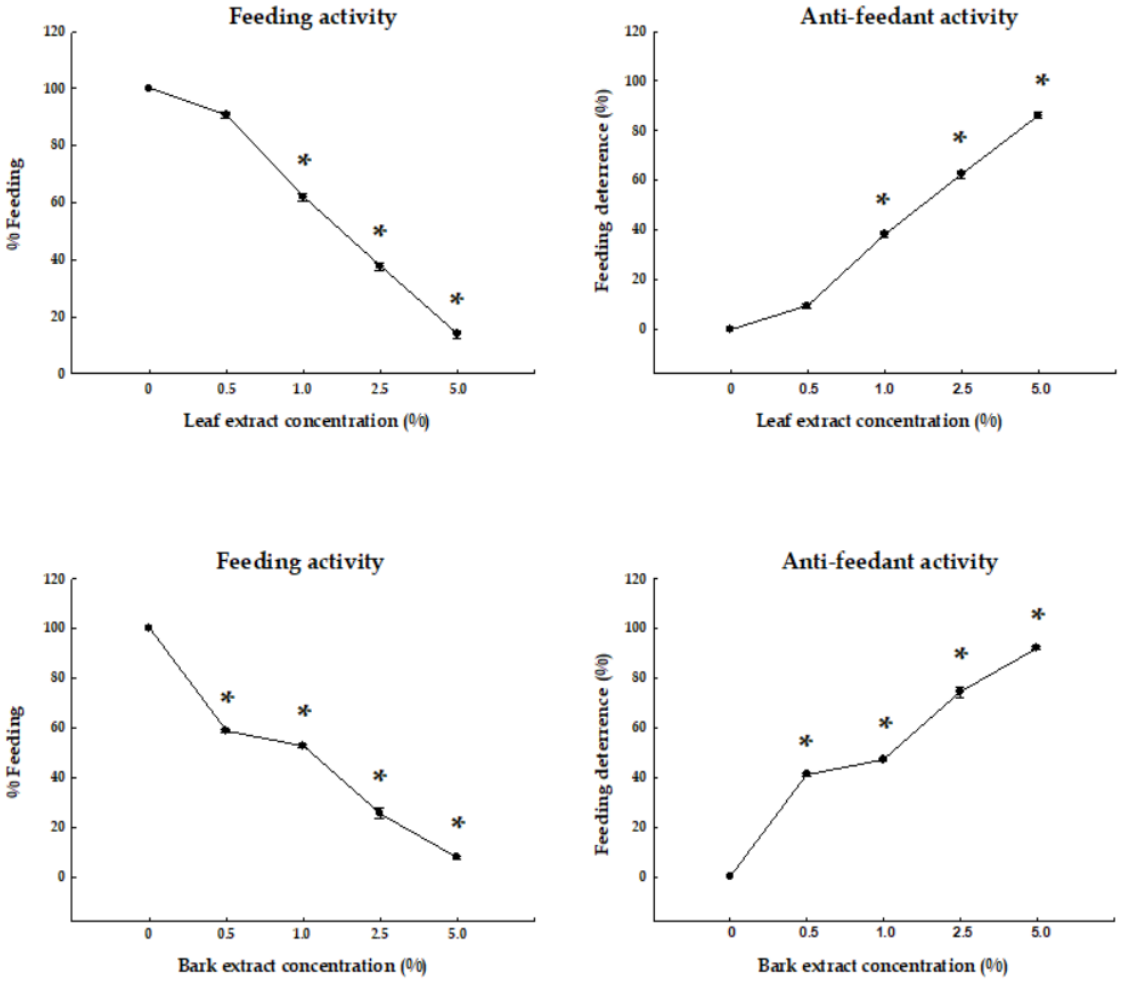


Figure – 4.4: Feeding activity and anti-feedant activities of leaf and bark methanol extracts of *C. laurifolius* against *Spodoptera litura*. The values are expressed as mean ± SE of four replicates per experiment. * Significantly different from respective controls at $p \leq 0.05$ by Dunnet's test.

4.1.2.4.2. Repellent activity

Repellent index (RI) of both leaf and bark extracts (100 mg/ml) of *C. laurifolius* was tested in third instar larvae of *S. litura*. Lleaf extracts of *C. laurifolius* showed an attractant (-25) activity. However, bark extract showed a significantly higher value (Figure - 4.5). The study indicated that the bark extract of *C. laurifolius* could be developed as a promising insect repellent. A dose dependent study of the bark extract may give a better understanding on the repellence. But leaf extract did not show any repellence rather it worked as an attractant.

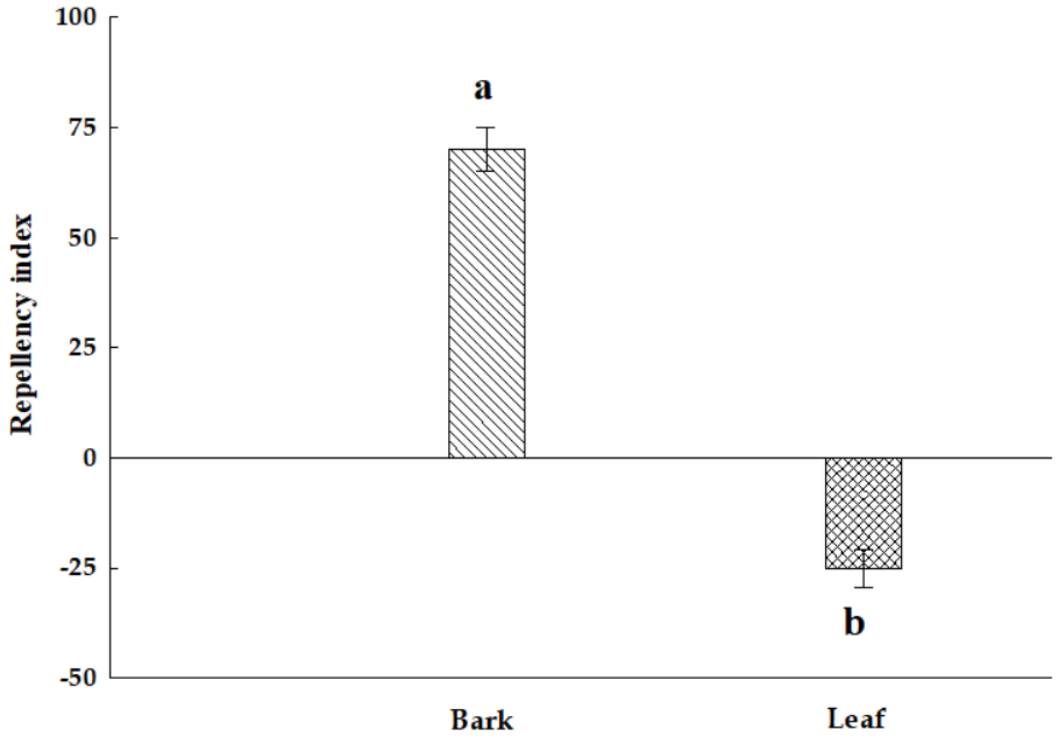


Figure – 4.5: Repellent activity of leaf and bark methanol extracts of *C. laurifolius* against *Spodoptera litura.* The values are expressed as mean ± SE of four replicates per experiment. Means followed by different alphabets are significantly different at $p \leq 0.05$ by Student-Newman-Keul's multiple comparison test.

4.1.2.4.3. Contact toxicity

The contact toxicity of leaf extract of *C. laurifolius* was 92.5 µg/ml and bark was only 15.25 µg/ml (Figure - 4.6). The bark extract showed higher toxicity compared to leaf extract, antifeedant and repellent activities of bark extract were also much higher than that of leaf extract. This gives a promising opportunity to use bark extract as a natural bio-pesticide.

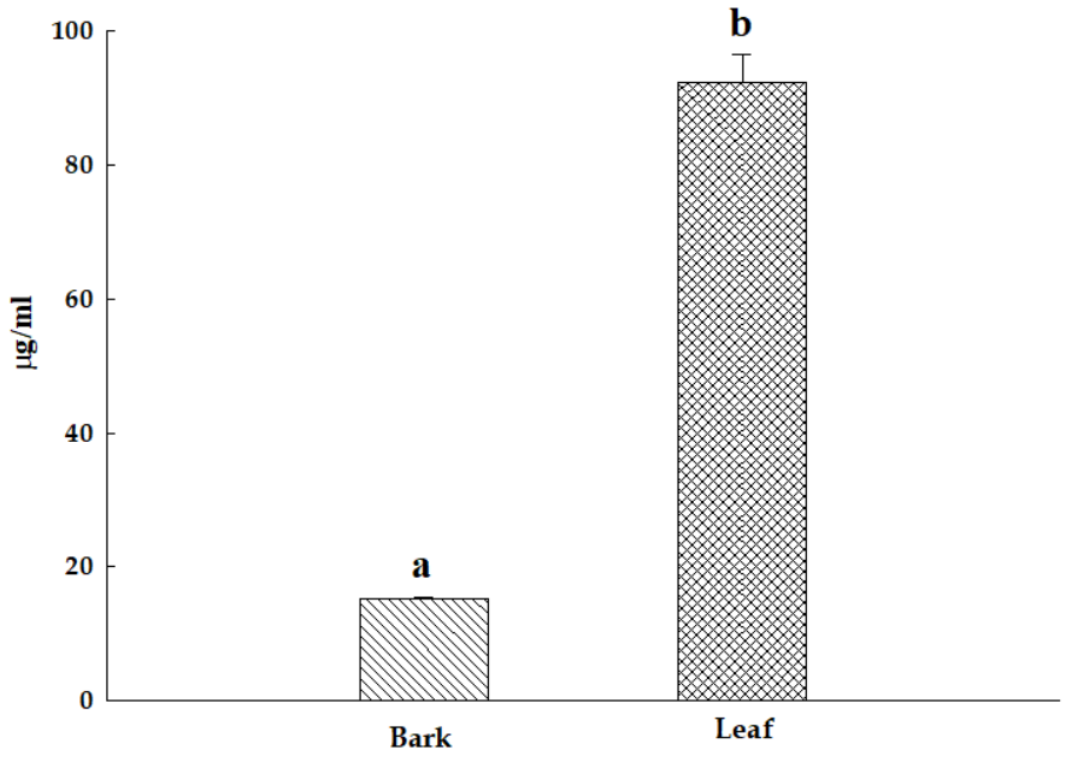


Figure - 4.6: Contact toxicity of leaf and bark methanol extracts of *C. laurifolius* against *Spodoptera litura*. The values are expressed as mean ± SE of four replicates per experiment. Means followed by different alphabets are significantly different at $p \leq 0.05$ by Student-Newman-Keul's multiple comparison test.

4.2. Bioactivity guided fractionation

4.2.1. Column chromatography of the chloroform bark extract

The principle involved in this separation of constituents is adsorption at the interface between solid and liquid. The component must have various degree of affinity towards adsorbent and also reversible interaction to achieve successful separation. No two compounds are alike in the above aspect. Low affinity compounds will elute first. The columns of different sizes were used for the present

studies. Since chloroform extract was found to possess significant pharmacological activity when compared to other extracts, an attempt was made to fractionate the chloroform extract by column chromatography.

The partial purification of chloroform extract was carried out by the sequential purification through column chromatography. Activated silica gel (pore size 100-200) was used as a stationary phase and toluene, chloroform and methanol in sequence were used as a mobile phase. A total 36 fractions were collected with following combinations ; Toluene, Toluene : Chloroform (75:25, 50:50), Toluene : Chloroform (25:75 – 3 fractions), Chloroform (9 fractions), Methanol : Chloroform (10:90 -7 fractions, 20:80 – 3 fractions , 50:50 -3 fractions, 75:25 -2 fractions, 90:10 -3 fractions, Methanol -2 fractions as given below;

Table – 4.3 : Column elution conditions for column chromatography

Fraction	Solvents	Ratio
1	Toluene	100
2	Toluene : Chloroform	75:25
3	Toluene : Chloroform	50:50
4	Toluene : Chloroform	25:75
5	Toluene : Chloroform	25:75
6	Toluene : Chloroform	25:75
7	Chloroform	100
8	Chloroform	100
9	Chloroform	100
10	Chloroform	100
11	Chloroform	100
12	Chloroform	100
13	Chloroform	100
14	Chloroform	100
15	Chloroform	100
16	Chloroform: Methanol	90:10
17	Chloroform: Methanol	90:10
18	Chloroform: Methanol	90:10
19	Chloroform: Methanol	90:10
20	Chloroform: Methanol	90:10

Fraction	Solvents	Ratio
21	Chloroform: Methanol	90:10
22	Chloroform: Methanol	90:10
23	Chloroform: Methanol	20:80
24	Chloroform: Methanol	20:80
25	Chloroform: Methanol	20:80
26	Chloroform: Methanol	20:80
27	Chloroform: Methanol	50:50
28	Chloroform: Methanol	50:50
29	Chloroform: Methanol	50:50
30	Chloroform: Methanol	25:75
31	Chloroform: Methanol	25:75
32	Chloroform: Methanol	10:90
33	Chloroform: Methanol	10:90
34	Chloroform: Methanol	10:90
35	Methanol	100
36	Methanol	100

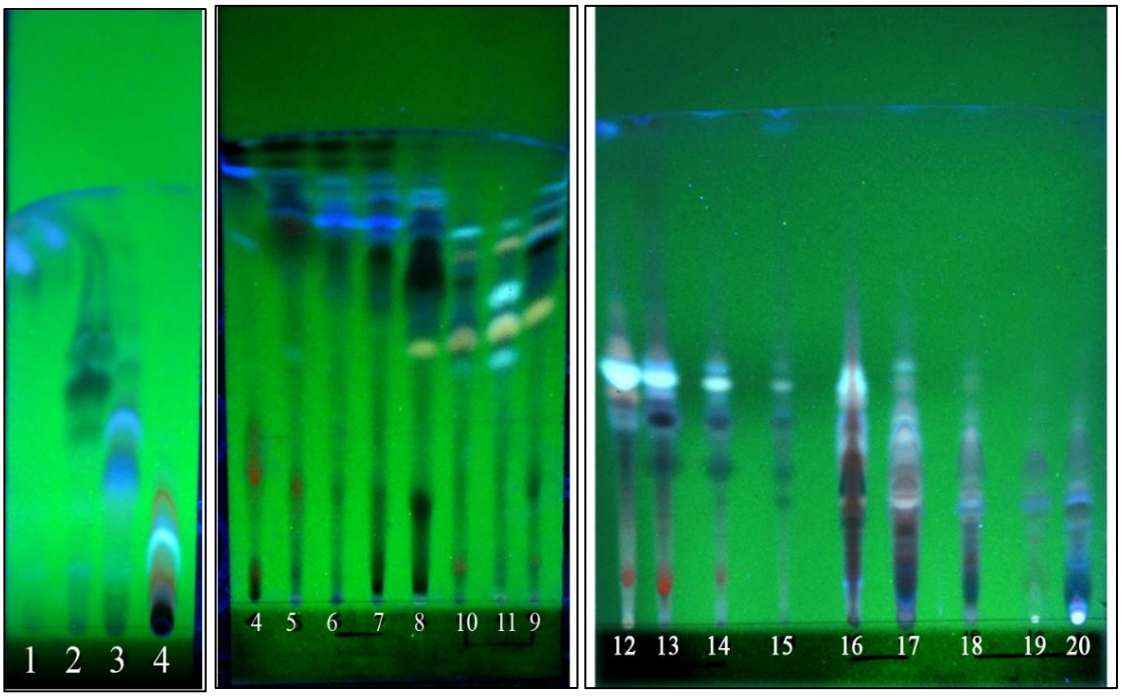
4.2.2. Thin layer chromatography of the fractions

The fractions collected through column chromatography was subjected to TLC to have a comparable chemical composition among the fractions (Figure – 4.7 A&B). Solvents used in the TLC are as given in Table – 4.4.

Table – 4.4 : TLC mobile phase conditions

Fractions	Mobile phase Used	Composition
1-4	Toluene: Chloroform	8:2
5-11	Toluene: Chloroform	5:5
12-18	Toluene: Chloroform: Methanol	4:5.8:0.2
19-29	Toluene: Chloroform: Methanol	4:5.5:0.5
28-36	Toluene: Chloroform: Methanol	3:6:1

(A)



(B)

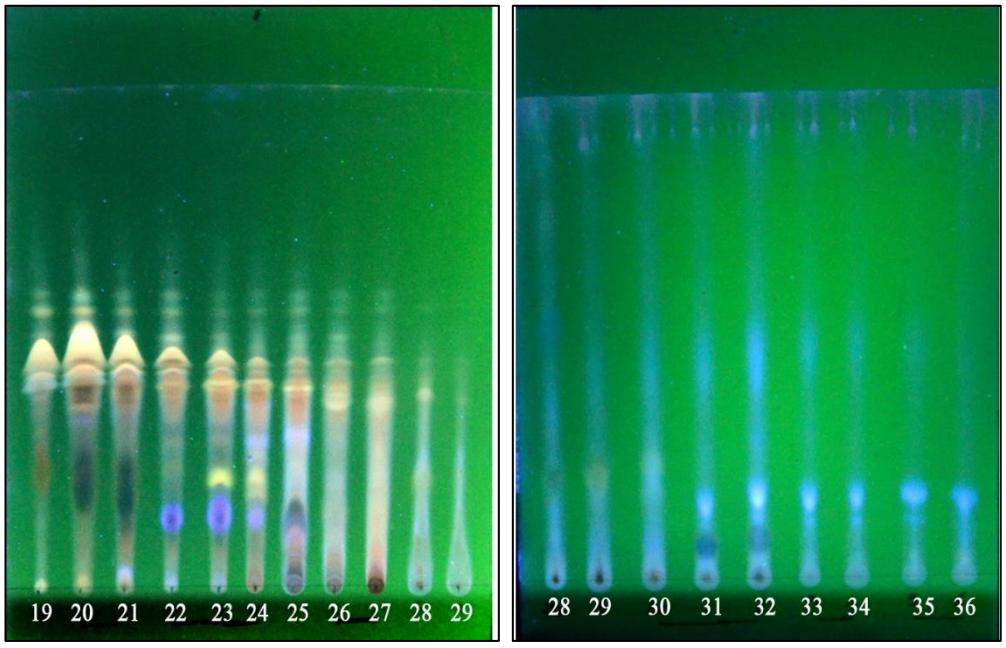


Figure – 4.7 : TLC analysis of column fractions. The column fractions were subjected to TLC analysis in pre-coated plates and separated using different mobile phases using mobile phases given in Table – 4.4.

Based on the pattens visible in the TLC analysis, the fractions with similar band patterns were identified and these fractions were combined. There were 21 combined fractions selected for further analysis. The details of the 21 fractions were given in Table – 4.5

Table – 4.5 : Details of fractions combined based on TLC

Fraction ID	Solvents	Combined fractions ID
1	Toluene	1
2	Toluene: Chloroform	2
3	Toluene: Chloroform	3
4	Toluene: Chloroform	4
5	Toluene: Chloroform	5
6	Toluene: Chloroform	6
7	Chloroform	
8	Chloroform	8
9	Chloroform	9
10	Chloroform	
11	Chloroform	11
12	Chloroform	
13	Chloroform	
14	Chloroform	
15	Chloroform	15
16	Chloroform: Methanol	16
17	Chloroform: Methanol	
18	Chloroform: Methanol	18
19	Chloroform: Methanol	
20	Chloroform: Methanol	20
21	Chloroform: Methanol	
22	Chloroform: Methanol	22
23	Chloroform: Methanol	
24	Chloroform: Methanol	
25	Chloroform: Methanol	25
26	Chloroform: Methanol	26
27	Chloroform: Methanol	
28	Chloroform: Methanol	28
29	Chloroform: Methanol	29
30	Chloroform: Methanol	30
31	Chloroform: Methanol	
32	Chloroform: Methanol	
33	Chloroform: Methanol	33
34	Chloroform: Methanol	
35	Methanol	35
36	Methanol	

4.3. Qualitative phytochemical analysis and bioactivity studies of fractions

4.3.1. Phytochemical evaluation of fractions

The qualitative phytochemical evaluation of the 21 fractions prepared based on the TLC analysis was carried out following standard procedures. The presence of alkaloids, tannins, saponins, steroids, glycosides, terpenoids, flavonoids, coumarins, fatty acids and quinones were found to present in many of the fractions with varying degrees (Table - 4.6 A&B). Though most of the phytochemicals were present in many of the fractions, high alkaloid content was noted in fractions 2, 5, 6, 8, 9, 11, 16, 18, 20, 22, 25, 26 and 28. Similarly terpenoid contents were also high in many of these fractions. Based on this, bioactivity in terms of cytotoxicity was performed in order to identify the most bioactive fraction.

Table – 4.6: Qualitative phytochemical evaluation of fractions

(A)

Phytochemicals	**Fractions**									
	1	**2**	**3**	**4**	**5**	**6**	**8**	**9**	**11**	**15**
Alkaloids	+++	+++	+	+	+++	+++	+++	+++	+++	+
Tannins	-	-	+	+	+	+	-	-	++	+
Saponins	-	+++	+	+	+	+	-	-	++	+
Steroids	++	+	+	+	+	+	-	-	++	+
Glycosides	+	+	+	-	-	++	++	-	-	+
Terpenoids	+++	+++	+	+	-	-	++	+++	+	+
Flavonoids	+	+	-	++	+	-	-	-	+	-
Coumarins	-	-	-	-	++	+	-	+	+	-
Fattyacids	+	-	-	-	++	+	++	+	+	+
Quinones	-	+++	-	++	+	-	+	+	++	-

(B)

Phytochemicals	Fractions										
	16	**18**	**20**	**22**	**25**	**26**	**28**	**29**	**30**	**33**	**35**
Alkaloids	+++	++++	+++	+++	+++	+++	+++	+	+	+	-
Tannins	+	+	++	+	+	+	++	+	+	-	-
Saponins	+	+	++	+	+	+	++	+	+	-	-
Steroids	+	+	++	+	+	+	++	+	+	-	-
Glycosides	-		+	+	-	-	+		-	++	+
Terpenoids	+	+++	-	++	+	-	-	++	+	+	-
Flavonoids	-	++	+	-	-	+	+	+	+	+	+
Coumarins	-	++	+		+	-	-	++	+	-	-
Fattyacids	-	++	+	+	+	-	-	-	++	+	-
Quinones	+	-	-	++	+	-	-	+	+	-	-

4.3.2. Cytotoxicity studies of combined fractions

The 13 fractions with high alkaloid and terpenoid content were subjected to cytotoxicity evaluation using MTT assay (Figure – 4.8). The HeLa cells were exposed with 30 µg/ml of all the fractions for 24 Hrs and assay was carried out. Only solvent was used as control. The optical density was measured and plotted against fraction ID's (Figure – 4.8). The fraction -18 showed the highest cytotoxicity with low optical density value. The OD value is of fraction-18 (0.434 ± 0.0262) was observed to be significantly lower than that of control (0.827 ± 0.012). Hence it is very clear that the fraction-18 possesses the activity due to the presence of biologically active molecules. Further purification of the fraction-18 is required to isolate the biologically active molecule.

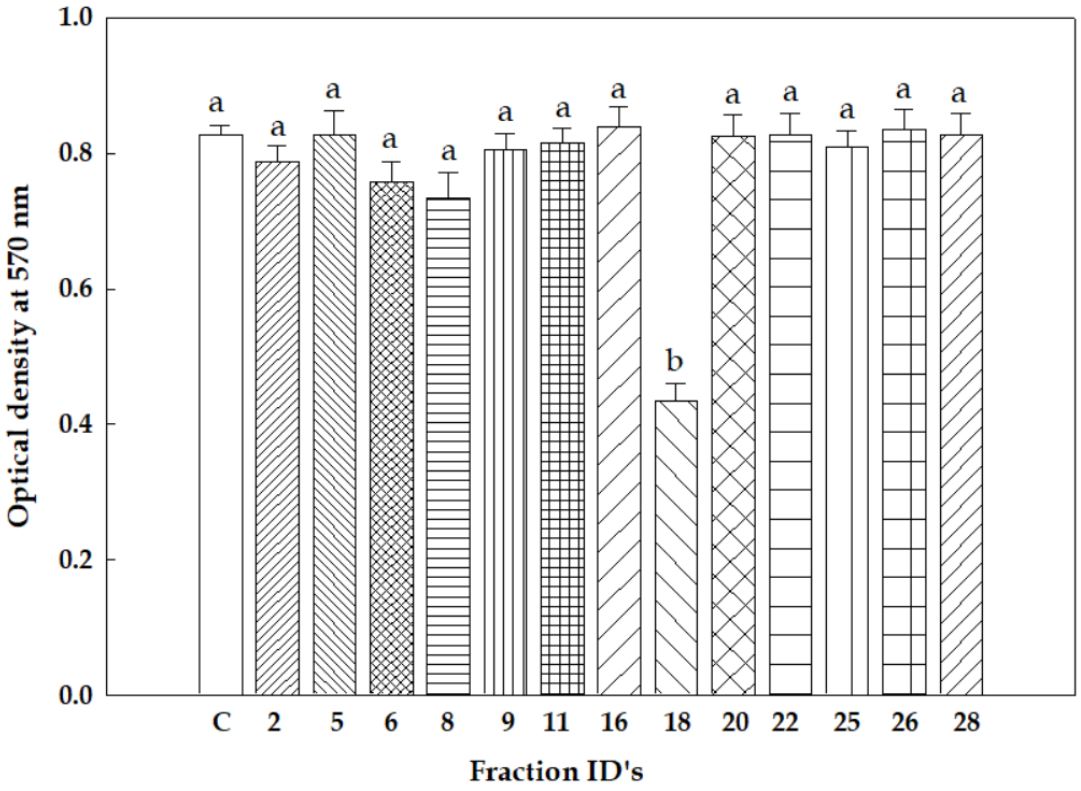


Figure – 4.8: Cytotoxicity activity of combined fractions of *C. laurifolius*. The values are expressed as mean ± SE of four replicates per experiment. Mean followed by different alphabets are significantly different at $p \leq 0.05$ by Student-Newman-Keul's multiple comparison test.

4.4. Mechanistic studies of the fractions

4.4.1. Cytotoxicity studies of Fraction ID – 18.

The fraction-18 was subjected to detailed cytotoxicity assay in order to ascertain the inhibitory concentration. The HeLa cells were exposed with different concentrations (0, 20, 40, 60, 80 & 100 µg/ml) of fraction – 18 for 24 Hrs and subjected to microscopic observations and MTT Assay. The microscopic observations showed a significant cytotoxic effect such as increased loss of microfilament structures and cellular blebbing with increase in doses of exposure (Figure – 4.9). A drastic decrease in the OD value was observed between 0 - 40 µg/ml and the IC50 was observed to be 35 µg/ml (Figure – 4.10) in MTT assay. The assay was carried out measuring the mitochondrial dehydrogenase activity in the treated cells. The NAD(P)H-dependent cellular dehydrogenase enzyme, under defined conditions, reflect the number of viable cells present. These enzymes are

capable of reducing the tetrazolium dye MTT 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide to its insoluble formazan, which has a purple colour.

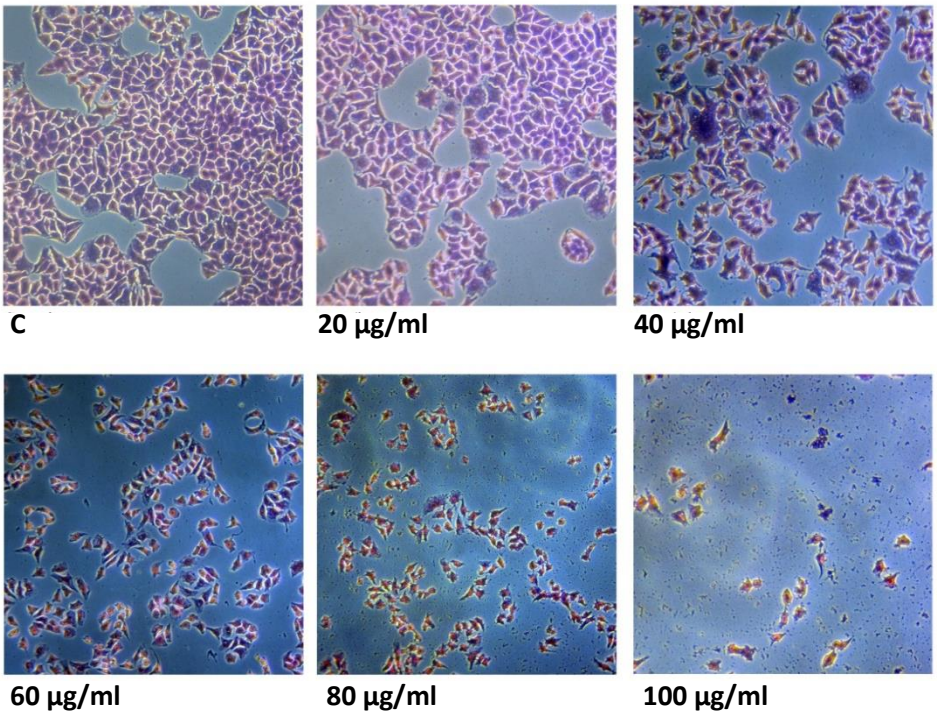


Figure – 4.9 – Cytotoxicity of the fraction - 18. The Human cervical cancer (HeLa) cells treated with different concentrations of fraction -18. The cells were stained with crystal violet dye and observed under light microscope (10X)

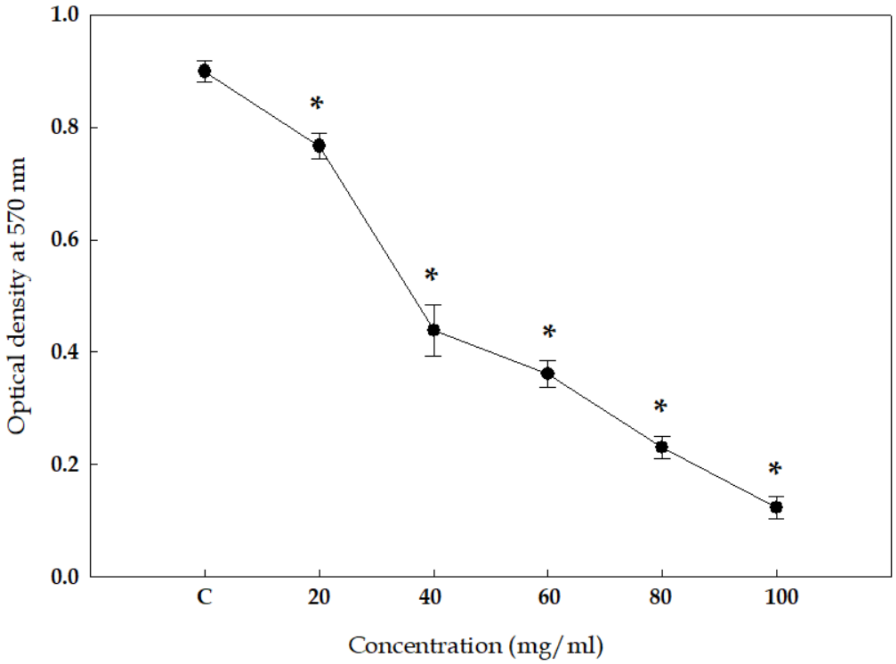


Figure – 4.10: Cytotoxicity assay of fraction -18 in Human cervical cancer (HeLa) cells. The values are expressed as mean ± SE of four replicates per experiment. * Significantly different from control at $p \leq 0.05$ by Dunnet's test.

MTT assay carried out in murine macrophage (RAW 264.7) cells also had a similar trend. The cells showed cell shrinkage, destruction of actin filaments and cellular structure (Figure – 4.11). The cells showed typical characteristic apoptotic morphology. However, the IC50 is found to be 200 µg/ml in RAW 264.7 (Figure – 4.12).

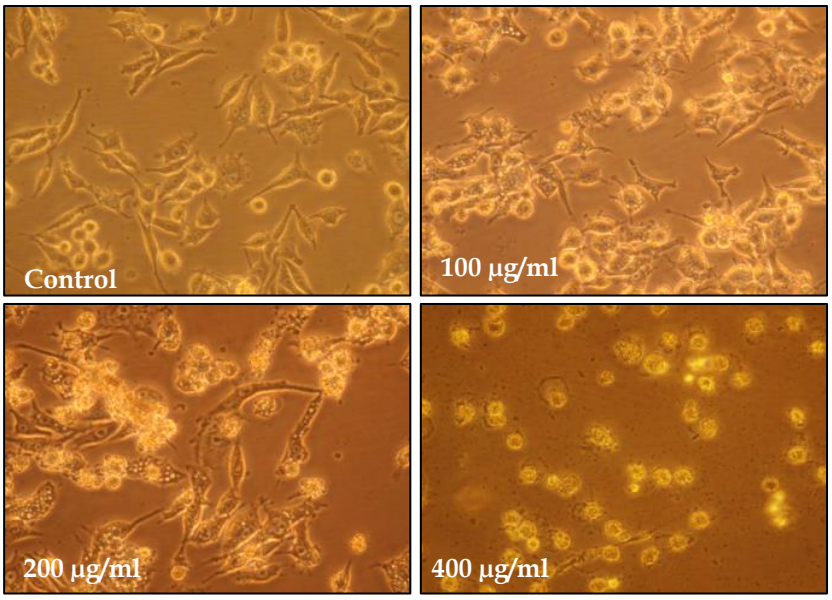


Figure – 4.11 – Cytotoxicity of the fraction - 18. The murine macrophage (RAW 264.7) cells treated with different concentrations of fraction -18. The cells observed under phase contract in microscope (10X)

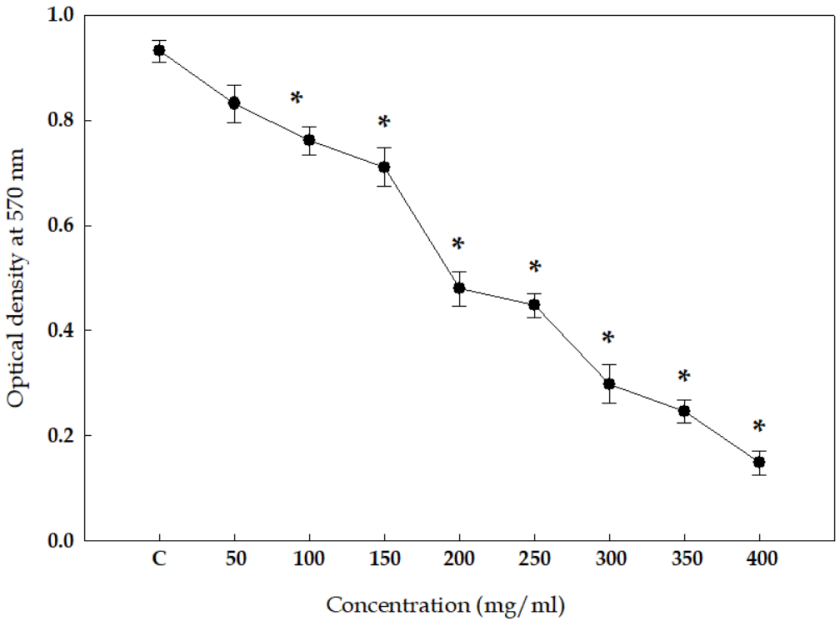


Figure – 4.12: Cytotoxicity assay of fraction -18 in murine macrophage (RAW 264.7) cells. The values are expressed as mean ± SE of four replicates per experiment. * Significantly different from control at $p \leq 0.05$ by Dunnet's test.

4.4.2. GCMS Analysis of Fraction ID – 18.

For qualitative identification of volatile molecules present in the fraction -18, GCMS analysis was carried out. Many prominent peaks were observed in the analysis and identified with library matches. Representative chromatogram obtained from the GC-MS analysis of Fraction ID-18 is given below (Figure - 4.13). The analysis confirmed the presence of 18 molecules as listed below; Phenol, 2,4 - Bis(1,1-Dimethylethyl; E-14-Hexadecenal, Nonadecane, E-15-Heptadecenal, 3-Hexadecanol, Isopropyl myristate, Eicosane, Hexadecanoic acid, methyl ester, 3-ethyl-3-undecanol, 1-octadecene, Furan, tetrahydro-2,2-dimethyl-5-(1-methylpropyl); 7-Hexadecenoic acid, methyl ester, (Z)- Methyl stearate, Docosane, Heptadecyl trifluoroacetate, Octadecyl trifluoroacetate; Docosyl trifluoroacetate and Solanesol. Many of these molecules are having bioactivities as detailed in Table - 4.7. 3-Hexadecanol (Cetyl alcohol) is used in the cosmetic industry as an opacifier or darkening agent in shampoos, or as a moisturizing agent, emulsifier or thickening agent in the manufacture of skin creams and lotions. Isopropyl myristate also reported to have the similar effects emulsifier or moisturizing agent. Furan is a precursor molecule in many of the chemical reactions. Solanesol is used as precursor in synthesis of high-value bio-chemicals such as Vitamin-K analogues and Co-enzyme Q10 (Co Q10). As starting material for Co Q 10, solanesol is useful in treatment of cancers. Co Q10 has been known to reduce size and number of tumours

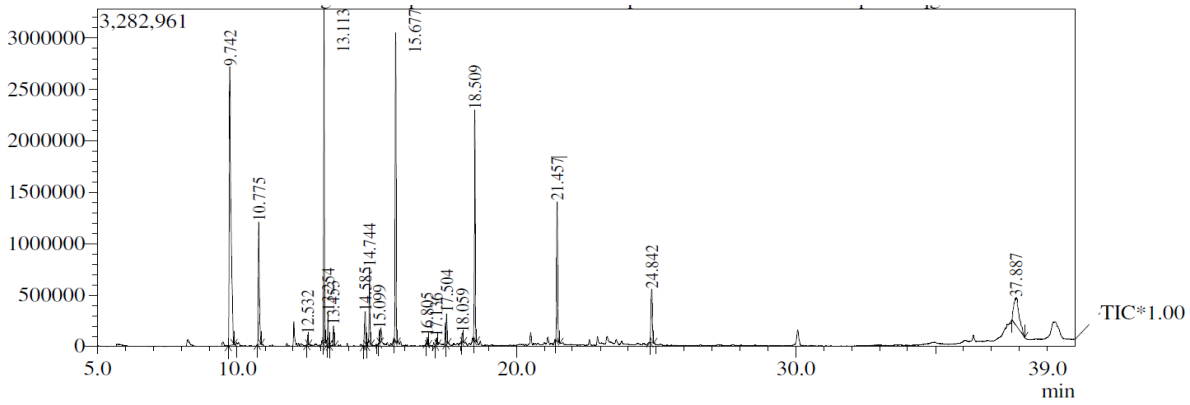


Figure – 4. 13 : The GCMS analysis of 18th fraction

Table – 4.7 : Phytochemicals identified from fraction ID-18 of *C. laurifolius* using GC-MS

No	Retention time	m/z ratio	Compound	Bioactivity
1.	9.742	191.20	Phenol, 2,4 -BIS(1,1-dimethylethyl	-
2.	0.775	55.05	E-14-Hexadecenal	-
3.	12.532	57.10	Nonadecane	-
4.	13.113	57.10	E-15-Heptadecenal	-
5.	13.254	59.05	3-Hexadecanol	Cetyl alcohol is used in the cosmetic industry as an opacifier in shampoos, or as an emollient, emulsifier or thickening agent in the manufacture of skin creams and lotions.
6.	13.453	60.05	Isopropyl myristate	Isopropyl Myristate is a synthetic oil used as an emollient, thickening agent, or lubricant in cosmetic products. It is easily absorbed by the skin, ensuring quick penetration of a formula's ingredients
7.	14.585	57.05	Eicosane	Icosane has little use in the petrochemical industry, as its high flash point makes it an inefficient fuel. *n*-Icosane (the straight-chain structural isomer of icosane) is the shortest compound found in paraffin waxes used to form candles.
8.	14.744	74.05	Hexadecanoic acid, methyl ester	-
9.	15.099	87.15	3-ethyl-3-undecanol	-
10.	15.677	57.10	1-Octadecene	-
11.	16.805	81.10	Furan, tetrahydro-2,2-dimethyl-5-(1-methylpropyl)	It is toxic and may be carcinogenic in humans. Furan is used as a starting point to other specialty chemicals.
12.	17.136	55.05	7-Hexadecenoic acid, methyl ester, (Z)-	-
13.	17.504	74.10	Methyl stearate	-
14.	18.059	57.10	Docosane	Docosane was used to investigate commercially available waxes in the form of thin disc samples as possible diffraction intensity standards for macromolecular crystallography synchrotron beamlines
15.	18.509	57.10	Heptadecyl trifluoroacetate	-
16.	21.457	57.10	Octadecyl trifluoroacetate	-
17.	24.842	57.05	Docosyl trifluoroacetate	-
18.	37.887	69.10	Solanesol	Solanesol is used as starting material in synthesis of high-value bio-chemicals such as Vitamin-K analogues and Co-enzyme Q10 (Co Q10). As starting material for Co Q 10, Solanesol is useful in treatment of cancers. Co Q10 has been known to reduce size and number of tumours

Some of the molecules identified from the fraction ID-18, have reported cytotoxic effects and are precursors of chemotherapeutic agents as detailed in the Table – 4.7. Though none of the molecules were reported to have pesticidal properties, our studies have indicated that the bark extracts of *C. laurifolius* showed significantly high insecticidal properties with high contact toxicity in *S. litura*.

DISCUSSION AND CONCLUSION

5. DISCUSSION AND CONCLUSION

Plants have important role in our lives due to the presence of unusual array of different classes of biomolecules with a variety of biological activities (Cotton, 1996). Plants are a rich source of chemical entities which can work against microbes, most of which are evolved as chemical defence against infection. Plants contain many phytochemical contents namely catechins, isocatechins, alkaloids, anthocyanin, isoflavones, flavonoids, tannins, saponins, lignins, phenols and coumarins. Due to the presence these molecules, plants exhibit strong biological activity including cytotoxicity, antimicrobial, antioxidant and pesticidal activities (Aqil et al., 2006).

Medicines from plants could be in the form of pure molecule with a strong and specific activity or could be in the form of crude preparations (extracts, tinctures, essential oils) containing a wide variety of compounds. In the present study, *C. laurifolius* - a plant used by tribal communities - was evaluated for biological activities. The preliminary phytochemical screening had shown the increased presence of alkaloids and terpenoids compared to other phytochemicals. High amount of alkaloids were observed in most of the extracts except in aqueous and petroleum ether extracts. Both bark and leaf extracts showed a similar trend. The plant is reported contain many alkaloids. Ten alkaloids of four different classes have been isolated from *C. laurifolius* leaves: erythrin (coculine, coculidine, coclaphine, coclafine hydrochloride), aporpfine (norisoboldine, isoboldine, talicmidine, and glaucine), *N*-oxidic (coculidine *N*-oxide (also erythrin type) and benzyltetraisoquinoline (coclaurine) (Tsakadze et al., 2005)

In addition, many other alkaloids were isolated from the roots of *C. laurifolius* including, α,α'-0-dioxo-70-O-demethylstebisimine, 70-O-demethylstebisimine, dihydroerysotrine, erythroculine, dihydroerysodine, dihydroerysovine, trilobine, isotrilobine, nortrilobine, O-methylthalmethine, tiliafunimine, stepharine, puertogaline B, stebisimine, (þ)-coclaurine and oblongine (Prelog et al., 1956; Cava et al., 1964; Barton et al., 1966; Johns et al., 1967; Millington et al., 1974; Juichi et al.,

1977; Wu et al., 1977; Ayim et al., 1977; Tackie et al., 1980; Ripperger et al., 1983; El-Kawi et al., 1984; Liu et al., 1996; Mahiou et al., 2000).

Though the isolation of phytochemicals form *C. laurifolius* were reported by various authors as mentioned above, scanty information is available on the bioactivity potential of this plant. In the present study, crude extracts were subjected to antioxidant evaluation and chloroform extract was found to be active among all the extracts. The trend was similar in both bark and leaf extracts. An earlier study with the bark chloroform extract showed significant DPPH free radical scavenging activity (Ajaib et al., 2017). The crude preparations of plants including extracts, tinctures or essential oils contains wide variety of compounds or pure molecules with a strong and specific activity. The high phenolic content in these extracts could be a factor for the antioxidant activity shown by them (Encalada et al., 2011). Plant secondary metabolites such as phenolics, flavonoids, and polyphenolics including condensed and hydrolysable tannins have shown potent antioxidant activities (Hagerman et al., 1998).

Cytotoxicity studies are a useful initial step in determining the potential toxicity of plant extracts or biologically active compounds isolated from plants. Many studies have confirmed that cytotoxic phytochemicals either induce apoptosis and necrosis or obstruct variety of cell-signaling pathways, thereby, leading to cell death or cell cycle arrest (Kishore et al., 2020). The initial screening of extracts had shown that bark chloroform extract is cytotoxic among others. After the fractionation, Fraction -18 is found be cytotoxic of all and morphologic studies in human cervical cancer cells showed typical symptoms of apoptotic mode of cell death including cell shrinking, membrane blebbing, destruction of actin filaments and cellular structure. Extracts from many plants had shown similar cytotoxic effects. Considering that over 60% of the anticancer drugs origins directly or through precursors from plants and most of them are cytotoxic phytochemicals, the evaluation of plants for cytotoxic compounds is very relevant.

Antimicrobial activity is another activity shown by many plant extracts. The antimicrobial activities of plant species have been widely researched, crude extracts of cinnamon, garlic, basil, curry, ginger, sage, mustard, and other herbs exhibit antimicrobial properties against a wide range of Gram-positive and Gram-negative bacteria (Alzoreky and Nakahara, 2003; Castro et al., 2008). It has been reported that the extracts from Chinese chives and cassia can effectively reduce the growth of *Escherichia coli* and other bacteria during storage of meat, juices, and milk (Mau et al., 2001). In the present study, the bark chloroform extract had shown potential antimicrobial activity against *Streptococcus aureus* and *Klebsiella pneumoniae*. A recent study with leaf and bark extracts of *C. laurifolius* showed significant to average inhibition against bacterial and fungal strains. Chloroform and methanol extracts of showed a maximum zone of inhibition against *S. aureus* (Ajaib et al., 2017). Phytochemicals with hydroxylated phenols, shown to be toxic to microorganisms. The site(s) and number of hydroxyl groups on the phenol group are thought to be related to their relative toxicity to microorganisms (Thomson, 1978). Cinnamic and caffeic acids are common representatives of a wide group of phenylpropane-derived compounds which are in the highest oxidation state and activity (Wild, 1994).

The folklore use of higher terrestrial plants by the natives of various parts of the world for pesticidal activities has been well known (Dalziel, 1937; Ayensu, 1978). Many plants such as tobacco (*Nicotiana tabacum*), Japanese plant, Roh-ten (*Rhododendron hortense*), *Chrysanthemum* and *Pyrethrum* were found to have pesticidal activities in the early days itself (Ayensu, 1978; Tooley, 1971; David, 2005). The pesticidal agents include insecticides (insect killers including toxicity to adults, ova, and larvae) insect repellents, antifeedants, molluscicides, fungicides and phytotoxins (herbicides). The methanolic extract of both leaf and bark extracts of *C. laurifolius* showed antifeedant, repellent activities and contact toxicity at varying degrees. The bark extract is found to be more active as antifeedant and repellent agents with significant contact toxicity. Earlier reports confirmed that herbal preparations or crude extracts can preserve the constituents to enhance synergism

and potency. Hence always preferred over pure molecules. However further bioassay guided fractionation can be used to obtain active compounds from plants (Mitscher, 1987).

The results of phytochemical screenings and bioactivity studies of a number of higher plants based on traditional knowledge strongly indicate that plants are endowed with various bioactivities including cytotoxicity, antioxidant and pesticidal properties that can be harnessed cost-effectively for the use of mankind as well as in agriculture related fields. The present study had brought out the cytotoxic, anti-microbial and pesticidal properties of *C. laurifolius*. Among this the cytotoxicity and pesticidal properties are reported for the first time. Isolation of the fraction with biological activity is a lead in separating the molecule with bioactive potential. Exploring the pesticidal properties of crude extract and developing appropriate formulations may help in developing cost-effective technologies for pest control in agriculture and related fields.

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6. BIBLIOGRAPHY

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