IN VITRO AND *IN VIVO* INVESTIGATION OF ANTI-CANCER ACTIVITY OF *ILEX KHASIANA* AND HPTLC FINGERPRINT PROFILING

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In partial fulfillment of the requirement for the degree of Doctor of Philosophy in Zoology of Mizoram University, Aizawl.

CERTIFICATE

I certify that the thesis entitled "***In vitro* and *in vivo* investigation of anti-cancer activity of *Ilex khasiana* and HPTLC fingerprint profiling**" submitted to the Mizoram University for the award of the degree of Doctor of Philosophy in Zoology Department by **Charles Lalnunfela** is a record of research work carried out during the period of 2016 – 2022 under my guidance and supervision, and that this work has not formed the basis for the award of any degree, diploma, associateship, fellowship or other titles in this University or any other University or Institution of higher learning.

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DECLARATION

I, **Charles Lalnunfela**, hereby declare that the subject matter of this thesis is the record of work done by me, that the contents of this thesis did not form the basis of the award of any previous degree to me or to do the best of my knowledge to anybody else, and that the thesis has not been submitted by me for any other University or Institute.

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KA LAWM E.

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CHAPTER – 1

GENERAL INTRODUCTION

The interdisciplinary science that deals with scientific exploration of bioactive compounds from a traditionally employed medicinal plants is known as Ethnopharmacology. Therefore, it has now become a new strategy for a pharmaceutical company to adopt a new perspective in their quests for new and potential sources of drug development. Thus, ethnopharmacology plays a vital role for the discovery and development of safe and inexpensive novel medicine **(Suntar, 2019)**.

The field of natural product chemistry made a giant leap since Serturner isolated morphine from opium in 1803 which was followed by isolation of novel compounds like quinine (*Cinchona officinalis* L.), emetine (*Carapichea ipecacuanha* (Brot.) L. Andersson), strychnine (*Strychnos nux vomica* L.), atropine (*Atropa belladonna* L.), colchicine (*Colchicum autumnale* L.), papaverine (*Papaver somniferum* L.), and salicin (*Salix* ssp.) **(Der Marderosian and Beutler, 2002; Allen and Hatfield, 2004; Siddiqui et al., 2014)**.

Traditional medicines contributed greatly into the discovery of modern drugs but the bioassay guided fractionation demanded a lot of time-consuming work which depends on the availability of convenient assay **(Cheng et al., 2006).** Fortunately, with the advancement in fractionation techniques for the isolation and purification of bioactive compounds both in chromatographic and analytical technique screening of bioactive compounds become more compatible with lower timescale and high-throughput results **(Wu et al., 2008; Harvey, 2007)**. With all these advancements, isolation and structure elucidation of bioactive compounds can be done within a short span of less than two weeks and using NMR techniques, less than 1 mg of compound is now sufficient to solve a complex structure **(Singh et al., 2006)**.

Structure elucidation of naturally derived compounds even when not employed directly served as a base structure for the development of new or improved drugs. This contributed enormously in organic chemistry for obtaining advanced synthetic methodology and obtaining analogues of the original compounds with improved pharmaceutical and pharmacological properties **(Newman, 2008; Sunazuka et al., 2008)**. These analogues or derivatives of natural compounds can be used to create a patentable novel drug with the application of new techniques while disclosing the original structure. This knowledge also helps in adapting naturally occurring antibiotics to produce more complex products. With the oxidative modification carried out by cytochrome P450 enzymes, macrocyclic compounds related to daptomycin and analogues of vancomycin and anti-cancer compound cryptophycin have been created using 'Mutasynthetic' method **(Kennedy, 2008; Lamb et al., 2007)**

Besides providing a foundation for novel drugs, natural products played a significant role in physiology. Bioactive compounds will be useful to study binding interaction with isolated proteins or serves as an inhibitory molecule in biochemical processes as the role of sodium-potassium- ATPase were discovered from digitalis (foxglove), tubocurarine, muscarine and nicotine revealed the different types of acetylcholine receptors **(Ganesan, 2008; Rishton, 2008; Stockwell, 2004)**.

Unfortunately, due to rapid industrialization, loss of ethnic culture, risk of extinction and endemic nature of plants, there is a decline in the knowledge of traditional plants and practices **(Gencler Ozkan and Koyuncu, 2005)**. Under this circumstances, production of semisynthetic and synthetic compounds became very prominent in the twentieth century. But, synthesis of compounds like vinca alkaloids, podophyllotoxin, taxol, etc. having molecular weight of more than 2650 Da and multiple chiral centers requires multiple steps and very expensive compared to natural product isolated from plants **(Beutler, 2009)**. Therefore, more than 60% of all the medicines in the industrialized nations are either natural products or secondary metabolites of a medicinal plants **(Eddershaw et al., 2004)**.

Structural diversity of bioactive compounds plays a vital role in developing semi-synthetic drugs, a hybrid of natural and synthetic sources such as antibiotic penicillin **(Oshiro, 1999)** and paclitaxel (an anticancer drug derived from the Pacific yew tree, *Taxus brevifolia*) **(Lahlou, 2013)**. Natural compounds serve as a starting materials and synthetic compound was employed to give a desirable end product through series of chemical reaction **(Cragg et al., 2013)**.

Naturally originated compounds play a vital role in drug discovery as well as in development. Between the year 1981 and 2016, 73% of the drugs available in the market are of natural origin and the rest are of synthetic origin. Drugs with natural origin can be categorized broadly into three groups: (a) unaltered natural products (b) defined mixture of natural products and natural product derivatives isolated from plants or other microorganisms like sponges, lichens, fungi (c) products altered by medicinal chemistry. Therefore, among the 1328 approved new drug entities (between 1981and 2016), 549 were derived from natural compounds, 326 were of biological entities (including therapeutic antibodies) and 94 were vaccines. In cancer therapy, among 136 approved anticancer drug (1981-2014), 23 drugs were synthetically obtained **(Newman and Cragg, 2016)**. Moreover, the natural compound structures serve as a starting material for many new drugs using fragment-based drug discovery approach **(Mortenson et al., 2018).** In 2018, among the molecules that are derived from natural compounds, plants (44.1%) contributed the highest followed by marine organisms (13.2%), microorganisms (12.9%), fungi and lichens (9.3%) and animals (2.6%) respectively **(Lautie et al., 2020).**

Drugs of natural origin accounted for one third of the drugs available in the clinic. Among which colchicine, morphine, semi-synthetic aspirin, taxol, penicillin, tetracyclines artesunate from artemisinin are the notable ones that are derived directly isolated, synthesized or semi-synthesized from the natural compound by structural alteration **(Sticher, 2014; Cragg et al., 2014)**. Surprisingly, WHO recommended falciparum malarial drug artesunate is also known to have both *in vitro* and *in vivo*

anticancer activity **(Krusche et al., 2013)**. The remarkable point is that in many cases the source extracts or materials have a superior activity over a single isolated compound which calls for the new approach in screening the natural product to consider the synergistic effects as well **(Verpoorte et al., 2005)**. Pathophysiological nature of many diseases is multifactorial and the treatment requires multitarget therapy, thus it is believed that drug combination plays a vital role in treating complex disease like cancer and HIV which also avoid drug resistance **(Zimmermann et al., 2007).**

Combination of synthetic drugs with a complex natural product is expected to have a synergistic effect to give positive or antagonistic result **(Peterson and Novick, 2007)**. Gene expression profile demonstrated that such combination has stable and reproducible output **(Panossian et al., 2013)**. This combined therapeutic agents that have a successful input in Germany. Multitargeting drugs like STW5, a combination of 9 plant extract for the treatment of bowel problem and Sinupret (consists of 5 plants extracts) which is used for the treatment of common cough and cold are the two prominent drugs with multiple combinations **(Ottillinger et al., 2013).** The combination of medicinal plants is known to have higher potency with lower dosage due to synergistic effects of the constituents. It also reduces the adverse effect of phytomedicines due to lower drug concentrations. Multiple drug combinations have several notable advantages like one drug (example: saponins) helping in transportation that enhance the other drug bioavailability, also enhancing permeability that divert the multi drug resistance mechanism in autoimmune disease or cancer. With all the complexities demanded by the synergistic effects of combination of synthetic and phytomedicines, its potential is still undeniable in the development of future medicine **(Ulrich-Merzenich, 2014)**.

In the presence of allergen, tissue injury, toxic substance and pathogenic invasion into human physiological system, the body reaction towards this hostility is called inflammation. Many cytokines are produced by immune cells as well as non-immune injured cells. It involves interaction of many antigen presenting cells or APCs,

lymphocytes as well as monocytes that differentiated in macrophages (**Latruffe, 2017).** Many *Ilex* species are known for their medicinal properties. *I. latifolia* and *I. kudingcha* are Chinese traditional medicines used for the treatment of swelling, pain, fever and diarrhea **(Yi et al., 2016)**. *I. paraguariensis, I. vomitoria, I. glabra* and *I. guayusa* are all reported to have antioxidant, anticancer and anti-inflammatory activities. The bioactive compounds found in many of the *Ilex* species are caffeoylquinic acids like chlorogenic acid as well as flavanols like quercetin and kaempferol and their glycosides which are believed to be associated with the medicinal properties of the genus *Ilex* **(Norato et al., 2011).**

More than 70% of the global total death is due to noncommunicable disease (NCDs) which is 63% in India and 9% of which is caused by cancer alone **(World Health Organization, 2018)**. Since 1982, India has a well-structured data from the population-based cancer registries (PBCRs) and hospital-based cancer registries (HBCRs) under the National Cancer Registry Programme (NCRP)–National Centre for Disease Informatics and Research (NCDIR) of the Indian Council of Medical Research (ICMR; ICMR-NCDIR-NCRP), Bengaluru. For PBCRs, the country was divided into six geographical zone - North (Delhi, Patiala), South (Hyderabad, Kollam, Thiruvananthapuram, Bangalore, and Chennai), East (Kolkata), West (Ahmedabad urban, Aurangabad, Osmanabad and Beed, Barshi rural, Mumbai, and Pune), Central (Wardha, Bhopal, and Nagpur) and Northeast (NE; Manipur, Mizoram, Sikkim, Tripura, West Arunachal, Meghalaya, Nagaland, Pasighat, Cachar, Dibrugarh,and Kamrup urban). Age adjusted rate (AAR) per 100,000 population using world standard population method was used for the study in which Aizawl district had the highest AAR (269.4) and mortality (152.7) rate among males. According to the findings, Aizawl and other NE regions one fourth of every male between the age of 0-74 years are prone to develop cancer at a particular point of time. Lung, breast, cervix, liver and stomach ulcer are the cancer having high incident rates in Northeastern part of India **(Mathur et al., 2020)**.

ILEX SPECIES – AN OVERVIEW ON ITS ETHNOPHARMACOLOGICAL PROPERTIES

Ilex, the largest genus of the family Aquifoliaceae are dioecious, evergreen trees or sometimes shrubs without not forming a large homogeneous population. They inhabit the tropical and temperate regions of the world consisting of about 600 species **(Hu, 1989)**. South East Asia and South America host majority of the species with only a few found in Indonesia, New Caledonia, North America and Central part of Pacific Islands. This disjunct pattern of distribution might be due to the extinction of *Ilex* species in Australia, Africa and central Eurasia. The distribution of *Ilex* species has two hypotheses. First one is based on continental drift theory, which states that *Ilex* has Gondwanic origin with living species in South America and South Eastern Asia **(Raven and Axelrod, 1974).** Second hypothesis suggested the first colonization location of *Ilex* to be South Eastern Asia **(Hu, 1967)**. Being a dioecious plant, the exact total number of species under *Ilex* genus is still uncertain probably due to misidentification and duplication of names **(Giberti, 1990).** Therefore, a new species report has been found from Peru, Colombia and Panama **(Hahn, 1996).** Sequencing of chloroplastic atpB-rbcl intergene spacer and rbcL gene is used for the analysis of *Ilex* phylogeny **(Manem et al., 1998)**.

According to many scientific reports the most common phytochemical constituents of the genus *Ilex* is saponins **(Ouyang et al., 1996; Taketa et al., 2000; Ouyang et al., 1998; Pires et al., 1997)**. Other reports cited the presence of important bioactive compounds like aldehydes **(Wen et al., 1996)**, flavonoids **(Martinez et al., 1997)**, pentyl esters, hexyl esters, other lipophilic compounds **(Van Genderen et al., 1988)**, alkanes, triterpenes **(Van Genderen et al., 1990)**, hemiterpene glycosides **(Jian et al., 2005; Fuchino et al., 1997)**, anthocyanins **(Ishikura, 1975)**. *Ilex* species like *I. crentata, I. cornuta, I. opaca* and *I. aquifolium* are commonly known as "Hollies", which are used as Christmas trees as well as for decoration purposes **(Hu, 1989)**. Some *Ilex* species play a huge role in serving as a beverage in many parts of the world,

I. paraguariensis (mate tree) is used for making the mate, a drink with high theobromine and caffeine content **(Filip et al., 1998)**. Apart from these polyphenolic compounds, yerba mate is also known to contain purine alkaloids like 3,4-dicaffeoylquinic acid, 3,5- dicaffeoylquinic acid and caffeic acid, polysaccharides, proteins, minerals (P, Fe, Ca, and Al), vitamins (C, B1, and B2) and flavonoids (rutin, quercetin, and kaempferol) **(Gullón et al., 2018).** This report suggested that yerba mate (*I. paraguariensis*) is a potent dietary source of antioxidants to combat free radicals in the system besides its hepatoprotective, central nervous system stimulant, diuretic and hypocholesterolemic properties. It aids the cardiovascular system, as it inhibits DNA oxidation, *in vitro* proliferation of colon cancer cells and lipid peroxidation of low-density protein **(de Mejía et al., 2010; Heck and de Mejía, 2007).** *I. tarapotina* and *I. vomitoria* are also known to contribute in stimulatory beverages **(Loizeau, 1994).**

Ilex species have a long history as a traditional medicine used in different parts of the globe. The medicinal properties of *Ilex* species are due to its wide range of bioactive compounds, that varies from species to species. In South America, *I. paraguariensis* (mate) is a source of a well-known stimulatory beverage which possess both anti-inflammatory and diuretic properties **(Kraemer et al., 1996).** *I. kudincha* is another famous beverage that is known to have diuretic, hypersensitive and CNS stimulant properties and is used for the treatment of sore throat and weight loss. Similarly, *I. cornuta* has been used for weight control and fertility besides its known curative effect against dizziness and hypertension. *I. latifolia* also has a long list of medicinal properties against toothache, hypertension, blood shot eyes and tinnitus **(Kothiyal et al., 2012)**. In China, root of *I. pubescens* have been employed for the treatment of coronary disease, myocardial infarction, hypercholesterolemia, Buerger's disease and cardiovascular **disease (Dictionary of Chinese Medicine, 1975)**. *I. rotunda* has a unique property for the treatment of snake bite apart from its wide application for treating burns, scalds, bleeding control, common cold, tonsilitis, intestinal ulcer, pyrexia and stomach ulcer **(Dictionary of Chinese Materia Medica, 1977; The Color Atlas**

of Chinese Herbs, 1987). In the Guangxi Province of China, *I. oblonga* has been used for the treatment of eczema, rheumatism, bruise and gumboil **(Zhu, 1973)**.

Ilex species namely *I. paraguariensis, I. breviscuspis, I. pseudobuxus, I. argentina, I. dumosa and I. theezans* were analyzed for its antioxidant activity, among them *I. paraguariensis* is known to possess the best antioxidant activity **(Filip et al., 2000)**. The presence of bioactive compounds like caffeoyl-derivatives, rutine, quercetin and kaempferol might be responsible for the plant antioxidant activity **(Ricco et al., 1995).** The presence of these bioactive compounds showed prominent variation depending upon the distribution of the genus. In the study of flavonoid distribution in 59 *Ilex* species, two flavones, apigenin and luteolin, and three flavanols namely isorhamnetin, quercetin and kaempferol were found. Among these flavones and flavonols, luteolin (*I. colchica*) and isorhamnetin made a new addition **(Ricco et al., 1995; Alikaridis, 1987).** *I. leucoclada* from Japan and *I. belizensis* from Guatemala were the only species containing flavones among the 59 species. Thus, difference in biogeographical distribution showed difference in aglycones accumulation. Likewise, Isorhamnetin frequency is found to be lesser in Asiatic region than in American region. *I. mitis* from Africa has quercetin but no isorhamnetin while Central American species has lesser kaempferol frequency. Due to this sporadic occurrence of flavones, it might be an interesting marker in determining the relationship between allied species **(Martinez et al., 1997)**.

The latest report on *Ilex* species showed that only 6% of the known species have been studies for its medicinal, ornamental, beverages and timber values which was 38 species of 669 known species **(Yao and Corlett, 2022)**. A full genome report of 727.10 Mb in length approximately was done on *Ilex polyneura* **(Yao et al., 2022).** China has the largest reports on Ilex species with South America being an enormous contributor on the chemistry and pharmacology of *Ilex* species. Many bioactive compounds were isolated from all parts of the plants including the fruits, bark, leaves and roots in which *I. pubescens* alone contributed 200 identified compounds **(Jiang et al., 2019).**

I. paraguariensis, I. breviscuspis, I. chinensis, I. rotunda, I. asprella and *I. hainanensis* are a few species that are well documented and major contributors on more than 172 patents made on drug-based products from *Ilex* species **(Chen et al., 2019).** The pharmacological properties of these species showed the antitumor, antimicrobial activity, protection of cardiovascular system and regulation of lipid metabolism **(Noureddine et al., 2018).** In spite of all these advances, the exploration of other species for its medicinal values and understanding the mechanism of the bioactive compounds is still a huge challenge **(Jiang et al., 2019).**

ILEX KHASIANA – ABOUT THE PLANT

Species of Ilex are well known in traditional Chinese medicine and have served as botanical sources of several compounds of health benefits **(Hao et al., 2013)**. *I. khasiana* is recorded as a holy species endemic to the Khasi Hills of Meghalaya, India, and classified as critically endangered under the IUCN Red List of Threatened Species **(IUCN Red List, 1998)**. It is an evergreen tree with an average height of 15–20 m and forming the sub-canopy in the humid subtropical forests at an elevation up to 1990 m above the sea level **(Haridasan and Rao, 1985)**. The tree starts flowering in summer during April-May, and fruits develop in winter during November-December. The fruits are purplish red with a size of 7–8 mm and the seeds, which are 3 mm long, are obovoid-ellipsoid or ellipsoid **(Adhikari et al., 2012)**. The aerial plant parts (mainly the fruit) serve as fodder for wild animals like palm civets, squirrels, and birds. Among the Khasi people, the bark and root decoction are used in the treatment of tuberculosis and severe cold **(Laloo et al., 2006)**. The species has also been identified from a localized area in Aizawl, Mizoram. Among the Mizo traditional healers, it is known as a good medicine but the exact ailment to which the plant is used remains unknown **(Sawmliana, 2013)**.

Taxonomic classification of the plant:

Kingdom	Plantae
Phylum	Tracheophyta
Class	Magnoliopsida
Order	Aquifoliales
Family	Aquifoliaceae
Genus	*Ilex*
Species	*khasiana*
Binomial name: *Ilex khasiana*	

Collection of plant:

Ilex khasiana is available only at one area in Mizoram, India, as naturally propagated tree at Luangmual, Aizawl (location **23°44.556'N** and **92°41.956'E**).

Authentication of the plant:

The plant specimen was authenticated at the Botanical Survey of India, Eastern Circle, Shillong, Meghalaya. The herbarium is catalogue with an accession number **BSI/EC/Tech./2008/577** in the Department of Pharmacy, Regional Institute of Paramedical and Nursing Sciences, Zemabawk, Mizoram, India.

Extraction of the plant:

Extraction of active compounds include pretreatment approaches like chemical, biological and mechanical processes for a better lignin removal, generation of toxic compounds, lignin structure alteration, increase accessible surface area and decrystallization of cellulose **(Zhao et al., 2020)**. Thus, grinding is properly done for size reduction to gain larger surface area and Soxhlet extraction method was employed to get higher and better yields using appropriate solvents **(Mohammad Azmin et al., 2016).** The collected leaves were washed and air dried in shade at ambient temperature

(23–25°C). Air drying method was used over forced drying the plant material at high temperature to preserve heat-labile compounds. The dried leaves were ground into a powdered form using electric grinder. This method increased the surface contact of the sample with selected extraction solvents. These ground samples were loaded in Soxhlet apparatus (5 liters) and cotton pads were placed on both the bottom and upper side of the tube to avoid dispersion of the sample along the process. Extraction solvent was poured on the sample until it overflowed and sufficient amount was collected in the round bottom flask for the extraction cycles. Petroleum ether, chloroform and methanol were used as the extraction solvent respectively. This method separated the bioactive compounds that was soluble in that particular solvent and the extraction was maintained using a condenser for 72 hours each. The crude extract was concentrated by evaporating and recovering the solvent in a rotary vacuum evaporator (Buchi Rotavapor® R-215). The initial extracts obtained were a mixture of phytochemicals like alkaloids, glycosides, phenolics, terpenoids, flavonoids, etc. **(Azwanida, 2015).**

The three main extracts namely *Ilex khasiana* Pet ether extract (IKP), *Ilex khasiana* Chloroform extract (IKC) and *Ilex khasiana* Methanol extract (IKM) were used for further analysis.

Therefore, now it is obvious that *Ilex* species have possessed a good and remarkable bioactive compound that are widely used in pharmaceutical developments. Unfortunately, *Ilex khasiana* a critically endangered species did not get enough attention and not much studies have been done on this particular holly plant. So, this dissertation will focus on an in-depth study on *I. khasiana* by following the objectives mentioned below:

1) Collection, extraction and HPTLC fingerprinting of *I. khasiana*.

2) Study of the *in vitro* and *in vivo* anticancer activity.

3) Evaluation of antioxidant and anti-inflammatory activity.

4) Study of anti-microbial activity.

Figure 1.1: Photograph of *Ilex khasiana* from its natural habitat.

REFERENCES

Adhikari, D., Barik, S. K., & Upadhaya, K. (2012). Habitat distribution modelling for reintroduction of *Ilex khasiana* Purk., a critically endangered tree species of northeastern India. Ecological Engineering, 40, 37-43.

Alikaridis, F. (1987). Natural constituents of *Ilex* species. Journal of Ethnopharmacology, 20(2), 121-144.

Allen, D.E., Hatfield, G. (2004) Medicinal plants in folk tradition: an ethnobotany of Britain and Ireland. Timber Press, Cambridge, p 431.

Azwanida NN (2015). A review on the extraction methods used in medicinal plants, principle, strength and limitation. Medicinal and Aromatic Plants. 4(196):2167-0412.

Beutler, J.A. (2009) Natural products as a foundation for drug discovery. Curr Protoc Pharmacol 46:1–9.

CAMP Workshops on Medicinal Plants, India. *Ilex khasiana*. The IUCN Red List of Threatened Species 1998: e.T31239A9618655.

Chen, W., Zhou, L., Qiao, Y., Qi, J., Fu, G., & Xia, X. (2019). Quality Evaluation of *Ilex asprella* Based on Simultaneous Determination of Five Bioactive Components, Chlorogenic Acid, Luteoloside, Quercitrin, Quercetin, and Kaempferol, Using UPLC–Q-TOF MS Study. Journal of AOAC International, 102(5), 1414-1422.

Cheng, Y., Wang, Y., & Wang, X. (2006). A causal relationship discovery-based approach to identifying active components of herbal medicine. Computational biology and chemistry, 30(2), 148-154.

Ching Su New Medical College (ed.), Dictionary of Chinese Materia Medica; Shanghai Scientific Technology Publishers: Shanghai, 1977, 2096.

Cragg, G. M., & Newman, D. J. (2013). Natural products: a continuing source of novel drug leads. Biochimica et Biophysica Acta (BBA)-General Subjects, 1830(6), 3670-3695.

Cragg, G. M., Grothaus, P. G., & Newman, D. J. (2014). New horizons for old drugs and drug leads. Journal of natural products, 77(3), 703-723.

de Mejía, E. G., Song, Y. S., Heck, C. I., & Ramírez-Mares, M. (2010). Yerba mate tea (*Ilex paraguariensis*): Phenolics, antioxidant capacity and in vitro inhibition of colon cancer cell proliferation. Journal of Functional Foods, 2(1), 23-34.

Der Marderosian, A., Beutler, J.A. (2002) The review of natural products, 2nd edn. Facts and Comparision, Seattle, pp 13–43.

Eddershaw, P. J., Beresford, A. P., & Bayliss, M. K. (2000). ADME/PK as part of a rational approach to drug discovery. Drug discovery today, 5(9), 409-414.

Filip, R., Lopez, P., Coussio, J., & Ferraro, G. (1998). Mate substitutes or adulterants: study of xanthine content. Phytotherapy Research: An International Journal Devoted to Pharmacological and Toxicological Evaluation of Natural Product Derivatives, 12(2), 129-131.

Filip, R., Lotito, S. B., Ferraro, G., & Fraga, C. G. (2000). Antioxidant activity of *Ilex paraguariensis* and related species. Nutrition research, 20(10), 1437-1446.

Fuchino, H., Tachibana, H., & Tanaka, N. (1997). Three new hemiterpene glycosides from Ilex macropoda. Chemical and pharmaceutical bulletin, 45(9), 1533-1535.

Ganesan, A. (2008). The impact of natural products upon modern drug discovery. Current opinion in chemical biology, 12(3), 306-317.

Gencler Ozkan, A.M., Koyuncu, M. (2005) Traditional medicinal plants used in Pinarbasi area (Kayseri-Turkey). Turk J Pharm Sci 2:63–82.

Gencler Ozkan, A.M., Koyuncu, M. (2005). Traditional medicinal plants used in Pinarbasi area (Kayseri-Turkey). Turk J Pharm Sci 2:63–82.

Giberti, G. (1990). Los parientes silvestres de la yerba mate y el problema de su adulteración. Dominguezia, 7(1), 3-22.

Gullón, B., Eibes, G., Moreira, M. T., Herrera, R., Labidi, J., & Gullón, P. (2018). Yerba mate waste: A sustainable resource of antioxidant compounds. Industrial Crops and Products, 113, 398-405.

Hahn, W. J. (1996). Notes on Costa Rican Aquifoliaceae. Novon, 181-184.

Hao, D., Gu, X., Xiao, P., Liang, Z., Xu, L., & Peng, Y. (2013). Research progress in the phytochemistry and biology of *Ilex* pharmaceutical resources. Acta Pharmaceutica Sinica B, 3(1), 8-19.

Haridasan, K., & Rao, R. R. (1985). Forest flora of Meghalaya. Dehra Dun India.

Harvey, A. L. (2007). Natural products as a screening resource. Current opinion in chemical biology, 11(5), 480-484.

Harvey, A. L. (2008). Natural products in drug discovery. Drug discovery today, 13(19-20), 894-901.

Heck, C. I., & De Mejia, E. G. (2007). Yerba Mate Tea (*Ilex paraguariensis*): a comprehensive review on chemistry, health implications, and technological considerations. Journal of food science, 72(9), R138-R151.

Hu, C.Y. (1989). Holly (*Ilex* spp.). In: Biotechnology in Agriculture and Forestry. 5, Trees II Y.P.S Bajaj, Ed. Springer, Verlag, Berlin ; 412-427.

Hu, S. Y. (1967). The Evolution and Distribution of the Species of Aquifoliaceae in the Pacific Area (1) & (2).

Ishikura, N. (1975). A further survey of anthocyanins and other phenolics in *Ilex* and *Euonymus*. Phytochemistry, 14(3), 743-745.

Jang Su Medical College, Dictionary of Chinese Medicine, Shanghai People's Press: Shanghai, 1975, pp 441-443, 1288, 1521-1522.

Jiang, S., Cui, H., Wu, P., Liu, Z., & Zhao, Z. (2019). Botany, traditional uses, phytochemistry, pharmacology and toxicology of *Ilex pubescens* Hook et Arn. Journal of ethnopharmacology, 245, 112147.

Jiang, Z. H., Wang, J. R., Li, M., Liu, Z. Q., Chau, K. Y., Zhao, C., & Liu, L. (2005). Hemiterpene Glucosides with Anti-Platelet Aggregation Activities from *Ilex pubescens*. Journal of natural products, 68(3), 397-399.

Kennedy, J. (2008). Mutasynthesis, chemobiosynthesis, and back to semi-synthesis: combining synthetic chemistry and biosynthetic engineering for diversifying natural products. Natural product reports, 25(1), 25-34.

Kothiyal, S., C Sati, S., SM Rawat, M., D Sati, M., K Semwal, D., B Semwal, R., & Kumar, A. (2012). Chemical constituents and biological significance of the genus *Ilex* (Aquifoliaceae). The Natural Products Journal, 2(3), 212-224.

Kraemer, K. H., Taketa, A. T., Schenkel, E. P., Gosmann, G., & Guillaume, D. (1996). Matesaponin 5, a highly polar saponin from *Ilex paraguariensis*. Phytochemistry, 42(4), 1119-1122.

Krusche, B., Arend, J., & Efferth, T. (2013). Synergistic inhibition of angiogenesis by artesunate and captopril in vitro and in vivo. Evidence-Based Complementary and Alternative Medicine, 2013.

Lahlou M: The Success of Natural Products in Drug Discovery. Pharmacol Pharm 4: 17-31, 2013.

Laloo, R. C., Kharlukhi, L., Jeeva, S., & Mishra, B. P. (2006). Status of medicinal plants in the disturbed and the undisturbed sacred forests of Meghalaya, northeast India:

population structure and regeneration efficacy of some important species. Current science, 225-232.

Lamb, D. C., Waterman, M. R., Kelly, S. L., & Guengerich, F. P. (2007). Cytochromes P450 and drug discovery. Current opinion in biotechnology, 18(6), 504-512.

Latruffe, N. (2017). Natural products and inflammation. Molecules, 22(1), 120.

Lautie, E., Russo, O., Ducrot, P., & Boutin, J. A. (2020). Unraveling plant natural chemical diversity for drug discovery purposes. Frontiers in pharmacology, 11, 397.

Loizeau, P.A. (1994). Les Aquifoliaceaes péruviennes. Boissiera, 48, 1-306.

Manen, J. F., Cuénoud, P., & Martinez, M. D. (1998). Intralineage variation in the pattern of rbc L nucleotide substitution. Plant systematics and evolution, 211(1), 103-112.

Martinez, M. A. D. P., Pelotto, J. P., & Basualdo, N. (1997). Distribution of flavonoid aglycones in *Ilex* species (Aquifoliaceae). Biochemical Systematics and Ecology, 25(7), 619-622.

Martinez, M. A. D. P., Pelotto, J. P., & Basualdo, N. (1997). Distribution of flavonoid aglycones in *Ilex* species (Aquifoliaceae). Biochemical Systematics and Ecology, 25(7), 619-622.

Mathur, P., Sathishkumar, K., Chaturvedi, M., Das, P., Sudarshan, K. L., Santhappan, S., & ICMR-NCDIR-NCRP Investigator Group. (2020). Cancer statistics, 2020: report from national cancer registry program, India. JCO Global Oncology, 6, 1063-1075.

Mohammad Azmin, S. N. H., Abdul Manan, Z., Wan Alwi, S. R., Chua, L. S., Mustaffa, A. A., & Yunus, N. A. (2016). Herbal processing and extraction technologies. Separation & Purification Reviews, 45(4), 305-320.

Mortenson, P. N., Erlanson, D. A., De Esch, I. J., Jahnke, W., & Johnson, C. N. (2018). Fragment-to-lead medicinal chemistry publications in 2017: mini perspective. Journal of medicinal chemistry, 62(8), 3857-3872.

Newman, D. J. (2008). Natural products as leads to potential drugs: an old process or the new hope for drug discovery?. Journal of medicinal chemistry, 51(9), 2589-2599.

Newman, D. J., & Cragg, G. M. (2016). Natural products as sources of new drugs from 1981 to 2014. Journal of natural products, 79(3), 629-661.

Noratto, G. D., Kim, Y., Talcott, S. T., & Mertens-Talcott, S. U. (2011). Flavonol-rich fractions of yaupon holly leaves (*Ilex vomitoria*, Aquifoliaceae) induce microRNA-146a and have anti-inflammatory and chemopreventive effects in intestinal myofribroblast CCD-18Co cells. Fitoterapia, 82(4), 557-569.

Noureddine, T., El Husseini, Z., Nehme, A., & Massih, R. A. (2018). Antibacterial activity of *Ilex paraguariensis* (Yerba Mate) against Gram-positive and Gram-negative bacteria. The Journal of Infection in Developing Countries, 12(09), 712-719.

Oshiro, B. T. (1999). The semisynthetic penicillins. Primary care update for ob/gyns, 6(2), 56-60.

Ottillinger, B., Storr, M., Malfertheiner, P., & Allescher, H. D. (2013). STW 5 (Iberogast®)—a safe and effective standard in the treatment of functional gastrointestinal disorders. Wiener Medizinische Wochenschrift, 163(3), 65-72.

Ouyang, M. A., Liu, Y. Q., Wang, H. Q., & Yang, C. R. (1998). Triterpenoid saponins from *Ilex latifolia*. Phytochemistry, 49(8), 2483-2486.

Ouyang, M. A., Wang, H. Q., Chen, Z. L., & Yang, C. R. (1996). Triterpenoid glycosides from *Ilex kudincha*. Phytochemistry, 43(2), 443-445.

Panossian, A. G., Hamm, R., Kadioglu, O., Wikman, G. C., & Efferth, T. (2013). Synergy and antagonism of active constituents of ADAPT-232 on transcriptional level of metabolic regulation of isolated neuroglial cells. Frontiers in neuroscience, 7, 16.

Peterson, J. J., & Novick, S. J. (2007). Nonlinear blending: a useful general concept for the assessment of combination drug synergy. Journal of Receptors and Signal Transduction, 27(2-3), 125-146.

Pires, V. S., Guillaume, D., Gosmann, G., & Schenkel, E. P. (1997). Saponins from Ilex dumosa, an erva-maté (*Ilex paraguariensis*) adulterating plant. Journal of Agricultural and Food Chemistry, 45(4), 1027-1031.

Raven, P. H., & Axelrod, D. I. (1974). Angiosperm biogeography and past continental movements. Annals of the Missouri Botanical Garden, 61(3), 539-673.

Ricco, R. A., Wagner, M. L., Giberti, G. C., & GURNI, A. A. (1995). Antocianos foliares de *Ilex paraguariensis* St. Hil. Acta Farm. Bonaerense, 14(2), 87-90.

Rishton, G. M. (2008). Natural products as a robust source of new drugs and drug leads: past successes and present-day issues. The American journal of cardiology, 101(10), S43-S49.

Sawmliana, M. (2013). The Book of Mizoram Plants (2nd edition). Aizawl, India: Zakhuma; p. 142.

Siddiqui, A.A., Iram, F., Siddiqui, S., Sahu, K. (2014) Role of natural products in drug discovery process. Int J Drug Dev Res 6(2):172–204.

Singh, S. B., & Barrett, J. F. (2006). Empirical antibacterial drug discovery—foundation in natural products. Biochemical pharmacology, 71(7), 1006-1015.

Sticher, O. (2014). Natural products–How have they contributed to health and society. Planta Medica, 80(10), IL18.

Stockwell, B. R. (2004). Exploring biology with small organic molecules. Nature, 432(7019), 846-854.

Sunazuka, T., Hirose, T., & Omura, S. (2008). Efficient total synthesis of novel bioactive microbial metabolites. Accounts of chemical research, 41(2), 302-314.

Süntar, I. (2020). Importance of ethnopharmacological studies in drug discovery: role of medicinal plants. Phytochemistry Reviews, 19(5), 1199-1209.

Taketa, A. T. C., Schmittmann-Schlager, T., Guillaume, D., Gosmann, G., & Schenkel, E. P. (2000). Triterpenoid glycosides and a triterpene from Ilex brevicuspis. Phytochemistry, 53(8), 901-904.

The Chinese Ministry of Public Heath: The Institute of Drug Control; The Colour Atlas of Chinese Herbs, Science Press, China, 1987, 245.

The Plant List. Ilex [Internet]. [cited 2010]. Available from: http://www.theplantlist.org/browse/A/Aquifoliaceae/Ilex.

Ulrich-Merzenich, G. S. (2014). Combination screening of synthetic drugs and plant derived natural products—Potential and challenges for drug development. Synergy, 1(1), 59-69.

Van Genderen, H. H., & Jaarsma, J. (1990). Triterpenes and alkanes in developing variegated and albino leaves of *Ilex aquifolium* L.(Aquifoliaceae). Plant Science, 72(2), 165-172.

Van Genderen, H. H., Jaarsma, J., & Versluis, C. (1988). Long chain pentyl-and hexyl-esters and other lipoids in leaf wax of *Ilex aquifolium* L.(Aquifoliaceae). Plant Science, 55(3), 231-238.

Verpoorte, R., Choi, Y. H., & Kim, H. K. (2005). Ethnopharmacology and systems biology: a perfect holistic match. Journal of ethnopharmacology, 100(1-2), 53-56.

Wen, D. X., & Chen, Z. L. (1996). A dimeric sinapaldehyde glucoside from Ilex rotunda. Phytochemistry, 41(2), 657-659.

World Health Organization. (2018). World health statistics 2018: monitoring health for the SDGs, sustainable development goals. World Health Organization.

Wu, S., Yang, L., Gao, Y., Liu, X., & Liu, F. (2008). Multi-channel counter-current chromatography for high-throughput fractionation of natural products for drug discovery. Journal of chromatography A, 1180(1-2), 99-107.

Yao, X., Lu, Z., Song, Y., Hu, X., & Corlett, R. T. (2022). A chromosome-scale genome assembly for the holly (*Ilex polyneura*) provides insights into genomic adaptations to elevation in Southwest China. Horticulture Research, 9.

Yao, X., Zhang, F., & Corlett, R. T. (2022). Utilization of the Hollies (*Ilex* L. spp.): A Review. Forests, 13(1), 94.

Yi, F., Zhao, X. L., Peng, Y., & Xiao, P. G. (2016). Genus *llex* L.: phytochemistry, ethnopharmacology, and pharmacology. Chinese Herbal Medicines, 8(3), 209-230.

Zhai, R.K. Iconographia Cormophytorum Sinicoru, 641, Beijing Science Publishing House, Beijing. 1985

Zhao, C., Qiao, X., Shao, Q., Hassan, M., Ma, Z., & Yao, L. (2020). Synergistic effect of hydrogen peroxide and ammonia on lignin. Industrial Crops and Products, 146, 112177.

Zhu, M. W. (1973). Chinese Medicinal Handbook of Guangxi.

Zimmermann, G. R., Lehar, J., & Keith, C. T. (2007). Multi-target therapeutics: when the whole is greater than the sum of the parts. Drug discovery today, 12(1-2), 34-42.

CHAPTER – 2

DETERMINATION OF ANTI-OXIDANT AND ANTI-MICROBIAL ACTIVITY OF *ILEX KHASIANA.*

INTRODUCTION

Antioxidants are man-made or natural substances that inhibits oxidation preventing damages caused by free radicals. Certain oxidative metabolism in human physiological system produced these free radicals including reactive oxygen species (ROS). The free radicals when acted upon vital molecules like DNA, proteins and lipids altered or inhibited the normal functions of these molecules. The exceptional function of antioxidant may be in synergistic effect of endogenous and exogenous, and aqueous and lipid soluble components, and intracellular and extracellular in terminating the ROS chain **(Stanner, 2013)**. Pathogenesis of various maladies including immune disorder, diabetes, cancer, cardiovascular disease, aging and neurodegeneration are often times related to oxidative stress. Plants are a rich source of antioxidants suppressing oxidative stress associated with different ailments in the form of dietary fruits and vegetables **(Szymanska et al., 2018)**.

Natural antioxidants are broadly classified into phenolic acids, flavonoids, lignans, stilbenes and tannins having wide contribution in human health. Gallic acid has an anti-apoptotic activity as well as anti-inflammatory properties **(Lu et al., 2010)** Ferulic acid revealed chemopreventive activity against oral cancer **(Mori et al., 1999)**. Interestingly, the number of hydroxy moieties attached to the aromatic ring of benzoic molecules effect the radical scavenging efficacy of phenolic acids **(Amarowicz et al., 2019).**

Many *Ilex* species are known for their high antioxidant contents. Flavonoids like quercetin, kaempferol, formononetin and isorhamnetin are found to be present in *Ilex cornuta* leave extract **(Si-Xiang et al., 2012)**. *Ilex paraguariensis* (yerba mate) and *Ilex brasiliensis* have a remarkable antioxidant property by protecting the myocardium against ischemia–reperfusion injury and attenuates oxidative damage **(Schinella et al., 2009)**. Methanol extract and Ethyl acetate fraction of *Ilex kudingcha* showed good free radical scavenging potency and anti-lipid peroxidation properties against mitochondrial oxidation **(Thuong et al., 2009)**.

Moreover, plants are well known for their antimicrobial activity against certain pathogenic and spoilage microbes which may be associated with its secondary metabolites-phenols and their derivatives **(Hayek et al., 2013).** Saponins, tannins, coumarins, terpenoids, phenolic acids, phenolics, quinones and alkaloids are the major compounds responsible for plants' antimicrobial activity. The structural differences and chemical composition ensued different mode of antimicrobial actions **(Lai and Roy., 2004 and Savoia, 2012).**

This is where *Ilex khasiana* comes in, having a long lineage of remarkable contribution to human health in the form of natural remedies. Its closely related *Ilex* species such as *I. pubescens, I. cornuta, I. ficoidea,* and *I. centrochinensis* are known to have antipyretic, anti-inflammatory, analgesic, anti-obesity, cardiovascular and circulatory activities.

There are few studies done on the antioxidant and antimicrobial activities in which *Ilex* species are known to have significant activity respectively. *I. paraguariensis* is known to possess antimicrobial activity against selected food pathogens such as *Staphylococcus aureus, Listeria monocytogenes, Salmonella enteritidis* and *Escherichia coli* **(Buris et al., 2011)**. The ethanol, ethyl acetate, chloroform, and n-hexane extracts of *I. aquifolium* were found to be effective against *E. coli, S. aureus, E.aerogenes, P. vulgaris, S. typhimurium,* and *C. albicans* **(Erdemoglu et al., 2009)**.

MATERIALS AND METHODS

Chemicals and reagents

Bovine serum albumin (BSA), nicotinamide adenine dinucleotide (NADH), 1,1-diphenyl-2-picrylhydrazyl radicals (DPPH), di-sodium hydrogen phosphate, n-butyl alcohol, 2-thiobarbituric acid (TBA), methanol, ferric chloride, sodium nitrite, alluminium chloride, hydrogen peroxide (H2O2), and glacial acetic acid were obtained from HiMedia Laboratories Pvt. Ltd. Mumbai, India). Quercetin, gallic acid and Ascorbic acid standards were purchased from Sigma-Aldrich, USA. Ferrous chlorides, potassium ferricyanide were obtained from LobaChemie Pvt., Ltd. Mumbai, India. Trichloroacetic acid (TCA), Folin-ciocalteu's reagent, sodium hydroxide and sodium carbonate were obtained from SD finechem Ltd. (Mumbai, India). Ceftriaxone and Clotrimazole standards were obtained from Sigma-Aldrich, USA. L-spreader was purchased from Tarson, Kolkata, India. Mueller-Hinton agar, Nutrient agar and Sabouraud Dextrose Agar were procured from HiMedia Pvt. Ltd, Mumbai, India,

PRELIMINARY PHYTOCHEMICAL SCREENING

Different extracts were subjected to preliminary phytochemical analyses according to standard protocol to identify the presence of phytoconstituents as follows **(Gokhale et al., 2017)**:

Test for Alkaloids:

a) **Mayer's test**: 2 ml of each extract solution was taken in a test tube. 0.2 ml of dilute hydrochloric acid and 0.1ml of Mayer's reagent were added. Formation of yellowish buff colored precipitate gave positive test for alkaloid.

b) **Dragendorff's test**: 0.1 ml of dilute hydrochloric acid and 0.1 ml of Dragendorff's reagent were added in 2 ml of each extract solution in a test tube. Development of orange brown colored precipitate suggested the presence of alkaloid.

c) **Wagner's test**: 2 ml of extract solution was treated with dilute hydrochloric acid and 0.1 ml of Wagner's reagent. Formation of reddish-brown precipitate indicated a positive response for alkaloid.

d) **Hager's test**: To 2 ml of each extract solution, 0.2 ml of dilute hydrochloric acid and 0.1 ml of Hager's reagent were added. A yellowish precipitate suggested the presence of alkaloid.

Detection of phytosterols:

a) Liebermann-Burchards's test: Each extract was dissolved in acetic anhydride, heated to boil, cooled and then 1 ml of Conc. H_2SO_4 was added along the side of test tube. Formation of red, pink or violet color at the junction indicated the presence of steroids or triterpenoids.

b) Salkowski reaction: To 2 ml of each extract, 2 ml of chloroform was added. 2 ml of the conc. H_2SO_4 was then added slowly at the side of the test tube and shaken and then allowed to stand for some time. Formation of red color in the lower layer indicated the presence of steroids and formation of yellow colored lower layer indicated the presence of triterpenoids.

Detection of flavonoids:

a) Shinoda test (Magnesium Hydrochloride reduction test): To the extract solution, few fragments of magnesium ribbon and conc. HCl was added drop wise. Formation of pink, scarlet, crimson red or occasionally green to blue color indicated the presence of flavonoids.

b) Zinc-Hydrochloride reduction test: To each extract solution, mixture of Zinc dust and conc. HCl was added. Formation of red color after few minutes indicated the presence of flavonoids.

Test for reducing sugars:

a) To 5 ml of each extract solution, 5 ml of Fehling's solution was added and boiled for 5 minutes. Formation of brick red colored precipitate indicated a positive presence of reducing sugars.

a) To 5 ml of the extract solution, 5 ml of Benedict's solution was added in a test tube and boiled for few minutes. Development of brick red precipitate confirmed the presence of reducing sugars.

Test for Tannins:

a) To 5 ml of extract solution, 1 ml of 5% ferric chloride solution was added. Formation of greenish black color indicated the presence of tannins.

b) 5 ml of the extract solution was treated with 1 ml of 10 % aqueous potassium dichromate solution. Formation of yellowish-brown precipitate suggested the presence of tannins.

c) 5 ml of the extract solution was mixed with 1 ml of 10 % lead acetate solution. Yellow color precipitation confirmed the presence of tannins.

Detection of Saponins:

Foam test: Small quantity of each extract was dissolved in 20 ml distilled water. The suspension was shaken in a graduated cylinder for 15 minutes. Formation of 2 cm layer of foam or froth which is stable for 10 minutes indicated the presence of saponins.

Detection of amino acids:

About 100 mg of each extract was dissolved in 10 ml of distilled water and filtered with Whatman filter paper no. 1 and the filtrate was further subjected to test amino acids:

a) Ninhydrin test: 2 drops of ninhydrin solution were added to 2 ml of aqueous filtrate. Formation of purple color indicated the presence of amino acids.

Detection of Glycosides:

For the detection of glycosides, about 50 mg of extract was hydrolyzed with Conc. HCl for 2 h in a water bath, filtered and was subjected to the following test:

a) Keller Killiani's test: 1 ml of glacial acetic acid containing traces of ferric chloride and 1 ml Conc. H_2SO_4 was added to the extract solution. Formation of reddish-brown color at the junction of two layers and bluish green in the upper layers indicated the presence of glycosides.

b) Legal's test: About 50 mg of the extract was dissolved in the solvent. Sodium nitroprusside solution was added and made alkaline using 10% sodium hydroxide solution. Presence of glycoside was indicated by a characteristic pink color.

Detection of carbohydrates:

100 mg of each extract was dissolved in 5 ml of distilled water and filtered. The filtrate was subjected to the following tests:

a) Molisch's test: 2 drops of alcoholic solution of α-naphthol were added to 2 ml of the filtrate. The mixture was shaken and 1ml of Conc. H_2SO_4 was added along the side of the test tube and then cooled in ice water and allowed to stand, a violet ring at the junction indicated the presence of carbohydrates.

b) Fehling's test: 1ml of the filtrate was boiled in water bath with 1 ml each of Fehling's solution A and B, formation of red precipitate confirms the presence of sugar.

c) Barfoed's test: To 1 ml of the filtrate, 1 ml of Barfoed's reagent was added and heated in a boiling water bath for 2 minutes. Formation of red precipitate indicated the presence of sugar.

d) Benedict's test: To 1 ml of the filtrate, 0.5 ml of Benedict's reagent was added, and the mixture was heated in a water bath for 2 minutes. Formation of colored precipitate indicated the presence of sugars.

IN-VITRO ANTIOXIDANT ACTIVITIES

Determination of diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity:

Free radical scavenging potency of *Ilex khasiana* was determined by a method developed by Blois **(1958)** with slight modification. In short, DPPH (2,2-diphenyl-1-picrylhydrazyl), a free radical was used as a substrate and butylated hydroxytoluene (BHT) was used as a reference standard. The plant extract namely petroleum ether (IKP), chloroform (IKC) and methanol (IKM) were prepared in different concentration (10, 20, 30, 40, 50, 80, and 100 µg/ml). 1 ml of 0.1 mM of DPPH in methanol was mixed with 3 ml of extracts and was left at room temperature for 30 minutes. A blank solution was prepared in the same manner without the extract. The mean value of absorbance

was obtained by measuring at 517 nm against control in a UV-visible spectrophotometer (LABTRONICS LT 2700).

The inhibition percentage (%) = (Abs control–Abs sample) / Abs control × 100

Hydroxyl radical scavenging assay:

Hydroxyl radical scavenging activity of *Ilex khasiana* was assessed according to the standard protocol **(Halliwell, 1991).** 1 ml each of different concentrations (10, 20, 40, 60, 80 and 100 µg/ml) of the standard Ascorbic Acid (AA) and all three extracts IKP, IKC and IKM were placed in separate tubes, 0.1 ml of 1 mM ethylenediaminetetraacetic acid (EDTA), 0.01 ml of 10 mM $FeCl_3$, 0.1 ml of 10 mM H_2O_2 and 0.36 ml of 10 mM deoxyribose were added. To this solution, 0.33 ml of phosphate buffer (pH 7.4) and 0.1 ml of 0.1 mM ascorbic acid were added and then incubated for an hour at 37°C. After an hour of incubation, the solutions were mixed with 1ml of trichloroacetic acid (TCA) and 1 ml of 0.5% thiobarbituric acid (TBA). The solutions were then heated at 80°C to develop pink chromogen for 10-20 minutes. After cooling at room temperature, the absorbances were taken at 532 nm using double beam UV-Vis spectrophotometer (LABTRONICS LT 2700). The results were calculated as percent inhibition of the deoxyribose degradation using the following formula:

Inhibition % = {(Absorbance of control – Absorbance of sample) / Absorbance of control} X 100.

Reducing power

The reducing power of *Ilex khasiana* was determined using a standard protocol with minor modifications **(Oyaizu, 1986).** Three extracts petroleum ether (IKP), chloroform (IKC) and methanol (IKM) of *Ilex khasiana* were used. These extracts after dissolving in their own respective solvent was mixed with 2.5 mL of 0.2 M phosphate buffer (pH 6.6) and 2.5 mL of 1% potassium ferricyanide solution. After the solution was incubated at 50 ^oC for 20 minutes, 2.5 mL of 10% TCA was added into it. Then, the mixture was centrifuged for 20 minutes at 3000 rpm. 2.5 ml of the supernatant was mixed with 0.5 mL of 1% ferric chloride solution and 2.5ml of distilled H_20. Absorbance

was taken at 700 nm using double beam UV-Vis Spectrophotometer (LABTRONICS LT 2700). Reducing power of the extract was indicated by increase in absorbance.

Total antioxidant activity:

The total antioxidant activity of *Ilex khasiana* was determined using a standard protocol **(Prieto et al., 1999).** The reduction potency of the three extracts IKP, IKC and IKM on phosphomolybdate was estimated against Ascorbic acid as the standard drug. The reagent solution contains 0.6 M H_2SO_4, 28 mM sodium phosphate and 4 mM ammonium molybdate from which 1 ml was taken and mixed with 1 ml of the sample solution. The solution was incubated for 90 minutes at 95°C and absorbance was taken at 695 nm using double beam UV-Vis Spectrophotometer (LABTRONICS LT 2700).

Determination of total phenolic content

The total phenolic content of *Ilex khasiana* was determined by following standard protocol **(Singleton and Rossi, 1965).** 1ml of the plant extracts IKP, IKC and IKM (50µg/ml) and gallic acid was taken from 10, 20, 30, 40, 60, 80 and 100µg/ml. A tenfold diluted 5ml Folin–Ciocalteu reagent was added to all. After three minutes, 4ml of 0.7 M sodium carbonate solution was added to the mixture and incubated for 1 hour at room temperature. The absorbance was taken at 756 nm using double beam UV-Vis Spectrophotometer (LABTRONICS LT 2700). All determinations were carried out in triplicate. Absorbance of gallic acid at different concentrations was used to plot a standard curve from which the total phenolic content of the plant extracts was calculated and expressed as milligrams of gallic acid equivalent (GAE) per g of the dried extract.

Determination of the total flavonoids

Quercetin was used as a standard to estimate the total flavonoid content of *Ilex khasiana* extract using aluminium chloride ($AlCl_3$) assay **(Zhishen et al., 1999).** Into 1ml each of IKP, IKC and IKM (50µg/ml), 2ml of distilled water was added. After 5 minutes incubation at room temperature, 3ml of 5% sodium nitrite ($NaNO_2$) and 0.3 ml of 10% $AlCl_3$ were added to the mixture. After 6 minutes, 2 ml of NaOH (1M) was

added and the volume was made up to 10ml with distilled water. 1 hour after the incubation absorbance reading was taken at 510 nm using double beam UV-Vis Spectrophotometer (LABTRONICS LT 2700). The same procedure was employed to the quercetin standard having a series of concentration (5, 10, 20, 40, 60, 80, and 100 μg/ml). The absorbance of different concentration of quercetin provided the standard curve and the total flavonoid content was expressed as milligrams of quercetin equivalent (QE) per gram of the dried extract. All tests were performed in triplicate.

ANTIMICROBIAL ACTIVITY OF *ILEX KHASIANA*

The antimicrobial activity of *Ilex khasiana* was performed following the protocol and permission provided by the Mizoram University Biosafety Committee with a permission no: **MZUBS/2021/01.**

Antibacterial activity

The antibacterial potency of different extracts of *Ilex khasiana* was determined using Kirby-bauer disk diffusion method with slight modification **(Bauer et al., 1966)**. Six bacteria namely *Bacillus subtilis* (ATCC-6051), *Pseudomonas aeruginosa* (ATCC-15442), *Escherichia coli* (ATCC-25922), *Klebsiella pneumoniae* (ATCC-BAA-1705), *Micrococcus luteus* (ATCC-10240), *Salmonella typhi* (ATCC-51812) were subjected to the test. The test organism inocula were allowed to grow overnight in nutrient broth at 37^0C. These overnight cultures were the sub-cultured into fresh nutrient broth for experiment after adjusting the turbidity at 0.5 McFarland Standard ($1x10^8$ CFU/ml). The Mueller-Hinton agar was autoclaved at 120 ^{O}C for 20 minutes in Erlenmeyer flask closed with cotton plug. The agar was poured on sterilized culture plate allowed to cool down at 45^OC and 0.2ml of microorganism was pipetted on the molten agar and spread evenly using a sterilized L-spreader. Two concentrations of *Ilex khasiana* Chloroform (IKC) and *Ilex khasiana Methanol* (IKM) extract were prepared, i.e., 10 mg/ml and 20 mg/ml respectively. Two different concentrations of these extracts were placed on sterile disks and were placed on the culture disk along with Ceftriaxone standard disk

(10µg) and the extract solvent served as the negative control). The plates were incubated at 37±1°C for 24 hours and the corresponding zones of inhibitions were measured using vernier calliper. The zone of inhibition of the extracts were measured and compared with the reference drug.

Determination of Minimum Inhibitory Concentration (MIC)

The standard agar disk diffusion protocol was used for estimation of Minimum Inhibitory Concentration (MIC). The extract was incorporated into nutrient agar using sterile paper disk at concentrations of 2, 4, 6 and 8 mg/ml. A control without the extract was also set up. 0.2ml of microorganism, previously diluted to give 10^8 cfu/ml was used to inoculate the plates and spread evenly using a sterilized L-spreader. These were incubated at 37 ^{O}C for 24 hours before the results were recorded after observing for growth. The agar plate with the lowest concentrations without any growth were regarded as minimum inhibitory concentrations (MICs) of the extract for each test microorganism.

Antifungal Activity of *Ilex khasiana*

The antifungal activity of Ilex khasiana was performed using the poisoned food method against *Candida albicans* (ATCC-10321) **(Euloge et al., 2012).** Methanol (IKM) and chloroform (IKC) extract of I. khasiana were used for the assessment with Clotrimazole (0.1%) as a reference standard. Different concentration (5, 10, 15 and 20 mg/20ml of SDA) were prepared for both the extracts by adding the appropriate amount of plant extract to molten Sabouraud Dextrose Agar (approximately 45^{O}C) and mixed thoroughly in sterile Erlenmeyer flask. 20ml of Sabouraud Dextrose Agar was dispensed into sterile petri dishes (9 cm in diameter) without any bubble formation and were allowed to solidify at room temperature (23 ± 2 ^{O}C). *Candida albicans* along with the agar media were cut from an actively growing 7 days old pure culture. The control plates were prepared using the same procedure but without containing the extracts. The plates were incubated at 28 ^{O}C for 7 days. The diameter of the fungal colony was measured

using vernier caliper on three different sides to get the mean colony size and the mycelial growth was measured using the following formula **(Philippe et al., 2012):**

$$\text{Inhibition of mycelial growth (\%)} = (dc - dt/dc) \times 100$$

where dc is mean diameter of colony in the control sample and dt is mean diameter of colony in the treated sample.

Statistical analysis

Data are expressed as mean ± standard error of the mean. GraphPad Prism software ver. 8.0.2 was employed to perform One-way analysis of variance (ANOVA) followed by Tukey multiple comparison of means on phytochemical contents, antioxidant activities, and antimicrobial activities of treatment groups. A p value <0.05 was considered statistically significant. Student's t-test was employed to calculate the variation in IC_{50} of the extracts.

RESULTS

Preliminary phytochemical screening

The bioactive phytochemicals were screened using the standard protocol showing the presence of alkaloids, phytosterols, flavonoids, reducing sugars, saponins, glycosides and carbohydrates. The petroleum ether extract (IKP) showed least number of phytochemical present while both methanol extract (IKM) showed the highest followed by chloroform extracts (IKC). Both tannins and amino acids were absent in all the three extracts. IKC and IKM were tested positive for the presence of phytosterols in both Liebermann-Burchard and Salkowski tests and the presence of flavonoids was confirmed on both the extracts by Shinoda test and Zinc Hydrochloride reduction test. Alkaloids was confirmed in both IKP and IKM by Mayer's test, Dragendorff's test, Wagner's test and Hager's test and was absent in IKC **(Table 2.1)**.

Diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity

The antioxidant property of *Ilex khasiana* was assessed using DPPH radical scavenging activity. The extracts namely IKP, IKC and IKM were prepared to have different concentrations of 10, 20, 40, 60, 80 and 100 µg/ml in which the scavenging activity increases with an increase in concentration **(Figure:2.1).** The percentage scavenging activity of the extracts were plotted against log-doses used for calculation of IC_{50}. After screening the three given extracts, IKC showed the highest scavenging activity by giving IC_{50} of 17.22±1.86 µg/ml, followed by IKM (26.93±1.14 µg/ml) and IKP (37.16±1.38 µg/ml) when compared to the standard BHT (8.312±0.71). When comparing the scavenging activity of all the samples (IKP, IKC and IKM) with the standard (BHT), all the samples are found to be statistically significant at ($p>0.05$) **(Figure: 2.2).**

Hydroxyl radical scavenging activity

The hydroxyl radicals scavenging potential of different extracts of *Ilex khasiana* was estimated using Ascorbic acid as standard. The different concentration concentrations (10, 20, 40, 60, 80 and 100 µg/ml) of the extracts IKP, IKC and IKM including Ascorbic acid showed better scavenging activity with the increase in concentration. Among the plant extracts IKM showed higher scavenging activity with an IC_{50} of 39.26±0.89 µg/ml followed by IKC with an IC_{50} of 156.7±1.38 µg/ml and IKP (242.66±0.99 µg/ml) which were comparatively much higher than the standard Ascorbic acid (19.55 ±0.96 µg/ml). The percentage scavenging activity was plotted against log-dose for the calculation of IC_{50} **(Figure: 2.3).** Statistically, at ($p>0.05$) all the extracts were found to significant after ANOVA followed by Tukey's test was performed **(Figure: 2.4).**

Reducing power

The reduction of Fe^{3+} by the three extracts IKP, IKC and IKM into Fe^{2+} was estimated by observing the absorbance given by different concentration (10, 20, 40, 60, 80 and 100 µg/ml) at 700 nm using Ascorbic acid as reference standard. The plant extracts showed concentration dependent reducing activity **(Figure: 2.5)** in which IKC showed highest reducing activity at 100 µg/ml concentration (0.093±0.088) and IKM (0.032±0.088) which is comparatively lower than the standard Ascorbic acid (0.210±0.088). Interestingly, IKP did not show any significant reducing property even at the highest concentration.

Total antioxidant activity

The total antioxidant activity of the *I. khasiana* was estimated from three different solvent extracts. The value was presented as milligrams of ascorbic acid equivalent (AE) per gram of the dried extract **(Figure: 2.6 A)**. It was determined that IKP showed 44.27 AE mg/g, IKC showed 17.08 AE mg/g, and IKM showed 76.42 AE mg/g.

Total phenolic content

The total phenolic content of the plant extracts was calculated from the standard curve of gallic acid and expressed as milligrams of gallic acid equivalent (GAE)/g of the dried extract **(Figure: 2.6 B)**. It was found that IKM contained **3.46 GAE mg/g** and IKC contained **1.450 GAE mg/g**. However, IKP did not show any activity.

Total flavonoid content

The total flavonoid content of the plant extracts was estimated against quercetin as a standard antioxidant, and the value is expressed as milligrams of quercetin equivalent (QE) per gram of the dried extract **(Figure: 2.6 C)**. From the standard curve, it was calculated IKM had **30.8 QE mg/g** and IKC had **41.9 QE mg/g**. IKP again did not show any activity.

ANTIMICROBIAL ACTIVITY OF *ILEX KHASIANA*

Antibacterial activity

The antibacterial activity of IKM and IKC was performed on six bacteria namely *Bacillus subtilis* (ATCC-6051), *Pseudomonas aeruginosa* (ATCC-15442), *Escherichia coli* (ATCC-25922), *Klebsiella pneumoniae* (ATCC-BAA-1705), *Micrococcus luteus* (ATCC-10240), *Salmonella typhi* (ATCC-51812). Both IKM and IKC showed concentration-dependent antimicrobial activity **(Figure: 2.7 and Figure: 2.9)**. IKC has the best inhibitory effect against *E. coli* with a zone of inhibition 14±0.57 (mm) at 20 mg/ml followed by *P. aeruginosa* (13.33±0.33) while IKC did not show any significant inhibition against *M. luteus* and *S. typhi.* IKM has the highest antimicrobial activity against *B. subtilis* with a zone of inhibition of 20±0.57 and least effective against *M. luteus* 10.75±0.63 at 20 mg/ml. Even though IKM exhibited antibacterial activity against all the selected microorganisms, IKC has shown lower IC_{50} in comparison to IKC except for *P. aeruginosa* and *K. pneumoniae* **(Figure: 2.8 and Figure: 2.10)**. When comparing the IC_{50} of both IKC and IKM, no significant variation was observed at

$p>0.05$ (ANOVA followed by Tukey's test) **(Figure: 2.11)**. Interestingly, IKP has no significant antimicrobial activity against the selected microbes.

Minimum Inhibitory Concentration (MIC)

MIC was calculated for both IKC and IKM by incorporating the extract into molten nutrient agar using sterile paper disk at concentrations of 2, 4, 6 and 8 mg/ml. The two extracts have notable inhibitory concentration for each organism except for IKC against *Salmonella typhi* and *Micrococcus luteus* **(Table: 2.2)**

Antifungal activity

The mycelial growth of *Candida albicans* was inhibited in a dose dependent manner by both IKC and IKM. The plant extracts were divided into four different concentrations (5, 10, 15 and 20 mg/20ml of SDA), in which the highest concentration exhibited the highest growth inhibition **(Figure: 2.12 and Figure: 2.14)**. At 20mg, IKC showed 56.99±0.39 inhibition which is much higher than IKM at 20 mg/20ml of SDA which was 44.16±0.69 when compared to 1% Clotrimazole standard which gave 60.49±0.30 **(Figure: 2.13 and Figure: 2.15)**. No significant inhibition was found on IKP extract. IC_{50} of the two extracts showed significant variation at $p>0.05$ (Student's t-test) (**Figure: 2.16)**.

DISCUSSION

Antioxidants that are found naturally have a protective potency against several ailments like cardiovascular disease and cancer. Redox homeostasis is maintained when antioxidant acts against reactive oxygen species (ROS)/ reactive nitrogen species (RNS) **(Shirwaikar et al., 2011)**. These natural antioxidants are found in the form of polyphenols, carotenoids, vitamin E and C. The significances of antioxidants are beyond doubt and their role in combating diseases are beyond measure. However, the utilization of antioxidants as a therapeutic agents need wider and deeper research **(Balsano and Alisi, 2009)**.

The antioxidant property of flavonoids is marked by its direct oxygen free radical scavenging, chelating or inhibition of oxidative enzymes **(Kumar and Pandey, 2013; Terao et al., 2009).** Besides inhibiting acetylcholinesterase, dietary flavonoids are also known to decrease risk of cardiovascular disease and hypertension **(Hügel et al., 2016).** DPPH is a common stable free radical used in determining antioxidant activity of a compound. Decolorization of DPPH occurred when electron is taken up by substance **(Bhagat et al., 2011)**. The scavenging activity of *I. khasiana* might be the result of phenolic compounds present in the extracts. One of the most reactive oxygen species is hydroxyl radical. Thus, scavenging these free radicals by plant extracts will really be a great relief for human physiological system as it causes lipid peroxidation (**Halliwell et al., 1987)**. Reductones are special class of organic compounds breaking the chain of free radicals by hydrogen atom donation. Consequently, the antioxidant properties of a plant are directly related to its reducing potential **(Gülçin et al., 2010)**.

In normal physiological system, reactive oxygen species (ROS) and reactive nitrogen species (RNS) are the by-products of many metabolic and biochemical processes. They are important in cell signaling, cell growth regulation, phagocytosis and energy production at normal level but oxidative stress occurred when over produced **(Sen et al., 2013)**.

Synthetic antioxidants are popularly used because of their performance and higher stability **(Engin et al., 2011)**. Among the widely available synthetic antioxidants are butylated hydroxytoluene (BHT), propyl gallate (PG), 2-naphthol (2NL), 4-phenylphenol (OPP) and 2,4-dichlorophenoxyacetic acid (2,4-DA), butylated hydroxy anisole (BHA) and tert-butyl hydroquinone (TBHQ) **(Xiu-Qin et al., 2009).** In spite of their well-known application and uses, the synthetic antioxidants have their inverse effect on human body **(Jeong et al., 2005)**. Excessive and extensive use of these synthetic products is reported to induced health related issues. Allergies and increased risk of cancer are the prominent ones while BHT and BHA described to have unfavorable impact on liver and enhance carcinogenesis **(Botterweck et al., 2005).** The use of natural antioxidants is expected to increase in a much higher level as the demand to ceased the use of synthetic ones are increasing in medical and food industries **(Sebranek et al., 2005)**.

Remarkably, the phenolic compounds are the responsible substance for antimicrobial activity of certain plants other than its role as natural antioxidant. Phenolic compounds are plant secondary metabolites that can interact with bacteria cell membrane by its hydroxyl (-OH) groups that cause membrane disruption **(Xue et al., 2013)**. This -OH group acts as proton exchanger allowing electron delocalization depleting the ATP pool leading to cell death **(Ultee et al., 2002)**. The effectiveness of antimicrobial components is determined strongly by the position of -OH group. Thymol and carvacrol having the same chemical structures but different in its -OH position have astoundingly contrasted effect on both gram-positive and gram-negative bacteria **(Dorman and Deans., 2000)**. So, the presence of not only -OH group but also delocalized electrons (double bonds) is very crucial for antimicrobial activity. Among citronellol, geraniol, and nerol, the latter two having two double bonds have higher antimicrobial activity than citronellol having only one double bond **(Gochev et al., 2010).** Besides, the length of saturated side chain and position of -OH substitution in the -OH ring the antimicrobial activity of phenolic compounds depends on the alkyl or alkenyl group in which longer alkyl group mean greater antimicrobial activity **(Pelczar**

et al.,1988). Like its chemical composition, lipophilic nature of phenolic group, solubility as well as functional group potency play an important role in antimicrobial activity **(Dorman and Deans., 2000)**. Moreover, the extraction methods, size of inoculum, method adopted for determination and culture medium all play extensive role in determining the antimicrobial activity of a plant **(Tajkarimi et al., 2010).**

SUMMARY

The present work depicted the overall potency of *Ilex khasiana* extracts on its antioxidant properties as well as their antimicrobial activity. Remarkably among the three extracts, IKP has the lowest antioxidant properties while IKC and IKM have a notable free radical scavenging property when subjected to DPPH and Hydroxyl radicals. Thus, the plant is expected to be a good source of natural antioxidants. Both IKC and IKM extracts also portrayed a good reducing power of the plants in a dose dependent manner. The phenolic content 3.46 GAE mg/g (IKM)and 1.45 GAE mg/g (IKC) including the total flavonoid content of 30.8 QE mg/g (IKM) and 41.9 QE mg/g (IKC) might play a vital role in both the antioxidant as well as the antimicrobial properties of the plant. Comparatively, IKC and IKM have an antimicrobial activity against both gram positive and gram negative namely *Bacillus subtilis* (ATCC-6051), *Pseudomonas aeruginosa* (ATCC-15442), *Escherichia coli* (ATCC-25922) and *Klebsiella pneumoniae* (ATCC-BAA-1705). Notably, IKC showed no antibacterial effect on *Micrococcus luteus* (ATCC-10240) and *Salmonella typhi* (ATCC-51812) but IKM has a strong effect against both the bacteria. The antifungal activity of IKC showed 56.9% of inhibition at 20mg/20ml of SDA against *Candida albicans* (ATCC-10321) and IKM showed 44.16% inhibition which is comparatively significant with 1% clotrimazole which is 60.49%. This indicate that both the extract IKC and IKM has antimicrobial activity which is species specific as well as dose dependent. Therefore, the two extract IKC and IKM were used for the following parameters employed for this research.

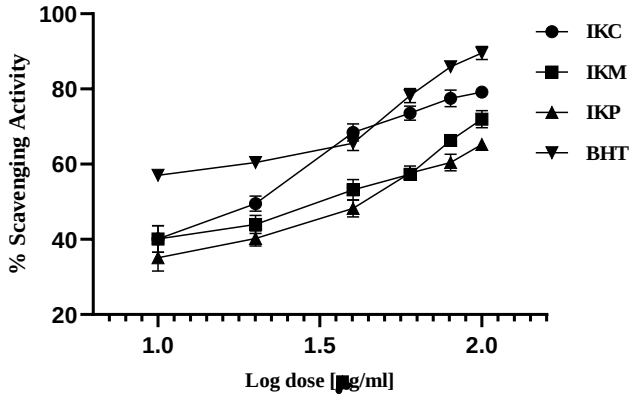


Figure 2.1: Percentage scavenging activity of IKP, IKC, IKM and the standard BHT against log-doses of the extracts on DPPH free radical scavenging activity (Mean ± SEM). P<0.05 was considered significant (ANOVA followed by Tukey multiple comparison test). (IKP= *Ilex khasiana* Pet ether extract, IKC= *Ilex khasiana* Chloroform extract, IKM= *Ilex khasiana* Methanol extract and BHT= Butylated hydroxytoluene).

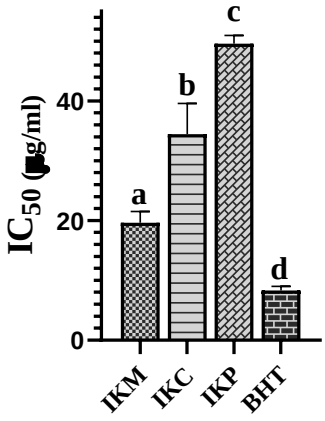


Figure 2.2: IC_{50} of BHT and different extracts of *I. khasiana* on DPPH scavenging activity (Mean ± SEM). Different letters indicated statistical significance at P<0.05 (ANOVA followed by Tukey multiple comparison test). (IKP= *Ilex khasiana* Pet ether extract, IKC= *Ilex khasiana* Chloroform extract, IKM= *Ilex khasiana* Methanol extract and BHT= Butylated hydroxytoluene).

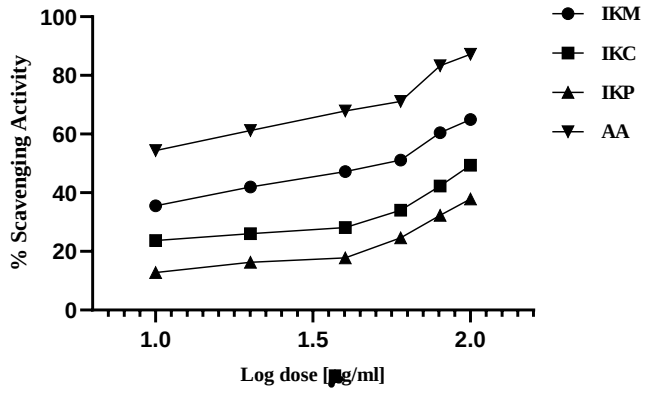


Figure 2.3: Percentage scavenging activity of IKP, IKC, IKM and the standard BHT against log-doses of the extracts on Hydroxyl free radical scavenging activity (Mean ± SEM). P<0.05 was considered significant (ANOVA followed by Tukey multiple comparison test). (IKP= *Ilex khasiana* Pet ether extract, IKC= *Ilex khasiana* Chloroform extract, IKM= *Ilex khasiana* Methanol extract and AA= Ascorbic acid).

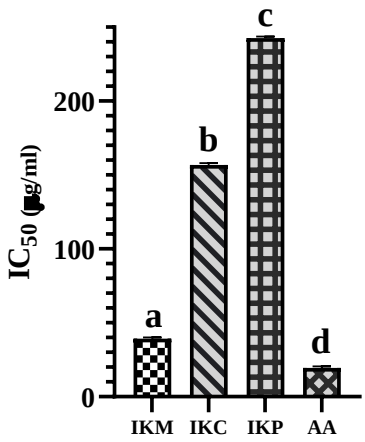


Figure 2.4: IC_{50} of AA and different extracts of *I. khasiana* on DPPH scavenging activity (Mean ± SEM). Different letters indicated statistical significance at P<0.05 (ANOVA followed by Tukey multiple comparison test). (IKP= *Ilex khasiana* Pet ether extract, IKC= *Ilex khasiana* Chloroform extract, IKM= *Ilex khasiana* Methanol extract and AA= Ascorbic acid).

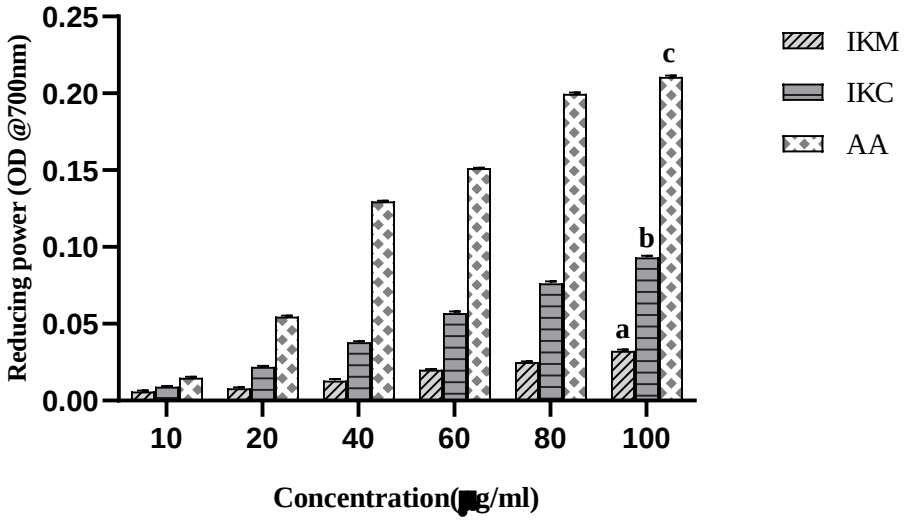


Figure 2.5: Reducing power of IKC and IKM at different concentration (10 – 100 µg/ml) and the standard ascorbic acid (AA). Values are expressed as Mean ± SEM, n=3. Different letters indicate significant variation. (IKC= *Ilex khasiana* Chloroform extract, IKM= *Ilex khasiana* Methanol extract and AA= Ascorbic acid).

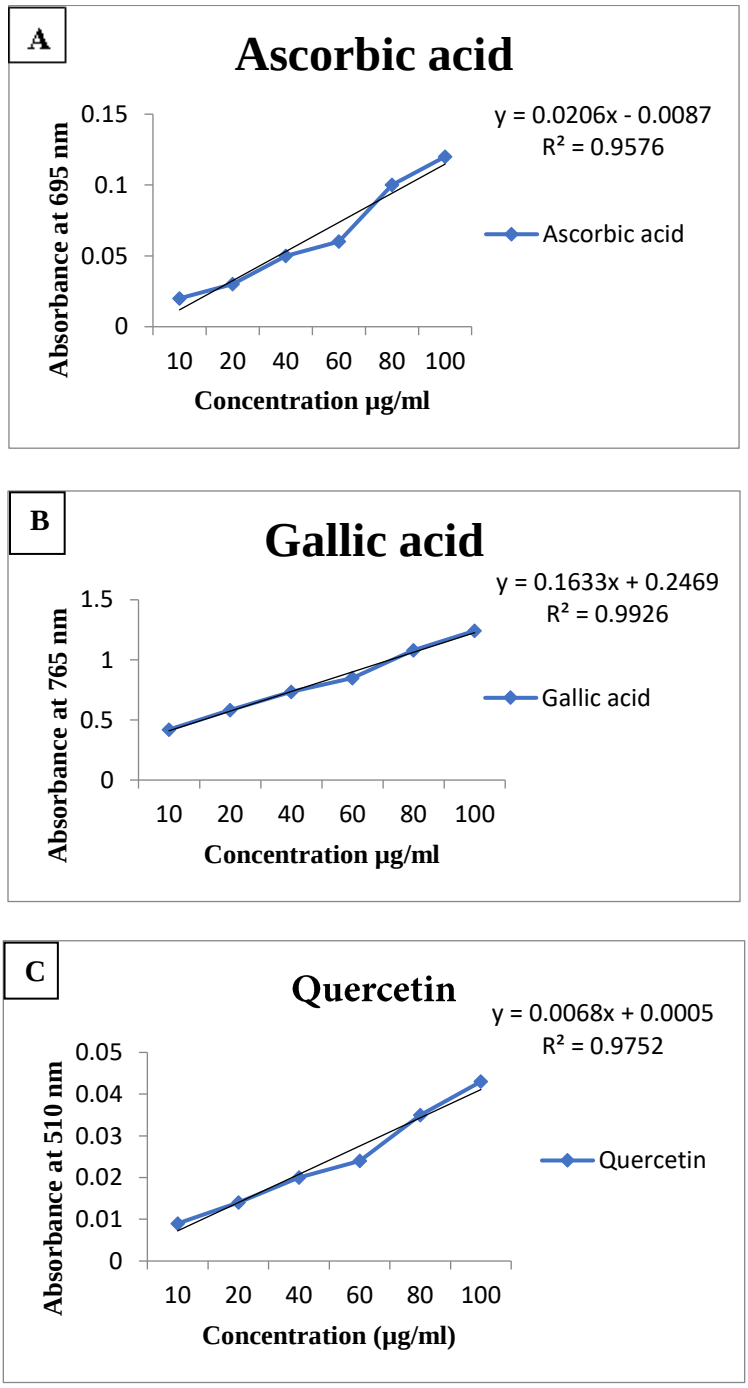


Figure 2.6: Standard curve of (A) Ascorbic acid for the estimation of total antioxidant, (B) Gallic acid for the estimation of total phenol and (C) Quercetin for the estimation of total flavonoid content at the concentration of 10 to 100 µg/ml.

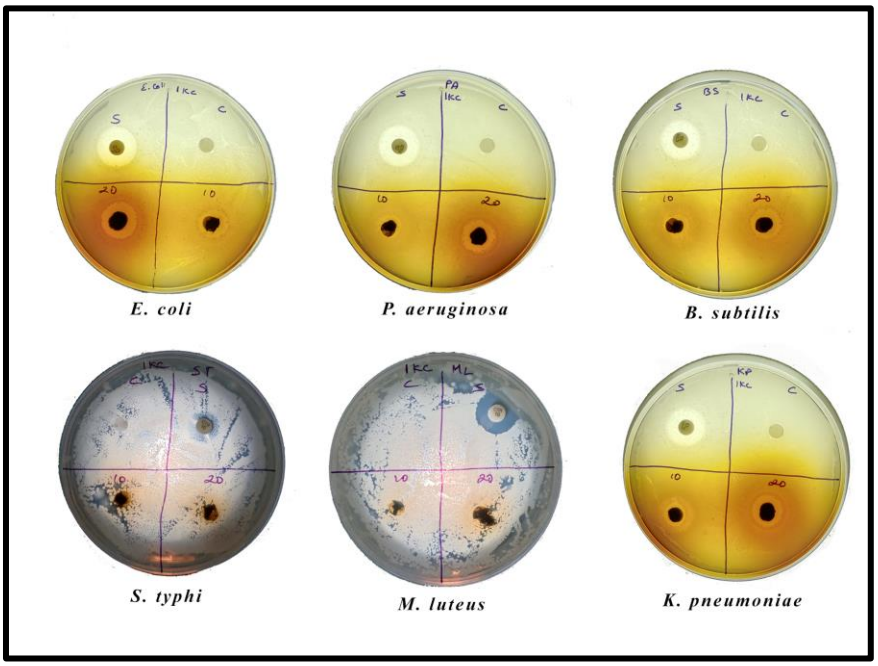


Figure 2.7: Growth inhibition of selected bacterial strains caused by *Ilex khasiana* Chloroform extract (IKC).

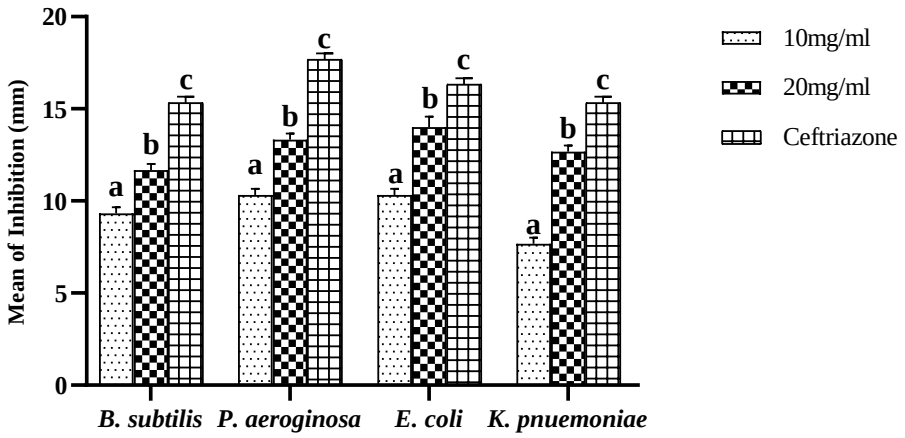


Figure 2.8: Mean inhibitory effect of IKC on selected bacteria. Values are Mean ± S.E.M. Different letters indicated significant variation at P<0.05 (ANOVA followed by post-hoc Tukey's test). (IKC = *Ilex khasiana* Chloroform extract)

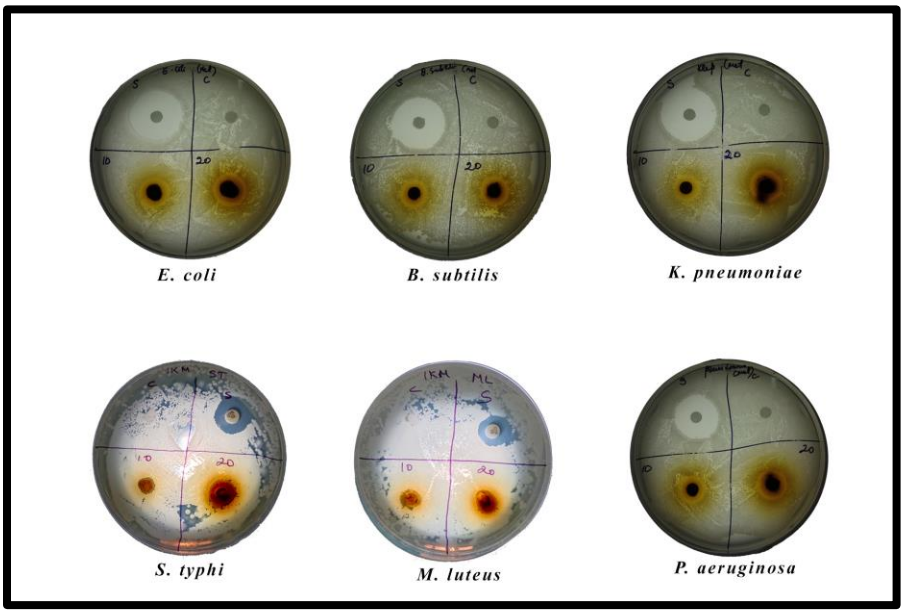


Figure 2.9: Growth inhibition of selected bacterial strains caused by *Ilex khasiana* methanol extract (IKM).

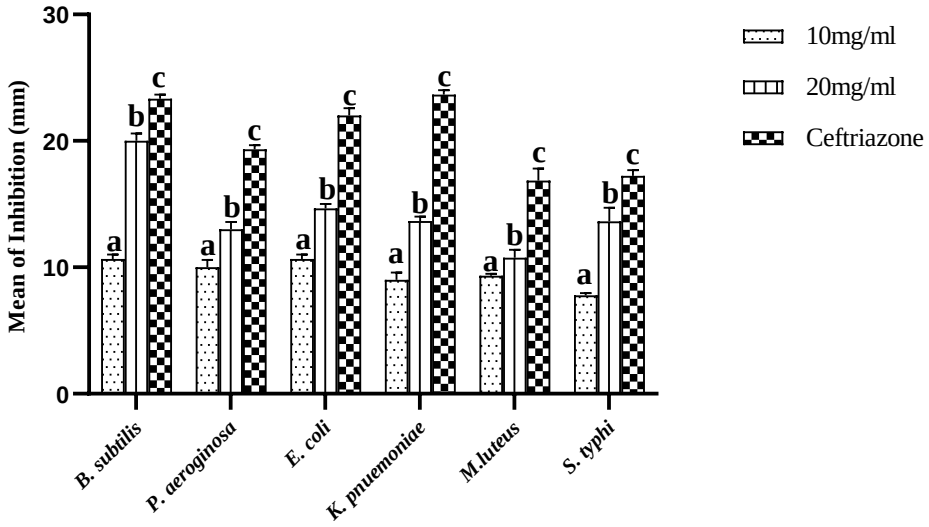


Figure 2.10: Mean inhibitory effect of IKM on selected bacteria. Values are Mean ± S.E.M. Different letters indicated significant variation at P<0.05 (ANOVA followed by post-hoc Tukey's test). (IKM= *Ilex khasiana* methanol extract).

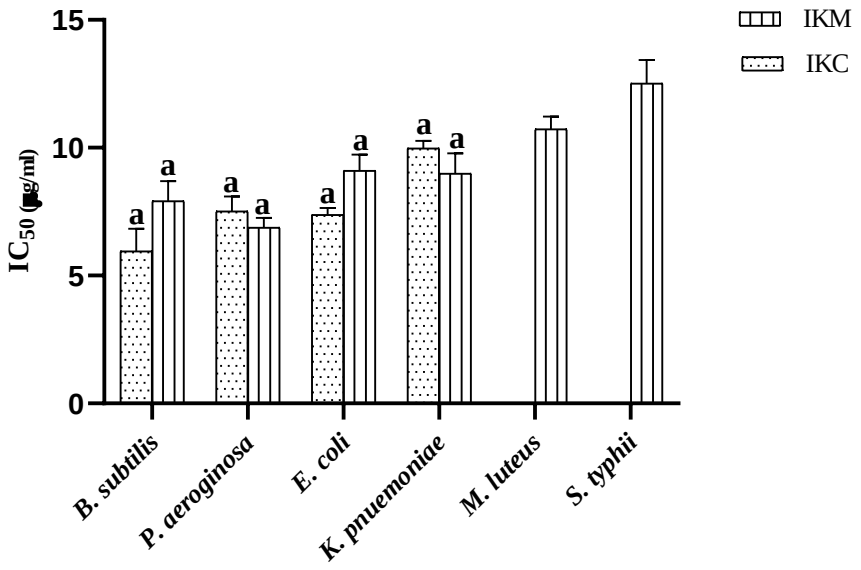


Figure 2.11: IC_{50} of IKC and IKM against selected bacteria. Data are represented as Mean ± S.E.M. Different letters indicated statistical significance at P<0.05 (ANOVA followed by Tukey multiple comparison test). (IKC= *Ilex khasiana* Chloroform extract and IKM= *Ilex khasiana* Methanol extract).

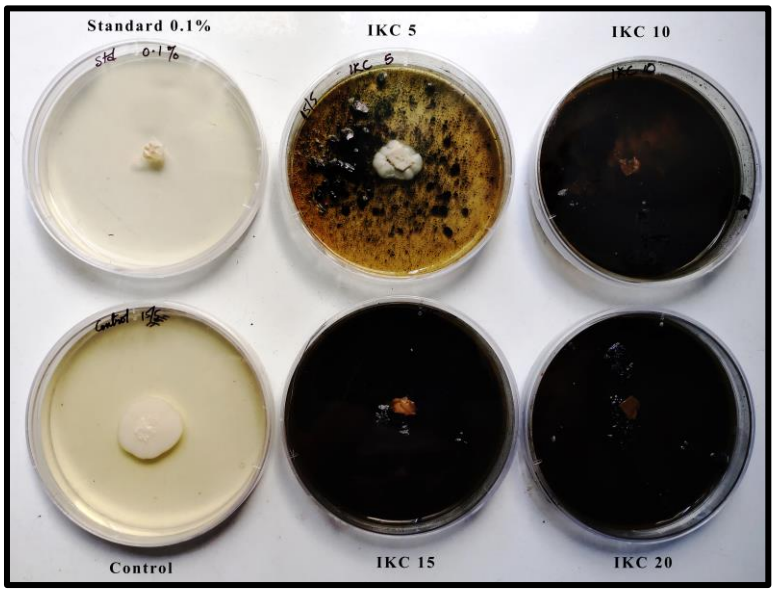


Figure 2.12: Inhibition of mycelia growth in *Candida albicans* by IKC after 7 days of incubation. (IKC= *Ilex khasiana* Chloroform extract)

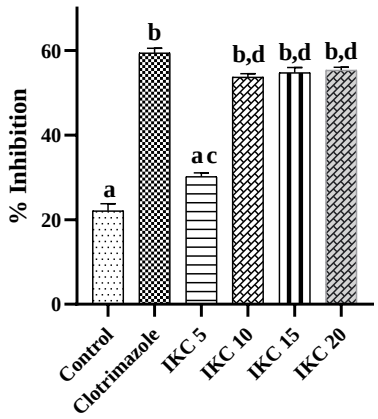


Figure 2.13: Anti-fungal effect of IKC on *Candida albicans.* Values are Mean ± S.E.M. Different letters indicated means that are statistically significant at P<0.05 (ANOVA followed by Tukey's test). (IKC= *Ilex khasiana* Chloroform extract).

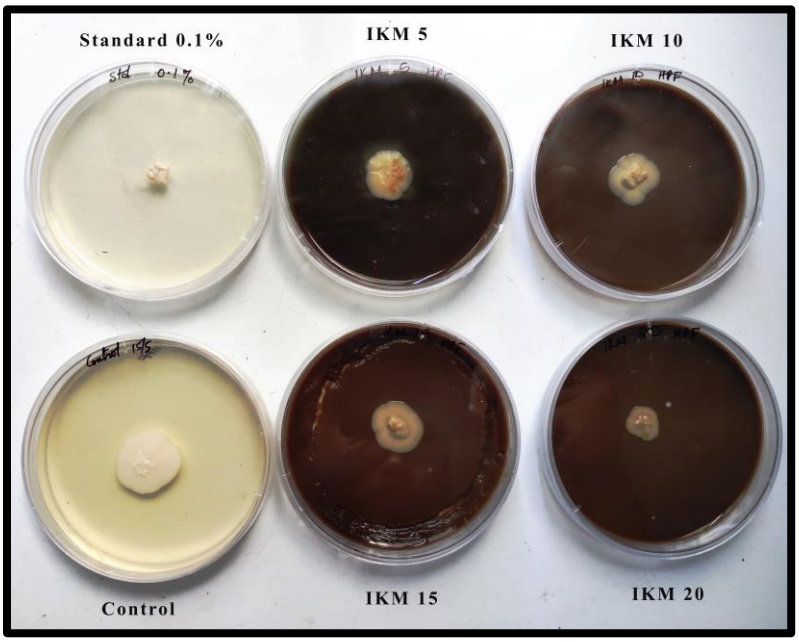


Figure 2.14: Inhibition of mycelia growth in *Candida albicans* by IKM after 7 days of incubation. (IKC= *Ilex khasiana* methanol extract)

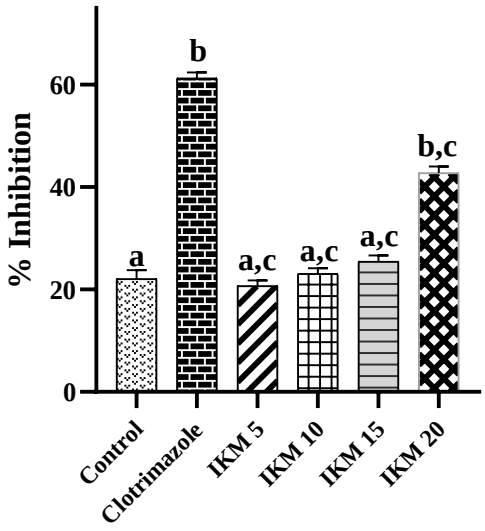


Figure 2.15: Anti-fungal effect of IKM on *Candida albicans.* Values are Mean ± S.E.M. Different letters indicated means that are statistically significant at P<0.05 (ANOVA followed by Tukey's test). (IKM= *Ilex khasiana* Methanol extract).

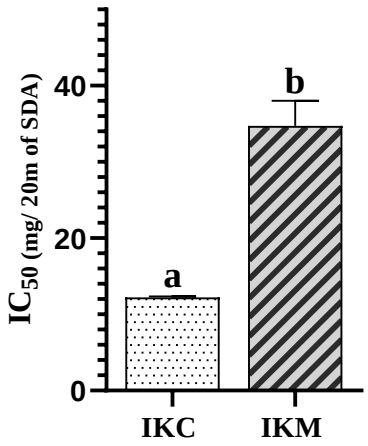


Figure 2.16: IC_{50} of IKC and IKM on *Candida albicans*. Data are represented as Mean ± S.E.M. Different letters indicate statistical significance at <0.005 (Student's t-test). (IKC= *Ilex khasiana* Chloroform extract, IKM= *Ilex khasiana* Methanol extract).

Table 2.1: Preliminary phytochemical screening of extracts of *Ilex khasiana* leaves.

Phytochemicals	Pet Ether	Chloroform	Methanol
Alkaloids	+	-	+
Phytosterols	-	+	+
Flavanoids	-	-	-
Reducing sugars	-	+	+
Tannins	-	-	-
Saponins	-	+	+
Amino acids	-	-	-
Glycosides	+	+	+
Carbohydrates	-	+	+

(+) = Present and (-) = Absent

Table2.2: Minimum inhibitory concentrations (MICs) of the ethanol total extract of IKC and IKM against test organisms**.**

Sl.no	Name of Organisms	Name of extracts	
		IKM	**IKC**
1	*Escherichia coli*	6 mg	6 mg
2	*Pseudomonas aeruginosa*	4 mg	6 mg
3	*Bacillus subtilis*	4 mg	4 mg
4	*Klebsiella pnuemoniae*	2 mg	4 mg
5	*Salmonella typhi*	2 mg	ND
6	*Micrococcus luteus*	4 mg	ND

ND= Not determined since there is no inhibition.

REFERENCES

Amarowicz, R., & Pegg, R. B. (2019). Natural antioxidants of plant origin. In Advances in food and nutrition research (Vol. 90, pp. 1-81). Academic Press.

Balsano, C., & Alisi, A. (2009). Antioxidant effects of natural bioactive compounds. Current pharmaceutical design, 15(26), 3063-3073.

Bauer, A. W., Kirby, W. M. M., Sherris, J. C., & Turck, M. (1966). Antibiotic susceptibility testing by a standardized single disc method. Am J clin pathol, 45(4), 493-496.

Bhagat, J., Lobo, R., Parmar, V., & Ballal, M. (2011). In vitro Free Radical Scavenging Potential of Indian Habitant *Anisochilus carnosus* (LF) Wall. Chinese journal of natural medicines, 9(6), 456-460.

Blois, M.S. (1958). Antioxidant determinations by the use of stable free radical. Nature;181:1199-200.

Botterweck, A. A., Verhagen, H., Goldbohm, R. A., Kleinjans, J., & Van den Brandt, P. A. (2000). Intake of butylated hydroxyanisole and butylated hydroxytoluene and stomach cancer risk: results from analyses in the Netherlands cohort study. Food and Chemical Toxicology, 38(7), 599-605.

Burris, K. P., Davidson, P. M., Stewart, Jr, C. N., & Harte, F. M. (2011). Antimicrobial activity of yerba mate (*Ilex paraguariensis*) aqueous extracts against *Escherichia coli* O157: H7 and Staphylococcus aureus. Journal of Food Science, 76(6), M456-M462.

Dorman, H. D., & Deans, S. G. (2000). Antimicrobial agents from plants: antibacterial activity of plant volatile oils. Journal of applied microbiology, 88(2), 308-316.

Engin, A. B., Bukan, N., Kurukahvecioglu, O., Memis, L., & Engin, A. (2011). Effect of butylated hydroxytoluene (E321) pretreatment versus l-arginine on liver injury

after sub-lethal dose of endotoxin administration. Environmental toxicology and pharmacology, 32(3), 457-464.

Erdemoglu, N., Iscan, G., Sener, B., & Palittapongarnpim, P. (2009). Antibacterial, antifungal, and antimycobacterial activity of *Ilex aquifolium* leaves. Pharmaceutical Biology, 47(8), 697-700.

Euloge, S. A., Kouton, S., Dahouenon-Ahoussi, E., Sohounhloue, D. C. K., & Soumanou, M. M. (2012). Antifungal activity of Ocimum canum essential oil against toxinogenic fungi isolated from peanut seeds in post-harvest in Benin. International Research Journal of Biological Sciences, 1(7), 20-26.

Gochev, V., Dobreva, A., Girova, T., & Stoyanova, A. (2010). Antimicrobial activity of essential oil from Rosa alba. Biotechnology & Biotechnological Equipment, 24(sup1), 512-515.

Gokhale MS, Kokate CK and Purohit AP (2017). Pharmacognosy II. Pune: Nirali Prakashan; Chapter 4, pp 4.9 – 4.16.

Gülçin, İ., Huyut, Z., Elmastaş, M., & Aboul-Enein, H. Y. (2010). Radical scavenging and antioxidant activity of tannic acid. Arabian journal of chemistry, 3(1), 43-53.

Halliwell, B. (1991) Reactive oxygen species in living systems: Source, biochemistry, and role in human disease. Am. J. Med.; 91, S14–S22.

Halliwell, B., Gutteridge, J. M., & Aruoma, O. I. (1987). The deoxyribose method: a simple "test-tube" assay for determination of rate constants for reactions of hydroxyl radicals. Analytical biochemistry, 165(1), 215-219.

Hayek, S. A., Gyawali, R., & Ibrahim, S. A. (2013). Antimicrobial natural products. In A. Mendez-Vilas (Ed.), Microbial pathogens and strategies for combating them: Science, technology and education (Vol. 2); (pp. 910e921). Formatex Research Center.

Hügel, H. M., Jackson, N., May, B., Zhang, A. L., & Xue, C. C. (2016). Polyphenol protection and treatment of hypertension. Phytomedicine, 23(2), 220-231.

Jeong, S. H., Kim, B. Y., Kang, H. G., Ku, H. O., & Cho, J. H. (2005). Effects of butylated hydroxyanisole on the development and functions of reproductive system in rats. Toxicology, 208(1), 49-62.

Kumar, S., & Pandey, A. K. (2013). Chemistry and biological activities of flavonoids: an overview. The scientific world journal, 2013.

Lai, P. K., & Roy, J. (2004). Antimicrobial and chemopreventive properties of herbs and spices. Current medicinal chemistry, 11(11), 1451-1460.

Oyaizu, M. (1986). Studies on products of browning reaction antioxidative activities of products of browning reaction prepared from glucosamine. The Japanese journal of nutrition and dietetics, 44(6), 307-315.

Pelczar, M. J., Chan, E. C. S., & Krieg, N. R. (1988). Control of microorganisms, the control of microorganisms by physical agents. Microbiology, 469, 509.

Philippe, S., Souaïbou, F., Guy, A., Sébastien, D. T., Boniface, Y., Paulin, A., ... & Dominique, S. (2012). Chemical Composition and Antifungal activity of Essential oil of Fresh leaves of Ocimum gratissimum from Benin against six Mycotoxigenic Fungi isolated from traditional cheese wagashi. Research Journal of Biological Sciences, 1(22-27).

Prieto, P., Pineda, M., & Aguilar, M. (1999). Spectrophotometric quantitation of antioxidant capacity through the formation of a phosphomolybdenum complex: specific application to the determination of vitamin E. Analytical biochemistry, 269(2), 337-341.

Robak, J., & Marcinkiewicz, E. (1995). Scavenging of reactive oxygen species as the mechanism of drug action. Polish journal of pharmacology, 47(2), 89-98.

Savoia, D. (2012). Plant-derived antimicrobial compounds: alternatives to antibiotics. Future Bicrobiol. 7: 979-990.

Schinella, G., Fantinelli, J. C., Tournier, H., Prieto, J. M., Spegazzini, E., Debenedetti, S., & Mosca, S. M. (2009). Antioxidant and cardioprotective effects of *Ilex brasiliensis*: A comparative study with *Ilex paraguariensis* (yerba mate). Food Research International, 42(10), 1403-1409.

Sebranek, J. G., Sewalt, V. J. H., Robbins, K., & Houser, T. A. (2005). Comparison of a natural rosemary extract and BHA/BHT for relative antioxidant effectiveness in pork sausage. Meat science, 69(2), 289-296.

Sen, S., De, B., Devanna, N., & Chakraborty, R. (2013). Total phenolic, total flavonoid content, and antioxidant capacity of the leaves of Meyna spinosa Roxb., an Indian medicinal plant. Chinese journal of natural medicines, 11(2), 149-157.

Shirwaikar, A., Patel, B., Kamariya, Y., Parmar, V., & Khan, S. (2011). In vitro Free Radical Scavenging Potential of Defatted Ethanolic Extract of the Seeds of *Lepidium sativum* Linn. Chinese Journal of Natural Medicines, 9(6), 435-440.

Singleton, V. L., & Rossi, J. A. (1965). Colorimetry of total phenolics with phosphomolybdic-phosphotungstic acid reagents. American journal of Enology and Viticulture, 16(3), 144-158.

Si-Xiang, Z. H. O. U., Zhi-Rong, Y. A. O., Jun, L. I., & Peng-Fei, T. U. (2012). Flavonoids from the leaves of *Ilex cornuta*. Chinese Journal of Natural Medicines, 10(2), 84-87.

Stanner, S., Weichselbaum, E. (2013). Antioxidants, Editor(s): Benjamin Caballero, Encyclopedia of Human Nutrition (3rd Edition) (pp 88-99). Academic Press.

Szymanska, R., Pospíšil, P., & Kruk, J. (2018). Plant-derived antioxidants in disease prevention 2018. Oxidative medicine and cellular longevity, 2018.

Tajkarimi, M. M., Ibrahim, S. A., & Cliver, D. O. (2010). Antimicrobial herb and spice compounds in food. Food control, 21(9), 1199-1218.

Terao, J. (2009). Dietary flavonoids as antioxidants. Food factors for health promotion, 61, 87-94.

Thuong, P. T., Su, N. D., Ngoc, T. M., Hung, T. M., Dang, N. H., Thuan, N. D., & Oh, W. K. (2009). Antioxidant activity and principles of Vietnam bitter tea *Ilex kudingcha*. Food Chemistry, 113(1), 139-145.

Ultee, A., Bennik, M. H. J., & Moezelaar, R. J. A. E. M. (2002). The phenolic hydroxyl group of carvacrol is essential for action against the food-borne pathogen *Bacillus cereus*. Applied and environmental microbiology, 68(4), 1561-1568.

Xiu-Qin, L., Chao, J., Yan-Yan, S., Min-Li, Y., & Xiao-Gang, C. (2009). Analysis of synthetic antioxidants and preservatives in edible vegetable oil by HPLC/TOF-MS. Food Chemistry, 113(2), 692-700.

Xue, J., Davidson, P. M., & Zhong, Q. (2013). Thymol nano emulsified by whey protein-maltodextrin conjugates: The enhanced emulsifying capacity and antilisterial properties in milk by propylene glycol. Journal of agricultural and food chemistry, 61(51), 12720-12726.

Zhishen, J., Mengcheng, T., & Jianming, W. (1999). The determination of flavonoid contents in mulberry and their scavenging effects on superoxide radicals. Food chemistry, 64(4), 555-559.

CHAPTER - 3

IN VIVO EVALUATION OF *ILEX KHASIANA* FOR ITS ANALGESIC AND ANTI-INFLAMMATORY ACTIVITY ON SWISS ALBINO MICE MODEL.

INTRODUCTION

Nociception is an irksome sensation caused by physical factors such as injury, temperature, pressure or collisions or chemically induced by cytokines, neutrophins and chemokines. The inflammatory mediators may aid in locating the target source in treating nociception and inflammation. Many series of responses against this painful sensation mediated by the nervous system **(Browne and Kelly, 2017).**

As 90% of the anti-inflammatory drugs are reported to have toxic effect and iatrogenic reactions, thus treatment based on bioactive compounds are the urgent cries in combating anti-inflammation **(Lanas, 2009).** These naturally obtained compounds are the true candidate for antinociceptive and anti-inflammatory treatment because of their diverse chemical nature. Different part of the plants including the leaves, roots, fruits and barks are reported to have medicinal values. The presence of secondary metabolites like polyphenols, flavonoids, carotenoids, coumarins and terpenoids may be associated with the plant's medicinal values **(Dzoyem et al., 2017).**

Natural products play a significant role in developing new drugs because of the large varieties of bioactive compounds. The rapid development in the pharmaceutical industries have led to massive improvement in drug discovery and production. Synthetic drugs have occupied vast majority of pharmaceutical company and help in improving healthcare. However, with the advancement in research there are many notable adverse effects in utilizing the synthetic drugs. Many eminent drugs like aspirin and other steroidal and non-steroidal anti-inflammatory drugs (NSAIDs) are used extensively for

the treatment of inflammation as well as nociception. Agonizingly, these well-known drugs were known to have limitation in curing chronic inflammation and suspected to induced blood clotting resulting in increased heart attack and stroke **(Dzoyem et al., 2017).**

Inflammation is a physiological response of a body to neutralize or counteract pathogens, invading organisms as well as a process of tissue repair. It a complex process in which involves numerous mediators and events like chemokines like interferon-α (IFN-α), IFN-γ, interleukin (IL)-1, IL-8, histamine, 5-hydroxytryptamine (5-HT), leukotrienes, prostacyclins, prostaglandins, lymphokines and tumor necrosis factor-α (TNF-α) **(Serhan and Savill, 2005).** Inflammation might initiate various disease like asthma, rheumatoid arthritis, osteoporosis, obesity, cancer, cardiovascular disease and nervous system related diseases like Alzheimer's disease, depression and Parkinson's disease **(Laveti et al., 2013).**

Many of the *Ilex* species are known for their contributions in anticancer, antinociceptive and anti-inflammatory effects. In *I. mamillata* and *I. pubescens* are known for their anti-inflammatory activity which is believed to be associated with saponins a subclass of terpenoids **(Wang et al., 2008)**. The anti-inflammatory mechanism might be associated with inhibition of proinflammatory cytokines and cyclooxygenase (COX)-2 protein also by increasing IL-4 and IL-10 an anti-inflammatory cytokine. Quercetin and saponins in *I. paraguariensis* collaboratively impede iNOS and COX-2 through NF*k*B pathways **(Puangpraphant et al., 2009)**. Regular consumption of this yerba mate demonstrated deduction in both lipid peroxidation and free radicals **(Matsumo et al., 2009)**. *I. centrochinensis* in China is known to have anti-inflammatory activities. Besides its choleretic property *I. breviscuspis* is known to have antioxidant activities **(Filip and Ferraro, 2003)**. In this study, *I. khasiana* is scrutinized for its analgesic and anti-inflammatory properties.

MATERIALS AND METHODS

Chemicals and reagents

Carboxymethyl cellulose, Formalin, Xylene and Acetic Acid were purchased from HiMedia Laboratories Pvt. Ltd. Mumbai, India. Diclofenac sodium standard was procured from Sigma-Aldrich, USA and Morphine Sulphate Injection was obtained from Rusan Healthcare Pvt. Ltd. Mumbai, India

Housing and Handling of the Animals.

Swiss albino mice (20 – 25g) of both sexes were maintained at Institutional Animal House, RIPANS. The animals were kept in polyvinyl cages under fully controlled environment of temperature (24-25 ºC), 50% humidity and light and dark (12 h each) cycle. The animals were fed with standard food pellets and water *ad libitum* according to OECD guidelines. The Institutional Animal Ethics Committee of Regional Institute of Paramedical and Nursing Sciences approved the entire study vide letter no. **IAEC/RIPANS/25**, Aizawl, India.

Preparation of Ethanolic Extract

The two semi-solid extracts *I. khasiana* Chloroform Extract (IKC) and *I. khasiana* Methanol Extract (IKM) were dissolved in Carboxymethyl cellulose (CMC) and Normal saline (NS)respectively to obtain stock concentration of 50mg/ml respectively.

The animals were divided into 5 groups consisting of six mice in each group as follows:

1. Group I: Vehicle control
2. Group II: Standard drug
3. Group III: 100 mg/kg b.w of IKC/IKM
4. Group IV: 250 mg/kg b.w of IKC/IKM
5. Group V: 500 mg/kg b.w of IKC/IKM

Acute Toxicity

The acute toxicity study of different extracts of *Ilex khasiana* was performed according to Organization for Economic Co-operation and Development (OECD) guidelines. The animals of both sexes were used for the study (n=6). The animals were fasted 3-4 hours but water *ad libitum* before giving different extract of *Ilex khasiana*. The animals were observed for 14 days for any behavioral or physiological changes. If mortality was observed on 2-3 animals, then the treatment was considered as toxic dose.

ANALGESIC ACTIVITY OF *ILEX KHASIANA*

Acetic acid writhing test

Analgesic property of *Ilex khasiana* was assessed by Acetic acid induced writhing test **(Koster, 1959)**. Briefly, animals were divided into 5 groups consisting of 6 mice in each group. The first group received morphine (10 mg/kg, i.p.), a standard analgesic drug and the second group being the vehicle control (Normal saline for IKM and CMC for IKC p.o.). The third, fourth and fifth group received the extracts (IKM and IKC) 100, 250 and 500 mg/kg body weight p.o. respectively. Treatments were given 30 minutes prior to intra-peritoneal injection of 0.6%, 10 ml/kg body weight acetic acid (i.e. 0.01 ml/g). Writhes or abdominal constrictions were counted from 5 minutes after the injection up to 20 minutes and the result was expressed as percent protection using the following formula:

$$\text{Protection (\%)} = (N_c - N_t / N_c) \text{ X } 100$$

Where N_c is number of writhing in control, and N_t is the number of writhing in test animals.

Tail immersion test

Evaluation of *Ilex khasiana* on its analgesic activity was performed as per standard protocols with slide modification (**Aydin et al., 1999)**. Animals were divided

into 5 groups with 6 animals in each group and treatment were given 30 minutes before the test as mentioned in the above experiment. Temperature of water was maintained at 55 ± 0.5°C into which 5 cm of the mouse tail was immersed. The reaction time was measured 30 minutes before and after the treatment, denoted by the time at which the mouse withdrew the tail from the water. The cut-off time was fixed at 10 seconds to avoid tissue damage. The test was continued every 30 minutes up to 120 minutes.

Percentage latency period was calculated as follows:

$$= (T_n\text{-}T_0)/ (10\text{-}T_0) \times 100$$

Where 10 is the cut-off time, T_0 is the latency time before treatment and T_n is latency after treatment.

Hot Plate Test

The hot plate test was used to calculate analgesic activity using the method explained by Eddy and Leimbach (**1953)** with minor modifications. In this test, mice were placed on a hot plate having a temperature of 55 ± 1^{o}C (UGO BASILE, 35100, Italy) before the treatment and at 30, 60, 90, 120 and 180 minutes after the administration of the treatment. The time taken by the mouse for licking or jumping were recorded in order to analyzed the analgesic effect of IKC and IKM with compared to Morphine, the standard drug on electrical heat-induced pain.

The percentage protection against thermal pain stimulus was calculated according to the following formula **(Turner., 1965)**:

Protection (%) against thermal stimulus

$$= \{\text{Test mean } (T_a) - \text{Control mean } (T_b)\}/\text{Control mean } (T_b) \times 100$$

ANTI-INFLAMMATORY ACTIVITY OF *ILEX KHASIANA*

Xylene induced ear edema model

The mice were divided into 5 groups (6 mice in each group) and xylene induced ear edema model was used to determine the anti-inflammatory activity of both IKC and IKM **(Cao., 1992).** Each group received treatment saline (p.o.), the IKM or IKC (100, 250 and 500 mg/kg, p.o.), diclofenac sodium (20 mg/kg, i.p.) 30 minutes before topical administration of 30 µl of xylene in the posterior and anterior surfaces of the right ear. The left ear served as control. After 15 minutes, the animals were euthanized and circular section (7 mm) were taken using cork-borer and the weight for both the sections. The weight difference was the edematous response. The percentage inhibition was calculated as

$$\text{Inhibition (\%)} = [1 - E_t / E_c] \times 100$$

where E_t and E_c are the average weight of the edemas in the sample-treated and control groups, respectively.

Formalin-induced paw edema test

The anti-inflammatory activity of *Ilex khasiana* extracts (IKC and IKM) was performed by using formal induced paw edema test following the standard protocol with minor modification **(Tjølsen et al., 1992)**. Treatments with saline (p.o.), the IKM or IKC (100, 250 and 500 mg/kg, p.o.), diclofenac sodium (20 mg/kg, i.p.) were given 1 hour prior to formalin injection (n = 6 per group). The paw thickness was measured bilaterally before injecting formalin. Subcutaneously, the paw was injected with 20 µl/paw of formalin ((2.5 % in 0.9 % sterile saline). Paw thickness was re-measured at 1, 2, 3, 4, 5 and 6 hour(s) after formalin injection to determine the edema using Plethysmometer (UGO BASILE, 7140, Italy).

The difference in paw diameters was taken as inflammatory response.

% Inhibition =

$$= \{(V_t - V_o)_{control} - (V_t - V_o)_{treated} / (V_t - V_o)_{control}\} \times 100$$

where $(V_t - V_o)_{control}$ is the difference in the volume displacement in Plethysmometer at different hours in control group, $(V_t - V_o)_{treated}$ is the difference in the volume displacement in Plethysmometer at different hours in treated group.

Carrageenan induced paw edema test

Carrageenan induced paw edema in mice a well-known method for screening anti-inflammatory potential of a bioactive compounds. The anti-inflammatory activity of IKC and IKM were observed by following a standard protocol with slight modification **(Winter., 1962**). The mice were pretreated 1 hour before administration of 50 µl of 1% λ carrageenan (in 0.9% saline) with diclofenac sodium (20 mg/kg), normal saline and 100, 250 and 500 mg/kg b.w of both IKC and IKM separately. The paw edema was measured hourly interval at 1, 2, 3, 4, 5, and 6 hours using vernier caliper. The paw size before the treatment served as the initial paw size/control. The inhibitory effect was determined by using following formula:

% Inhibition =

$$= \{(P_t - P_o)_{control} - (P_t - P_o)_{treated} / (P_t - P_o)_{control}\} \times 100$$

where $(P_t - P_o)_{control}$ is the difference in the paw size at different hours in control group, $(P_t - P_o)_{treated}$ is the difference in the paw size at different hours in treated group.

Data Analysis

Data were analyzed using statistical software GraphPad Prism version 8.0.2 One-way analysis of variance (ANOVA) test was used to ascertain the significance of variations followed by Tukey HSD post hoc test. Data are shown as mean ± S.E.M. All data were considered significant at $P < 0.05$.

RESULTS

Acute Toxicity

At the dose of 2000 mg/kg b.w, the extract did not show any sign of lethality and the animal behavior did not display any sign of discomfort.

Acetic acid Writhing test

Acetic acid induced abdominal constriction is a method that involves prostaglandin pathway mediated local peritoneal cells to analyze peripherally acting analgesics **(Ribeiro et al., 2000)**. The analgesic activity that is found in both IKM and IKC extracts might be the result of prostaglandin pathways acting mechanism of the extracts. Significant inhibition of abdominal nociception was observed upon oral treatment of IKC and IKM **(Figure 3.1 and Figure 3.2)**. At 250 mg/kg b.w, IKC showed 25.50% inhibition and IKM showed maximum inhibition of 30.95%. Among the treatment groups in both the extracts which is comparatively lower than the inhibition induced by morphine (56.47%) **(Table 3.2)**. The mean difference of each treated group in comparison with the standard drug showed that only IKM at 250 mg/kg b.w was found to have significant variation comparing with the control while all the other treatment groups in IKC and IKM were statistically significant when compared with the standard drug, Morphine **(Figure 3.1 and Figure 3.2)**.

Tail immersion test

The tail emersion test was employed to determine the antinociceptive activity of *I. khasiana.* The reaction time was measured for all the untreated control group, morphine and groups that received 100, 250 and 500 mg/kg b.w of IKC/IKM. Plots of changes in latency at different time intervals (30, 60, 90 and 120 minutes) for both the extract was given in **Figure 3.3 and Figure 3.4.** The results showed dose-dependent inhibition **(Table 3.3 and.** Among the two extracts, IKC (250 mg/kg b.w) showed highest inhibition 68.96 at 90 minutes but IKM showed highest inhibition 50% at 120 minutes after administration of the extracts **(Table 3.5 and Table 3.6).**

Total Analgesia Coverage (AUC) in Tail emersion Test

AUC of IKC

Area under the Curve (AUC) of response versus time was used to determine the total analgesia coverage time of both IKC in tail emersion method. As shown in **(Table 3.3)** Morphine showed its peak response at 60 minutes **(8.40 seconds)** while the extract IKC (250 mg/kg) showed it peak response at 90 minutes **(7.75 seconds).** Thus, the total area under the curve of morphine was 13.50, 5.65 for CMC (vehicle control), 7.86, 11.95, 9.63 secs for IKC at 100, 250, and 500mg/kg, respectively.

AUC of IKM

Total analgesia coverage was estimated for IKM using tail emersion test. Among the different dosages IKM (250 mg/kg) was found to be most active at 90 minutes (8.40 seconds) at which morphine latency period was 9 seconds at the same time point. The Area under the curve for IKM at different dose was 7.43, 13.24, 12.69 (100, 250, and 500 mg/kg, respectively) while 6.04 was for normal saline and 14.01 for morphine **(Table 3.4)**

Hot Plate Test

The latency time in Hot plate test showed dose dependent increase throughout the test time points **(Figure 3.5 and Figure 3.6)**. IKC and IKM were selected for the analgesic activity analysis of *I. khasiana* at three different doses (100, 250 and 500 mg/kg b.w). There were significant variations from the control group at 250 and 500 mg/kg b.w after 90 minutes of the treatment for both the extracts. Maximum inhibition for IKC (76.19%) was given by 500 mg/kg b.w at 180 minutes after the oral administration, while 250 mg/kg b.w of IKM exhibited maximum inhibition of 79.36% at 180 minutes after the treatment **(Table 3.9 and Table 3.10)**. Comparatively, IKM showed better inhibitory effect than IKC in the hot plate test.

Total Analgesia Coverage (AUC) in Hot plate Test

AUC of IKC

The analgesic potential of IKC was determined using Hot plate method **(Table 3.7).** Morphine was found to be the most effective when compared to the extracts giving the highest peak at 90 minutes (18.4 seconds). The area under curve were found to be 20.33 for CMC (vehicle control), 48.04 for morphine and 29.90, 41.05 & 40.64 for IKC at 100, 250 and 500 mg/kg respectively.

AUC of IKM

Total analgesia coverage was estimated for IKM using Hot plate test. Among the three dosage IKM (250mg/kg) gave the highest peak response at 17.4 seconds which was slightly lower compared to the standard morphine 18.4 seconds at 90 minutes. The Area under the curve for IKM at different dose was 33, 42.85 and 38.82 (100, 250, and 500 mg/kg, respectively) while 22.36 was for normal saline and 48.04 for morphine as given in **Table 3.8**.

Xylene induced Ear Edema.

The anti-inflammatory activity of IKC and IKM were shown in **Figure 3.7 and Figure 3.8.** The vehicle control group receiving CMC showed increase in ear weight up to 7.75 while the lowest mean of weight was observed in 500 mg/kg. Diclofenac gave the highest inhibition of 50.97% and oral treatment of IKC showed 9.68, 19.35 and 24.75 % at 100, 250 and 500 mg/kg respectively **(Table 3.11)**. While groups that received IKM exhibited inhibition percentage of 18.42, 36.84 and 31.58 at 100, 250 and 500 mg/kg respectively. Notably, 250 mg/kg dose has a higher inhibitory effect than the higher dose 500 mg/kg b.w in IKM treated group **(Table 3.12)**. On both the extracts at different doses, only IKM 250 mg/kg showed significant variation from the groups that received vehicle control.

Carrageenan induced paw edema

The inhibition potency of *I. khasiana* on carrageenan induced paw edema at different time points was given in **Figure 3.9 and 3.10** for IKC and IKM. At dose of 250 mg/kg b.w, IKC and IKM showed significant variation at 5 hours after the treatment compared to control group. IKC and IKM (250 mg/kg b.w) exhibited highest inhibition percentage of 28.16% and 36.21 at 6 hours respectively which was slightly lower when compared to reference drug diclofenac sodium 47.41% **(Table 3.15 and Table 3.16)**.

AUC of IKC

The total anti-inflammatory area coverage of IKM was represented by Area under coverage (AUC). The inflammation was highest at 2 hours after subcutaneous injection of carrageenan for all the treatment groups. The AUC for IKC was 23.78, 22.02 and 22.67 at 100, 250 and 500 mg/kg b.w which was much lower than the vehicle control (24.81) **(Table 3.13)**.

AUC of IKM

Total anti-inflammatory coverage was estimated for IKM using paw edema induced by carrageenan. The IKM (250mg/kg) showed the lowest area (20.91) among the treated groups which was slightly higher than diclofenac (19.78). IKM 100 and 500 showed 23.47 and 21.60 which was comparatively lower than 24.81 exhibited by normal saline treated group (**Table 3.14)**.

Formalin induced Mice Paw Edema

The anti-inflammatory activity of *I. khasiana* was determined using Formalin induced paw edema. Different doses (100, 250 and 500mg/kg) of IKC and IKM was administered orally. Mean volume change in the paw edema was displayed in **Figure 3.11** and **Figure 3.12** for both the extracts. The highest peak was observed at 2 hours after formalin administration and decline gradually in time. Diclofenac sodium (20mg/kg b.w) gave the highest inhibitory effect compared to the groups that received

both IKC and IKM. Animals that were treated with IKC revealed statistical difference at 250 and 500 mg/kg b.w at 4, 5 and 6 hours after sub-planter injection of formalin. In groups that received IKM, 250 mg/kg b.w showed statistical significance. The IKC extract (250 mg/kg b.w) revealed highest inhibitory percentage of 49.69 at 6 hours **(Table 3.17)** while 36.21% was the highest inhibition given by IKM (250 mg/kg b.w) at the same time point **(Table 3.18)**.

AUC of IKC

The total anti-inflammatory area coverage of IKC was shown in **Table 3.15.** The area coverage was smallest for diclofenac sodium which was 1.32, while at 100, 250 and 500 mg/kg b.w of the extract gave 1.84, 1.60 and 1.70. IKC at 250 mg/kg b.w showed the lowest area coverage, having the best anti-inflammatory effect.

AUC of IKM

The total area of inflammation was given by the vehicle control (2.02) and diclofenac gave the lowest area coverage (1.32). IKM at 250 mg/kg b.w showed the highest inhibitory effect (1.50), followed by 1.59 and 1.73 for both 500 and 100 mg/kg b.w respectively (**Table 3.16)**.

DISCUSSION

Inflammation resulted when the body is invaded by harmful foreign bodies or suffered tissue injuries to protect, repair, prevent and heal the body from damages. It showed symptoms like swelling, redness and severe heat which give rise to unpleasant sensation called pain **(Stewart and Beart, 2016).** Pro-inflammatory mediators like COX-2 and iNOS increase upon inflammation, which led to the rise in the level of cytokines such as TNF-α, IL1β, and IL-6 as well as PGE2 and NO too. This in turn played a crucial role in pathogenesis such as Parkinson's and Alzheimer's disease **(Shao et al., 2013)**. Thus, blocking the pro-inflammatory mediators using bioactive compounds is a long-time target, in which natural products like phenols are known to inhibit inflammation.

Inflammation is initiated with the up regulation of enzymes like iNOS and cyclooxygenase-2 (COX-2). Therefore, inhibition of COX pathway of arachidonic acid metabolism which produce prostaglandins is required for anti-inflammatory activity **(Oyedapo et al., 2008)**. Besides its role in causing edema and erythema, prostaglandins are known to be hyperalgesic and have vasodilating activity. Thus, anti-inflammatory drug must have analgesic activity as well. Many side effects are reported in using Nonsteroidal anti-inflammatory drugs (NSAIDs) like ulcers, and renal ailments due to their non-specific inhibition of COX-1 and COX-2 isoforms in spite of its celebrated anti-inflammatory and analgesic activity **(Kaushik et al., 2012)**.

Non-steroidal anti-inflammatory drugs (NSAIDs) are the most prominent clusters of drugs that are widely consumed because of their antipyretic, analgesic and anti-inflammatory properties. In 1982, Nobel Prize in Medicine was awarded to John R. Vane for describing the working mechanism of Acetylsalicylic acid (ASA) which was introduced under a brand name 'Aspirin' **(Robert, 2001)**. The blooming of NSAID drugs continue to rise with the discovery of ibuprofen and indomethacin in 1969 and 1964. Globally, 2.5% of the total dollar spent on prescription was on NSAID drugs serving people having arthritis Reiter's syndrome, systemic lupus erythematosus**,**

thrombosis, pericarditis, gout, gouty arthritis, ankylosing spondylitis, patent ductus arteriosus and Kawasaki disease **(Bleumink et al., 2003; Poddubnyy et al., 2009)**.

Many NSAIDs are metabolized by liver and glucuronidation by kidney enzymes may occur for some drugs like ibuprofen and ketoprofen **(Harirforoosh and Jamali, 2009)**. General classification of NSAIDs is based on two factors – selective and non-selective COX-2 inhibitors **(Warner et al., 1999)**.

A series of reaction called arachidonic acid cascade give rise to prostanoids that are mediators of inflammation including prostaglandins (PGs), prostacyclins and thromboxanes. The purpose of NSAIDs is to inhibit COX enzymes which produce prostaglandins from arachidonic acid **(Smyth et al., 2009)**. This converts arachidonic acid to prostaglandins (PG) G2 later converted to PGD2, PGE2, PGF2α, PGI2, and thromboxane A2. The inhibition of prostaglandins is the key to NSAID's anti-inflammatory properties **(Rao and Knaus, 2008)**. Even though the anti-inflammatory properties of NSAIDs are due to inhibition of PGE2 and PGI2, this also has an adverse effect on human physiological system **(Fitzgerald, 2003)**.

In spite of its medical importance, NSAIDs are known to have renal, gastrointestinal and cardiovascular side effect. There are two forms of renal failures (Acute and Chronic failure). Kidney is the hub for synthesizing all the major prostaglandins like PGE2, PGF2α and PGI2 **(Clària, 2003)**. PGE2 acts as vasodilator responsible for fluid and salt excretion, they are present in nephrons, collecting tubules and renal medullary interstitial cells **(Antonucci et al., 2009)**. Thus, inhibition of PGs results lower renal function, interstitial nephritis, hyperkalemia and electrolytes containing fluid retention **(Whelton and Watson, 1998)**.

Short term and reversible side effects of NSAIDs on GI was commonly reported which include dyspepsia, heartburn and nausea **(Sostres et al., 2010).** The side effects found in GI are usually in the upper GI and lower GI side effects are not thoroughly studied **(Davies and Jamali, 1997)**. In serious cases a condition called diaphragm

disease was reported **(De Petris and López, 2008).** Meanwhile, NSAIDs are also known to have cardiovascular effect. Higher incidence of hypertension was reported in using NSAIDs **(Forman et al., 2007)**. Using of COX-2 inhibitor without arachidonic acid tends to have higher cardiovascular related risks **(Belknap, 2002)**.

Selection of extracting solvents is crucial to obtain different plants derived natural products. Notably, crude extracts may show better activity than the isolated compounds where synergistic effect of bioactive compounds are attributed to the activity **(Umar et al., 2014)**. But in many cases, the isolated compounds like amentoflavone, pseudohypericin, and hyperforin are known to have better anti-inflammatory activity than the crude extract **(Hammer et al., 2007).** Thus, numerous isolated compounds occupied prominent place as an anti-inflammatory compound. Ursolic acid and oleanolic acid are isomers sharing several structural similarities with a good anti-inflammatory activity **(Gupta et al., 1969)**. Among the commonly available polyphenols – quercetin, hesperidin and rutin are confirmed to have a significant anti-inflammatory activity **(Azab et al., 2016)**. Thus, phenolic compounds not only exhibited antioxidants properties but also anti-inflammatory activity by modulating the signaling pathway of inflammation **(Costa et al., 2012)**.

Many *Ilex* species are known to have analgesic and anti-inflammatory properties. *I. paraguariensis* is known as Yerba mate, a common beverage in Southern Larin America is known to have bioactive compounds like saponins, methylxanthines and phenolic compounds. These bioactive compounds contributed enormously in the plants anti-inflammatory activity. *I. paraguariensis* reduced Adenosine deaminase (ADA) enzymes, an anti-inflammatory mediator besides down regulating NF-κB activation and p65 phosphorylation **(Luz et al., 2016)**.

I. cornuta leaves inhibited Nitric oxide synthase and cyclooxygenase-2 attenuating the production of NO and PGE2, inflammation mediator. Studies have shown that there are eight responsible compounds for its anti-inflammatory activity. Among them kaempferol and isoquercetin play a major role in suppressing IL-6, IL-1b,

and PGE2 production synergistically, as there is no report on anti-inflammatory effect of this individual compounds **(Kim et al., 2017)**. *I. vomitoria,* yaupon holly is known to contain quercetin and kaempferol 3-rutinosides besides other flavonols and caffeoylquinic acid derivatives. It exhibited anti-inflammatory activity by up-regulation of microRNA-146a (miR-146a) which down regulates pro-inflammatory NF-κB activation **(Noratto et al., 2011).**

The two extracts IKC and IKM exerted notable analgesic as well as anti-inflammatory activity when compared with the reference drugs morphine and diclofenac respectively. Among the two, IKM showed slightly higher activity in both the cases, which showed that methanol is more suitable solvent for extracting the compounds that are responsible for its anti-inflammatory and antinociceptive activity. In many situations, in both IKC and IKM, the treatment dose of 250 mg/kg b.w is found to be the most suitable dose as the efficacy of the plant did not increase upon increasing concentration. So, *I. khasiana* is expected to be loaded with phytochemical compounds like phenols, saponin and phytosterols like those that are found in other *Ilex* species.

SUMMARY

Therefore, the present studies deal with the analgesic and anti-inflammatory properties if *I. khasiana.* Dolefully, there is no record of any kind regarding the plant's medicinal values and phytochemical compositions. In this study, the two extract IKC and IKM showed astonishing analgesic and anti-inflammatory properties. In acetic acid induced abdominal writhing test, highest percentage of inhibition 30.95% was found in 250 mg.kg b.w dose of IKM followed by 25.50% in IKC at the same dose among the extract treatment groups. In tail emersion test highest analgesic percentage was found at 90 minutes in IKC (250 mg.kg b.w) 68.96, but IKM (250 mg.kg b.w) exhibited highest inhibition of 50% at 120 minutes. Surprisingly, IKC (500 mg.kg b.w) at 180 minutes gave the highest inhibition percentage of 76.19 but IKM (250 mg.kg b.w) at 180 minutes inhibited 79.36% of the pain which was slightly higher when compared to 60.60% of IKM (500 mg.kg b.w) at the same time point. The anti-inflammatory properties of IKC and IKM was determined by Xylene induced ear edema, formalin induced paw edema and carrageenan induced paw edema. As usual, the treatment dose of 250 mg/kg b.w seemed to be the best dose in both the extracts in all the three parameters. In xylene induced ear edema test IKM gave 36.84% of inhibition and it was higher than IKC (500 mg/kg b.w) which was found to be 24.73. After 6 hours in formalin induced edema, IKC (250 mg/kg b.w) gave 49.69% of inhibition which was higher than 36.21% inhibition exerted by IKM. In both the paw edema test, at 2 hours after inducing inflammation, the peak was comparatively high and reduced gradually as the time increases. Carrageenan paw edema was measured for 6 hours in an hourly interval. Inhibition of edema increased with time, IKM (250 mg/kg b.w) has a percentage of 36.21% and IKC at the same dose has 28.16% of inhibition. From the results stated above, *I. khasiana* being critically endangered species must get special attention and exploration of its medicinal values must be done extensively.

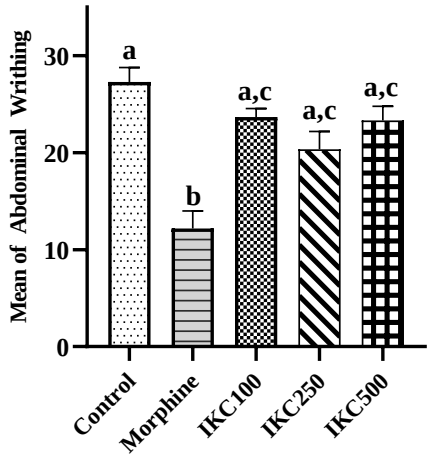


Figure 3.1: Analgesic effect of IKC on acetic acid induced writhing test. Values are expressed as Mean ± SEM. Different letters indicate significant variation at P<0.05 (ANOVA followed by Tukey multiple comparison test). (IKC= *Ilex khasiana* chloroform extract)**.**

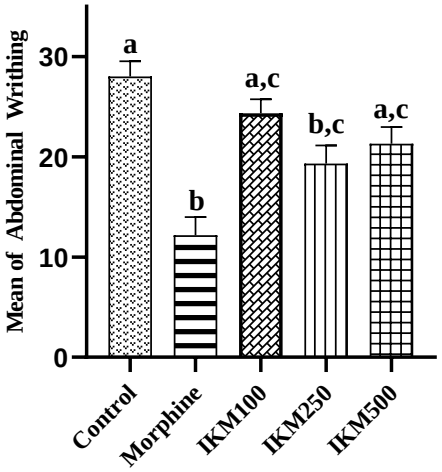


Figure 3.2: Analgesic effect of IKM on acetic acid induced writhing test. Values are expressed as Mean ± SEM. Different letters indicate significant variation at P<0.05 (ANOVA followed by Tukey multiple comparison test). (IKM= *Ilex khasiana* methanol extract).

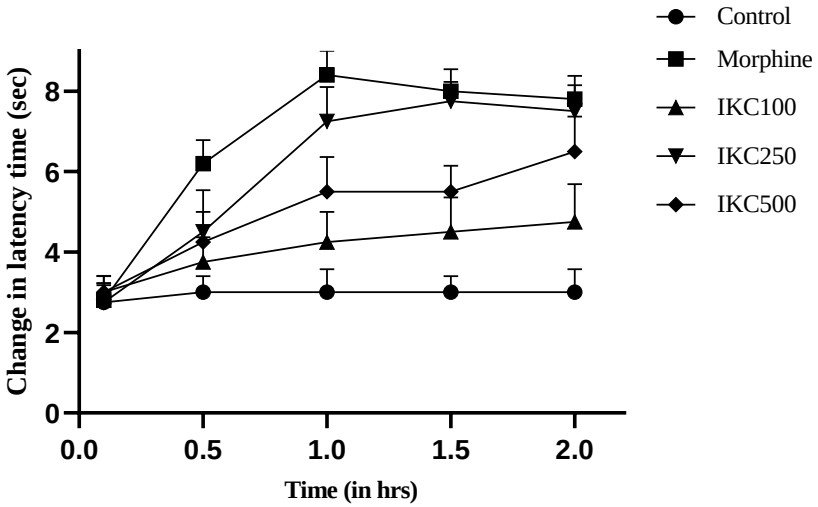


Figure 3.3: Analgesic effect of IKC on latency time in Tail emersion test. Values are expressed as Mean ± SEM. Different letters indicate significant variation at P<0.05 (ANOVA followed by Tukey multiple comparison test). (IKC= *Ilex khasiana* chloroform extract)**.**

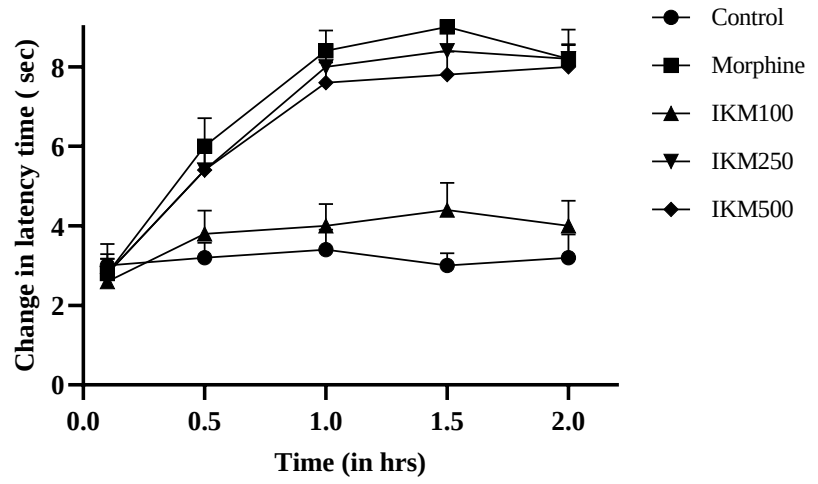


Figure 3.4: Analgesic effect of IKM on latency time in Tail emersion test. Values are expressed as Mean ± SEM. Different letters indicate significant variation at P<0.05 (ANOVA followed by Tukey multiple comparison test). (IKM= *Ilex khasiana* methanol extract)**.**

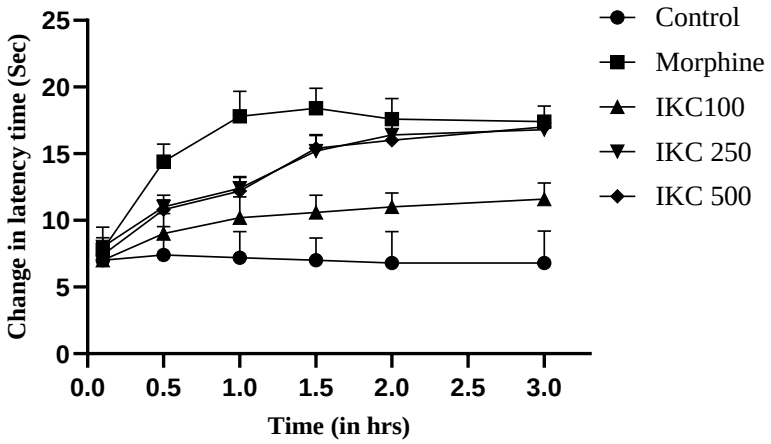


Figure 3.5: Analgesic effect of IKC on latency time in Hot plate test. Values are expressed as Mean ± SEM. Different letters indicate significant variation at P<0.05 (ANOVA followed by Tukey multiple comparison test). (IKC= *Ilex khasiana* chloroform extract)**.**

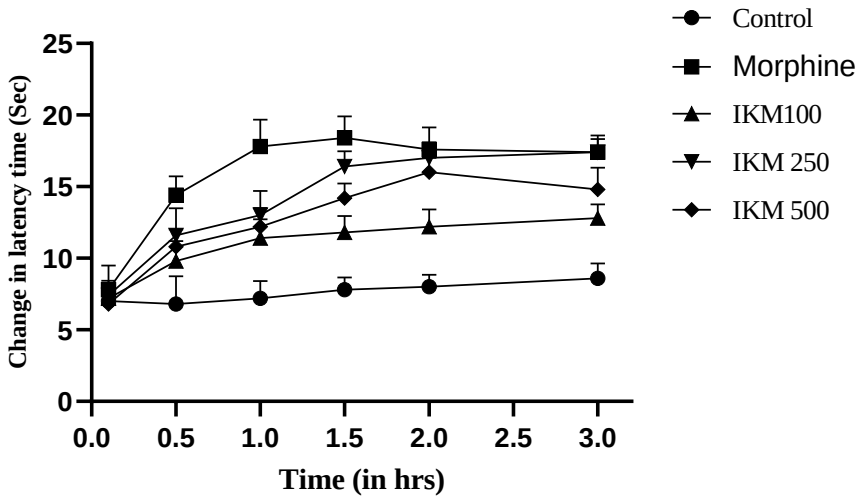


Figure 3.6: Analgesic effect of IKM on latency time in Hot plate test. Values are expressed as Mean ± SEM. Different letters indicate significant variation at P<0.05 (ANOVA followed by Tukey multiple comparison test). (IKM= *Ilex khasiana* methanol extract)**.**

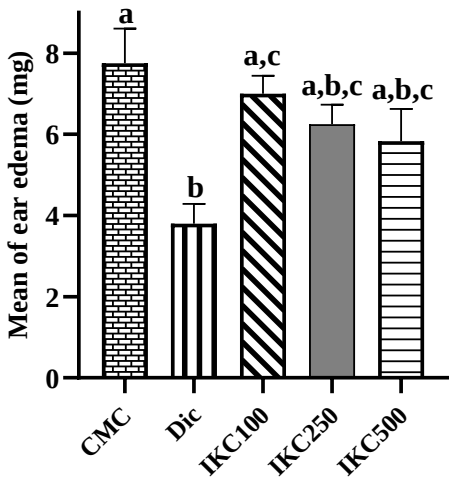


Figure 3.7: Anti-inflammatory effect of IKC in Xylene induced ear edema. Values are expressed as Mean ± SEM. Different letters indicate significant variation at P<0.05 (ANOVA followed by Tukey multiple comparison test). (IKC= *Ilex khasiana* chloroform extract)**.**

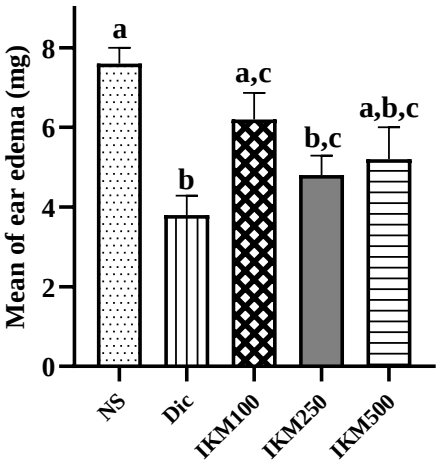


Figure 3.8: Anti-inflammatory effect of IKM in Xylene induced ear edema. Values are expressed as Mean ± SEM. Different letters indicate significant variation at P<0.05 (ANOVA followed by Tukey multiple comparison test). (IKM= *Ilex khasiana* methanol extract)**.**

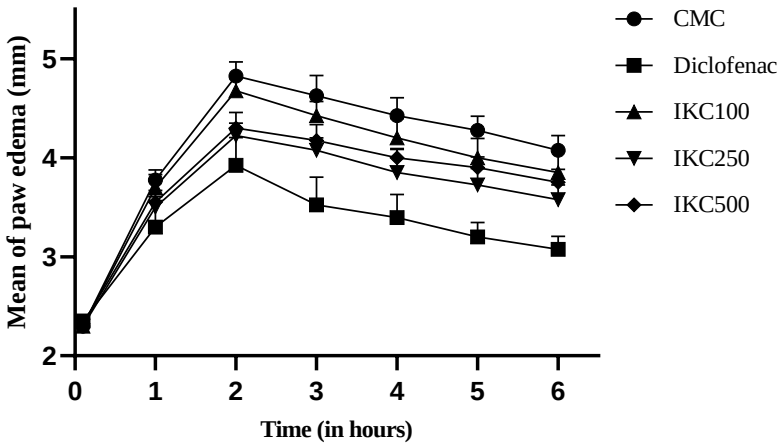


Figure 3.9: Anti-inflammatory effect of IKC in Carrageenan induced paw edema. Values are expressed as Mean ± SEM. Different letters indicate significant variation at P<0.05 (ANOVA followed by Tukey multiple comparison test). (IKC= *Ilex khasiana* chloroform extract)**.**

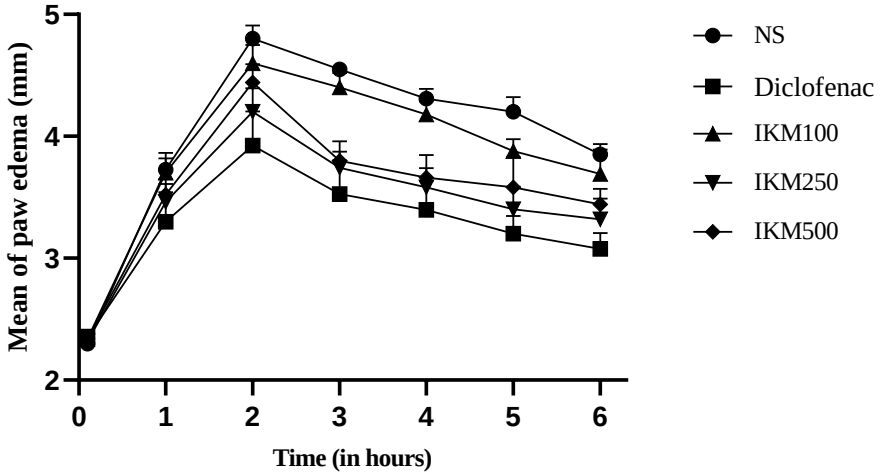


Figure 3.10: Anti-inflammatory effect of IKM in Carrageenan induced paw edema. Values are expressed as Mean ± SEM. Different letters indicate significant variation at P<0.05 (ANOVA followed by Tukey multiple comparison test). (IKM= *Ilex khasiana* methanol extract)**.**

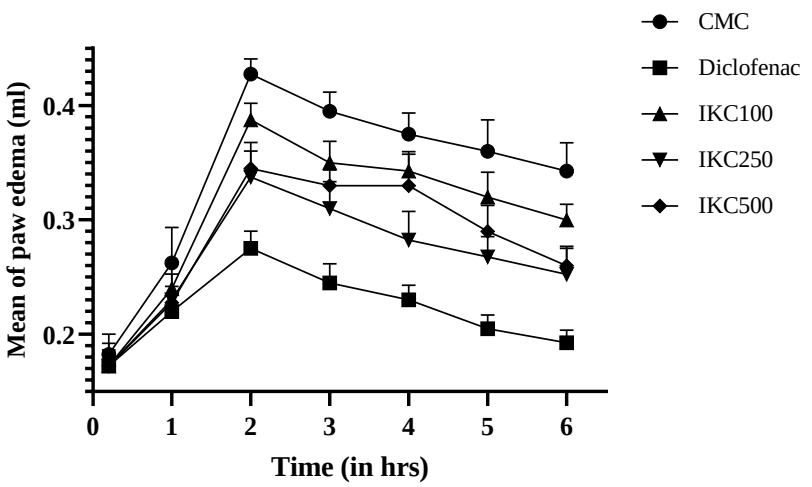


Figure 3.11: Anti-inflammatory effect of IKC in Formalin induced paw edema. Values are expressed as Mean ± SEM. Different letters indicate significant variation at P<0.05 (ANOVA followed by Tukey multiple comparison test). (IKC= *Ilex khasiana* chloroform extract)**.**

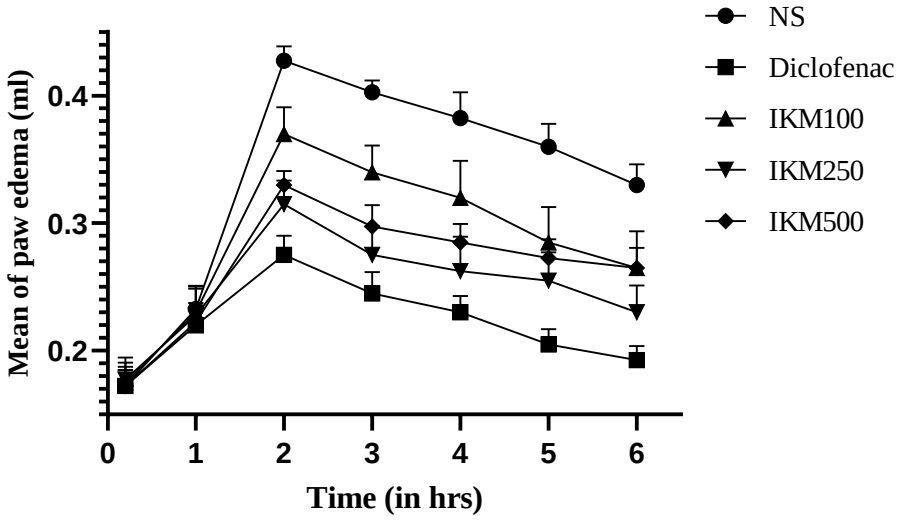


Figure 3.12: Anti-inflammatory effect of IKM in Formalin induced paw edema. Values are expressed as Mean ± SEM. Different letters indicate significant variation at P<0.05 (ANOVA followed by Tukey multiple comparison test). (IKM= *Ilex khasiana* methanol extract)**.**

Table 3.1: Effect of the IKC and morphine on pain threshold of mice on Acetic Acid writhing Test.

Treatment (mg/kg b.w)	**Time spent in Writhing (in seconds)**	**% Inhibition**
Control (CMC)	27.29±1.48 [a]	
Morphine$_{10}$	12.18±1.81 [b]	55.35
IKC$_{100}$	23.66±0.88 [a,c]	13.29
IKC$_{250}$	20.33±1.85 [a,c]	25.50
IKC$_{500}$	22.00±2.08 [a,c]	19.40

Values are Mean ± S.E.M.; (n=6). Different letters are significantly different at P<0.05 (ANOVA followed by Tukey's test)

Table 3.2: Effect of the IKM and morphine on pain threshold of mice on Acetic Acid writhing Test.

Treatment (mg/kg b.w)	**Time spent in Writhing (in seconds)**	**% Inhibition**
Control (NS)	28.00±1.52 [a]	
Morphine$_{10}$	12.18±1.81 [b]	56.47
IKM$_{100}$	24.33±1.45 [a,c]	13.09
IKM$_{250}$	19.33±1.85 [b,c]	30.95
IKM$_{500}$	25.58±1.68 [a,c]	22.61

Values are Mean ± S.E.M.; (n=6). Different letters are significantly different at P<0.05 (ANOVA followed by Tukey's test)

Table 3.3: Effect of IKC at different latency period in Tail emersion test.

Treatment (mg/kg)	Latency period					
	Pre treatment	**30 (minutes)**	**60 (minutes)**	**90 (minutes)**	**120 (minutes)**	**AUC**
Control (CMC)	2.75±0.48	3.00±0.40[a]	3.00±0.57[a]	3.00±0.40[a]	3.00±0.57[a]	5.65
Morphine$_{10}$	2.80±0.37	6.20±0.58[b]	8.40±0.60[b]	8.00±0.54[b]	7.80±0.58[b]	13.50
IKC$_{100}$	3.00±0.41	3.75±0.62 [a,b]	4.25±0.75 [a,c]	4.50±0.86 [a,c]	4.75±0.95 [a-d]	7.86
IKC$_{250}$	2.75±0.48	4.50±0.47 [a,b]	7.25±1.04 [b,c,d]	7.75±0.57 [b,d]	7.50±0.64 [b,c]	11.95
IKC$_{500}$	3.00±0.41	4.25±0.75 [a,b]	5.50±0.75 [a-d]	5.50±0.65 [a-d]	6.50±0.41 [b,d]	9.63

Values are Mean ± S.E.M.; (N=6). Different letters are statistically significant at P<0.05 (ANOVA followed by Tukey's test)

Table 3.4: Effect of IKM at different latency period in Tail emersion test.

Treatment (mg/kg)	Latency period					
	Pre treatment	**30 (minutes)**	**60 (minutes)**	**90 (minutes)**	**120 (minutes)**	**AUC**
Control (NS)	3.00±0.55	3.20±0.37[a]	3.40±0.51[a]	3.00±0.31[a]	3.20±0.58 [a]	6.04
Morphine$_{10}$	2.80±0.37	6.00±0.70[b]	8.40±0.77 [b]	9.00±0.31[b]	8.20±0.37 [b]	14.01
IKM$_{100}$	2.60±0.24	3.80±0.58 [a,b]	4.00±0.55 [a,c]	4.40±0.68 [a,c]	4.00±0.63 [a,c]	7.43
IKM$_{250}$	2.80±0.48	5.40±0.74 [a,b]	8.00±0.81 [b,d]	8.40±0.60 [b,d]	8.20±0.70 [b,d]	13.24
IKM$_{500}$	2.80±0.37	5.40±0.51 [a,b]	7.60±0.51 [b,d]	7.80±0.58 [b,d]	8.00±0.54 [b,d]	12.69

Values are Mean ± S.E.M.; (N=6). Different letters are statistically significant at P<0.05 (ANOVA followed by Tukey's test)

Table 3.5: Percentage inhibition of IKC different latency period in Tail emersion test**.**

Treatment (mg/kg)	**% Inhibition at 30 minutes**	**% Inhibition at 60 minutes**	**% Inhibition at 90 minutes**	**% Inhibition at 120 minutes**
Control (CMC)	-	-	-	-
Morphine$_{10}$	47.22	77.77	72.22	61.11
IKC$_{100}$	10.71	17.85	21.42	25.00
IKC$_{250}$	24.13	62.06	68.96	65.51
IKC$_{500}$	17.85	35.71	35.71	50.57

Table 3.6: Percentage inhibition of IKM different latency period in Tail emersion test.

Treatment (mg/kg)	**% Inhibition at 30 minutes**	**% Inhibition at 60 minutes**	**% Inhibition at 90 minutes**	**% Inhibition at 120 minutes**
Control (NS)	-	-	-	-
Morphine$_{10}$	44.44	72.22	63.88	61.11
IKM$_{100}$	16.21	18.92	24.32	18.92
IKM$_{250}$	25.00	38.88	47.22	50.00
IKM$_{500}$	22.22	36.11	44.44	41.66

Table 3.7: Effect of the IKC (po)and morphine (i.p.) on pain threshold of mice on Hot Plate test

Treatment (mg/kg)	Latency period						
	Pre treatment	30 (minutes)	60 (minutes)	90 (minutes)	120 (minutes)	180 (minutes)	AUC
Control (CMC)	7.00±1.41	7.40±2.12 [a]	7.20±1.94 [a]	7.00±1.67 [a]	6.80±2.35 [a]	6.80±2.38 [a]	20.33
Morphine$_{10}$	7.80±1.68	14.4±1.32 [b]	17.8±1.88 [b]	18.4±1.50 [b]	17.6±1.53 [b]	17.4±1.16 [b]	48.04
IKC$_{100}$	7.00±1.30	9.00±1.51 [b]	10.2±1.56 [a,c]	10.6±1.28 [a,d,e]	11.0±1.04 [a,b,d]	11.6±1.20 [a,b,d]	29.90
IKC$_{250}$	8.00±0.70	11.0±0.89 [b]	12.4±0.87 [a,b]	15.2±1.15 [b,c,e]	16.4±0.74 [b,c,d]	16.8±0.91 [b,c,d]	41.05
IKC$_{500}$	7.40±0.50	10.8±0.73 [b]	12.2±1.01 [a,b]	15.4±1.02 [b,c,e]	16.0±1.14 [b,c,d]	17.0±0.70 [b,c,d]	40.64

Values are Mean ± S.E.M.; (N=6)

Different letters are statistically significant at P<0.05 (ANOVA followed by Tukey's test)

Table 3.8: Effect of the IKM and morphine on pain threshold of mice on Hot Plate test

Treatment (mg/kg)	Latency period						
	Pre treatment	30 (minutes)	60 (minutes)	90 (minutes)	120 (minutes)	180 (minutes)	AUC
Control (NS)	7.00±0.70	7.05±1.93 [a]	7.20±1.20[a]	7.80±0.86 [a]	8.00±0.83 [a]	8.60±1.03 [a]	22.26
Morphine$_{10}$	7.80±1.68	14.4±1.32 [b]	17.8±1.88[b]	18.4±1.50 [b]	17.6±1.53 [b]	17.4±1.16 [b]	48.04
IKM$_{100}$	7.20±0.86	9.80±1.40 [a,b]	11.4±1.32 [a,c]	11.8±1.15 [a,d,e]	12.2±1.20 [a,d,e]	12.8±0.96 [a,b]	33.00
IKM$_{250}$	7.40±1.02	11.6±1.88 [a,b]	13.0±1.70 [a,b,c]	16.4±1.07 [b,c,e]	17.0±1.09 [b,c,e]	17.4±0.92 [b,c]	42.85
IKM$_{500}$	6.8±0.86	10.8±1.20 [a,b]	12.2±1.31 [a,b,c]	14.2±1.01 [b,c,e]	16.0±1.00 [b,c,e]	14.8±1.53 [b,c]	38.82

Values are Mean ± S.E.M.; (N=6).

Different letters are statistically significant at P<0.05 (ANOVA followed by Tukey's test)

Table 3.9: Percentage inhibition of IKC and morphine on pain threshold of mice on Hot Plate test

Treatment (mg/kg)	**% Inhibition at 30 minutes**	**% Inhibition at 60 minutes**	**% Inhibition at 90 minutes**	**% Inhibition at 120 minutes**	**% Inhibition at 180 minutes**
Control (CMC)	-	-	-	-	-
Morphine$_{10}$	54.09	81.96	86.88	80.32	78.69
IKC$_{100}$	15.38	24.61	27.69	30.76	35.38
IKC$_{250}$	25.00	36.67	60.00	70.00	73.33
IKC$_{500}$	26.98	38.09	63.49	68.25	76.19

Table 3.10: Percentage inhibition of IKM and morphine on pain threshold of mice on Hot Plate test

Treatment (mg/kg)	**% Inhibition at 30 minutes**	**% Inhibition at 60 minutes**	**% Inhibition at 90 minutes**	**% Inhibition at 120 minutes**	**% Inhibition at 180 minutes**
Control (NS)	-	-	-	-	-
Morphine$_{10}$	54.09	81.97	86.88	80.33	78.68
IKM$_{100}$	20.31	32.81	35.93	39.06	43.75
IKM$_{250}$	33.33	44.44	71.42	76.19	79.36
IKM$_{500}$	30.30	40.90	56.06	69.69	60.60

Table 3.11: Effect of IKC and diclofenac on Xylene induced ear edema.

Treatment (mg/kg)	**Mean±SEM**	**% Inhibition**
Control (CMC)	7.75±0.85[a]	
Dic$_{20}$	3.80±0.49[b]	50.97
IKC$_{100}$	7.00±0.45[a,c]	9.68
IKC$_{250}$	6.25±0.48[a,b,c]	19.35
IKC$_{500}$	5.83±0.79[a,b,c]	24.73

Values are Mean ± S.E.M.; (N=6). Different letters are statistically significant at P<0.05 (ANOVA followed by Tukey's test)

Table 3.12: Effect of IKM and diclofenac on Xylene induced ear edema.

Treatment (mg/kg)	**Mean±SEM**	**% Inhibition**
Control (NS)	7.60±0.40[a]	
Dic$_{20}$	3.80±0.49[b]	50
IKM$_{100}$	6.20±0.66[a,c]	18.42
IKM$_{250}$	4.80±0.49[b,c]	36.84
IKM$_{500}$	5.20±0.80[a,b,c]	31.58

Values are Mean ± S.E.M.; (N=6). Different letters are statistically significant at P<0.05 (ANOVA followed by Tukey's test)

Table 3.13: Effect of IKC and diclofenac on Carrageenan induced paw edema.

Treatment (mg/kg)	Paw size (at different hours)						
	1	2	3	4	5	6	AUC
Control (CMC)	3.78±0.10 [a]	4.83±0.14 [a]	4.63±0.20 [a]	4.42±0.17 [a]	4.27±0.14 [a]	4.07±0.15 [a]	24.81
Dic$_{20}$	3.38±0.30 [a]	3.90±0.27 [b]	3.53±0.27 [b]	3.39±0.23 [b]	3.20±0.14 [b]	3.08±0.13 [b]	19.78
IKC$_{100}$	3.70±0.13 [a]	4.67±0.13 [a,b]	4.42±0.14 [a,c,d]	4.20±0.19 [a,b]	4.00±0.19 [a,b,d]	3.85±0.21 [a,c,d]	23.78
IKC$_{250}$	3.55±0.02 [a]	4.22±0.13 [a,b]	4.07±0.13 [a,b,d]	3.85±0.23 [a,b]	3.72±0.16 [a,c,d]	3.57±0.15 [a,b,d]	22.02
IKC$_{500}$	3.56±0.11 [a]	4.30±0.16 [a,b]	4.17±0.16 [a,b,d]	4.00±0.09 [a,b]	3.90±0.11 [a,b,d]	3.75±0.13 [a,b,d]	22.67

Values are Mean ± S.E.M.; (N=6).

Different letters are statistically significant at P<0.05 (ANOVA followed by Tukey's test)

Table 3.14: Effect of IKM and diclofenac on Carrageenan induced paw edema.

Treatment (mg/kg)	Paw size (at different hours)						
	1	2	3	4	5	6	AUC
Control (NS)	3.72±0.09 [a]	4.83±0.11 [a]	4.55±0.11 [a]	4.30±0.17 [a]	4.27±0.09 [a]	4.20±0.12 [a]	24.36
Dic$_{20}$	3.30±0.30 [a]	3.93±0.27 [b]	3.53±0.27 [b]	3.40±0.24 [b]	3.20±0.15 [b]	3.08±0.13 [b]	19.78
IKM$_{100}$	3.70±0.13 [a]	4.67±0.13 [a]	4.42±0.14 [a,e]	4.20±0.19 [a,c,d]	4.00±0.19 [a,b,d]	3.85±0.21 [a,b]	23.47
IKM$_{250}$	3.46±0.08 [a]	4.20±0.19 [a]	3.74±0.13 [b,c,f]	3.58±0.16 [b,d]	3.40±0.19 [b,c,d]	3.32±0.17 [a,b]	20.91
IKM$_{500}$	3.52±0.13 [a]	4.44±0.15 [a]	3.80±0.16 [b,d,e]	3.66±0.19 [a,d]	3.58±0.25 [a,b,d]	3.44±0.13 [a,b]	21.60

Values are expressed as Mean ± S.E.M, n= 6

Statistically significant at P<0.05 are indicated by different letters (ANOVA HSD Post hoc Tukey Test).

Table 3.15: Percentage inhibition of IKC and diclofenac on Carrageenan induced paw edema.

Treatment (mg/kg)	**% Inhibition at 1 hr**	**% Inhibition at 2 hr**	**% Inhibition at 3 hr**	**% Inhibition at 4 hr**	**% Inhibition at 5 hr**	**% Inhibition at 6 hr**
Dic_{20}	32.20	35.64	47.31	48.47	54.43	56.33
IKC_{100}	5.08	5.94	8.60	10.58	13.92	12.67
IKC_{250}	18.64	23.76	23.65	27.05	27.84	28.16
IKC_{500}	16.61	21.58	20.21	20.94	20.00	19.43

Table 3.16: Percentage inhibition of IKM and diclofenac on Carrageenan induced paw edema.

Treatment (mg/kg)	**% Inhibition at 1 hr**	**% Inhibition at 2 hr**	**% Inhibition at 3 hr**	**% Inhibition at 4 hr**	**% Inhibition at 5 hr**	**% Inhibition at 6 hr**
Dic_{20}	30	30.86	32.78	35.54	37.21	47.41
IKM_{100}	9.33	11.72	17.78	18.78	14.42	16.38
IKM_{250}	16.67	16.67	20.56	23.35	25.12	36.21
IKM_{500}	18	16.05	21.67	22.35	24.18	32.76

Table 3.17: Effect of IKC and diclofenac on Formalin induced paw edema.

Treatment (mg/kg)	Paw size (at different hour)						
	1	2	3	4	5	6	AUC
Control (CMC)	0.26±0.03	0.42±0.02 [a]	0.39±0.03 [a]	0.37±0.02 [a]	0.36±0.02 [a]	0.34±0.01 [a]	2.04
Dic$_{20}$	0.20±0.30	0.22±0.02 [b]	0.23±0.01 [b]	0.25±0.02 [b]	0.26±0.02 [b]	0.24±0.01 [b]	1.32
IKC$_{100}$	0.24±0.02	0.38±0.03 [a,d,e]	0.35±0.02 [a,d,e]	0.34±0.02 [a,c,e]	0.32±0.02 [a,d,e]	0.30±0.02 [a,d,e]	1.84
IKC$_{250}$	0.23±0.02	0.33±0.02 [b,c,e]	0.31±0.02 [b,c,e]	0.28±0.02 [b,d,e]	0.27±0.03 [b,c,e]	0.25±0.03 [b,c,e]	1.60
IKC$_{500}$	0.22±0.01	0.34±0.02 [b,c,e]	0.33±0.02 [a,d,e]	0.31±0.03 [a,c,e]	0.29±0.02 [b,c,e]	0.26±0.01 [b,c,e]	1.70

Values are Mean ± S.E.M.; (N=6). Different letters are statistically significant at P<0.05 (ANOVA followed by Tukey's test)

Table 3.18.: Effect of IKM and diclofenac on Formalin induced paw edema.

Treatment (mg/kg)	Paw size (at different hour)						
	1	**2**	**3**	**4**	**5**	**6**	**AUC**
Control (NS)	0.23±0.02	0.42±0.02[a]	0.40±0.03 [a]	0.38±0.02 [a]	0.36±0.01 [a]	0.33±0.02 [a]	2.02
Dic$_{20}$	0.22±0.02	0.27±0.02 [b]	0.24±0.01 [b]	0.23±0.02 [b]	0.20±0.02 [b]	0.19±0.01 [b]	1.32
IKM$_{100}$	0.23±0.02	0.37±0.03 [a,d,e]	0.34±0.03 [a,d,e]	0.32±0.02 [a,b,d]	0.28±0.03 [a,b,d]	0.26±0.02 [a,b,d]	1.73
IKM$_{250}$	0.22±0.02	0.31±0.02 [b,c,e]	0.27±0.02 [b,c,e]	0.26±0.02 [b,c,d]	0.25±0.03 [b,c,d]	0.23±0.01 [b,c,d]	1.50
IKM$_{500}$	0.22±0.01	0.33±0.01 [b,c,e]	0.30±0.01 [b,c,e]	0.28±0.01 [a,b,d]	0.27±0.01 [a,b,d]	0.26±0.02 [a,b,d]	1.59

Values are Mean ± S.E.M.; (N=6). Different letters are statistically significant at P<0.05 (ANOVA followed by Tukey's test)

Table 3.19: Percentage inhibition of IKC and diclofenac in formalin induced paw edema.

Treatment (mg/kg)	% Inhibition at 1 hr	% Inhibition at 2 hr	% Inhibition at 3 hr	% Inhibition at 4 hr	% Inhibition at 5 hr	% Inhibition at 6 hr
Dic_{20}	40.63	58.16	65.88	70.13	81.69	87.50
IKC_{100}	15.63	12.24	16.47	11.69	16.90	20.31
IKC_{250}	27.50	32.45	35.06	42.60	46.20	49.69
IKC_{500}	18.75	29.59	25.88	18.18	33.80	45.31

Table 3.20: Percentage inhibition of IKM and diclofenac in formalin induced paw edema.

Treatment (mg/kg)	% Inhibition at 1 hr	% Inhibition at 2 hr	% Inhibition at 3 hr	% Inhibition at 4 hr	% Inhibition at 5 hr	% Inhibition at 6 hr
Dic_{20}	30.00	30.86	32.78	35.53	37.21	47.41
IKM_{100}	9.33	11.73	17.78	18.78	14.42	16.38
IKM_{250}	16.67	16.67	20.56	23.35	25.12	36.21
IKM_{500}	18.00	16.05	21.67	22.34	24.19	32.76

REFERENCES

Antonucci, R., Cuzzolin, L., Arceri, A., Dessì, A., & Fanos, V. (2009). Changes in urinary PGE2 after ibuprofen treatment in preterm infants with patent ductus arteriosus. European journal of clinical pharmacology, 65(3), 223-230.

Aydin, S., Demir, T., Öztürk, Y., & Başer, K. H. C. (1999). Analgesic activity of *Nepeta italica* L. Phytotherapy Research: An International Journal Devoted to Pharmacological and Toxicological Evaluation of Natural Product Derivatives, 13(1), 20-23.

Azab, A., Nassar, A., & Azab, A. N. (2016). Anti-inflammatory activity of natural products. Molecules, 21(10), 1321.

Belknap, S. (2002). studies on the cardiovascular effects of selective COX-2 inhibitors show mixed results. ACP journal club, 136(2), 53-53.

Bleumink, G. S., Feenstra, J., Sturkenboom, M. C., & Stricker, B. H. C. (2003). Nonsteroidal anti-inflammatory drugs and heart failure. Drugs, 63(6), 525-534.

Browne, A. S., & Kelly, C. R. (2017). Fecal transplant in inflammatory bowel disease. Gastroenterology Clinics, 46(4), 825-837.

Cao, B. J., Meng, Q. Y., & Ji, N. (1992). Analgesic and anti-inflammatory effects of *Ranunculus japonicus* extract. Planta medica, 58(06), 496-498.

Clària, J. (2003). Cyclooxygenase-2 biology. Current pharmaceutical design, 9(27), 2177-2190.

Costa, G., Francisco, V., C Lopes, M., T Cruz, M., & T Batista, M. (2012). Intracellular signaling pathways modulated by phenolic compounds: application for new anti-inflammatory drugs discovery. Current Medicinal Chemistry, 19(18), 2876-2900.

Davies, N. M., & Jamali, F. (1997). Pharmacological protection of NSAID-induced intestinal permeability in the rat: effect of tempo and metronidazole as potential free radical scavengers. Human & experimental toxicology, 16(7), 345-349.

De Petris, G., & López, J. I. (2008). Histopathology of diaphragm disease of the small intestine: a study of 10 cases from a single institution. American Journal of Clinical Pathology, 130(4), 518-525.

Dzoyem, J. P., McGaw, L. J., Kuete, V., & Bakowsky, U. (2017). Anti-inflammatory and anti-nociceptive activities of African medicinal spices and vegetables. In Medicinal spices and vegetables from Africa (pp. 239-270). Academic Press.

Eddy, N. B., & Leimbach, D. (1953). Synthetic analgesics. II. Dithienylbutenyl-and dithienylbutylamines. Journal of Pharmacology and Experimental Therapeutics, 107(3), 385-393.

Filip, R., & Ferraro, G. E. (2003). Researching on new species of "Mate": *Ilex brevicuspis.* European journal of nutrition, 42(1), 50-54.

Fitzgerald, G. A. (2003). Prostaglandins, aspirin and related compounds. Cecil Medicine, 23rd ed. (Philadelphia, PA: Saunders, Elsevier, 2007).

Forman, J. P., Rimm, E. B., & Curhan, G. C. (2007). Frequency of analgesic use and risk of hypertension among men. Archives of Internal Medicine, 167(4), 394-399.

Gupta, M. B., Bhalla, T. N., Gupta, G. P., Mitra, C. R., & Bhargava, K. P. (1969). Anti-inflammatory activity of natural products (I) Triterpenoids. European Journal of Pharmacology, 6(1), 67-70.

Hammer, K. D., Hillwig, M. L., Solco, A. K., Dixon, P. M., Delate, K., Murphy, P. A., & Birt, D. F. (2007). Inhibition of prostaglandin E2 production by anti-inflammatory *Hypericum perforatum* extracts and constituents in RAW264. 7 mouse macrophage cells. Journal of Agricultural and Food Chemistry, 55(18), 7323-7331.

Harirforoosh, S., & Jamali, F. (2009). Renal adverse effects of nonsteroidal anti-inflammatory drugs. Expert opinion on drug safety, 8(6), 669-681.

Kaushik, D., Kumar, A., Kaushik, P., & Rana, A. C. (2012). Analgesic and Anti-Inflammatory Activity of *Pinus roxburghii* Sarg. Advances in pharmacological sciences, 2012.

Kim, J., Kang, W., & Min, H. (2017). In vitro anti-inflammatory activity of *Ilex cornuta* extract mediated by inhibition of extracellular signal-regulated kinase phosphorylation. Journal of Medicinal Food, 20(10), 981-988.

Koster, R. (1959). Acetic acid for analgesic screening. In Fed proc (Vol. 18, p. 412).

Lanas, A. (2009). Nonsteroidal antiinflammatory drugs and cyclooxygenase inhibition in the gastrointestinal tract: a trip from peptic ulcer to colon cancer. The American journal of the medical sciences, 338(2), 96-106.

Laveti, D., Kumar, M., Hemalatha, R., Sistla, R., Gm Naidu, V., Talla, V., & Nagpal, R. (2013). Anti-inflammatory treatments for chronic diseases: a review. Inflammation & Allergy-Drug Targets (Formerly Current Drug Targets-Inflammation & Allergy) (Discontinued), 12(5), 349-361.

Luz, A. B. G., da Silva, C. H. B., Nascimento, M. V. P., de Campos Facchin, B. M., Baratto, B., Fröde, T. S., & Dalmarco, E. M. (2016). The anti-inflammatory effect of *Ilex paraguariensis* A. St. Hil (Mate) in a murine model of pleurisy. International Immunopharmacology, 36, 165-172.

Matsumoto, R. L. T., Mendonça, S., Moura de Oliveira, D., Souza, M. F., & Markowicz Bastos, D. H. (2009). Effects of maté tea intake on ex vivo LDL peroxidation induced by three different pathways. Nutrients, 1(1), 18-29.

Noratto, G. D., Kim, Y., Talcott, S. T., & Mertens-Talcott, S. U. (2011). Flavonol-rich fractions of yaupon holly leaves (*Ilex vomitoria*, Aquifoliaceae) induce

microRNA-146a and have anti-inflammatory and chemopreventive effects in intestinal myofribroblast CCD-18Co cells. Fitoterapia, 82(4), 557-569.

Oyedapo, O. A., Adewunmi, C. O., Iwalewa, E. O., & Makanju, V. O. (2008). Analgesic, antioxidant and anti-inflammatory related activities of 21-hydroxy-2, 41-dimethoxychalcone and 4-hydroxychalcone in mice. Journal of Biological Sciences, 8(1), 131-136.

Poddubnyy, D., Song, I. H., & Sieper, J. (2009). A systematic comparison of rheumatoid-arthritis and ankylosing spondylitis: Non-steroidal anti-inflammatory drugs. Clinical & Experimental Rheumatology, 27(4), S148.

Puangpraphant, S., & De Mejia, E. G. (2009). Saponins in yerba mate tea (*Ilex paraguariensis* A. St.-Hil) and quercetin synergistically inhibit iNOS and COX-2 in lipopolysaccharide-induced macrophages through NFκB pathways. Journal of Agricultural and Food Chemistry, 57(19), 8873-8883.

Rao, P., & Knaus, E. E. (2008). Evolution of nonsteroidal anti-inflammatory drugs (NSAIDs): cyclooxygenase (COX) inhibition and beyond. Journal of pharmacy & pharmaceutical sciences, 11(2), 81s-110s.

Ribeiro, R. A., Vale, M. L., Thomazzi, S. M., Paschoalato, A. B., Poole, S., Ferreira, S. H., & Cunha, F. Q. (2000). Involvement of resident macrophages and mast cells in the writhing nociceptive response induced by zymosan and acetic acid in mice. European journal of pharmacology, 387(1), 111-118.

Roberts, L. I. (2001). Analgesic-antipyretic and anti-inflammatory agents and drugs employed in the treatment of gout. Goodman and Gilman's the pharmacological basis of therapeutics.

Serhan, C. N., & Savill, J. (2005). Resolution of inflammation: the beginning programs the end. Nature immunology, 6(12), 1191-1197.

Shao, J., Li, Y., Wang, Z., Xiao, M., Yin, P., Lu, Y., & Liu, J. (2013). 7b, a novel naphthalimide derivative, exhibited anti-inflammatory effects via targeted-inhibiting TAK1 following down-regulation of ERK1/2-and p38 MAPK-mediated activation of NF-κB in LPS-stimulated RAW264. 7 macrophages. International immunopharmacology, 17(2), 216-228.

Smyth, E. M., Grosser, T., Wang, M., Yu, Y., & FitzGerald, G. A. (2009). Prostanoids in health and disease. Journal of lipid research, 50, S423-S428.

Sostres, C., Gargallo, C. J., Arroyo, M. T., & Lanas, A. (2010). Adverse effects of non-steroidal anti-inflammatory drugs (NSAIDs, aspirin and coxibs) on upper gastrointestinal tract. Best practice & research Clinical gastroenterology, 24(2), 121-132.

Stewart, A. G., & Beart, P. M. (2016). Inflammation: maladies, models, mechanisms and molecules. British Journal of Pharmacology, 173(4), 631-634.

Tjølsen, A., Berge, O. G., Hunskaar, S., Rosland, J. H., & Hole, K. (1992). The formalin test: an evaluation of the method. Pain, 51(1), 5-17.

Turner, R. A. (1965). Screening Methods in Pharmacology: Analgesic Agent, Academic Press, London, UK, vol. 13.

Umar, M. I., Asmawi, M. Z., Sadikun, A., Abdul Majid, A. M. S., Atangwho, I. J., Khadeer Ahamed, M. B.,& Ahmad, A. (2014). Multi-constituent synergism is responsible for anti-inflammatory effect of *Azadirachta indica* leaf extract. Pharmaceutical Biology, 52(11), 1411-1422.

Wang, J. R., Zhou, H., Jiang, Z. H., & Liu, L. (2008). Two new triterpene saponins from the anti-inflammatory saponin fraction of *Ilex pubescens* root. Chemistry & Biodiversity, 5(7), 1369-1376.

Warner, T. D., Giuliano, F., Vojnovic, I., Bukasa, A., Mitchell, J. A., & Vane, J. R. (1999). Nonsteroid drug selectivities for cyclo-oxygenase-1 rather than cyclo-

oxygenase-2 are associated with human gastrointestinal toxicity: a full *in vitro* analysis. Proceedings of the National Academy of Sciences, 96(13), 7563-7568.

Whelton, A., & Watson, A. J. (1998). Nonsteroidal anti-inflammatory drugs: effects on kidney function. Clinical Nephrotoxins, 203-216.

Winter, C. A., Risley, E. A., & Nuss, G. W. (1962). Carrageenin-induced edema in hind paw of the rat as an assay for anti-inflammatory drugs. Proceedings of the society for experimental biology and medicine, 111(3), 544-547.

CHAPTER - 4

STUDIES OF *IN VITRO* ANTICANCER ACTIVITY OF *ILEX KHASIANA* METHANOL EXTRACT AND ITS BIOACTIVE FRACTIONS ON A549 AND HCT116 CELL LINES.

INTRODUCTION

Cancer and cardiovascular disease (CVD) were the leading cause of premature death (ages between 30-70) in 127 countries, with cancer leading in 57 countries. In more than 50 countries cancer and CVD were among the top 3 causes with only 6 countries where these two were not in the top 3 causes of premature death. Noncommunicable diseases (NCDs) were accountable for the three-fourth of the 20.4 million premature death worldwide in which 3 out of 10 premature deaths due to NCDs were caused by cancer alone **(WHO, 2020)**. Thus, cancer is one of the most prominent barriers to growing in life expectancy in every country of the world **(Bray et al., 2021).** The Human Development Index (HDI) has direct impact on relative decline of infectious diseases in many countries worldwide. Moreover, there is remarkable decrease in CVDs in many of the high-HDI countries where cancer now is the leading cause of death. Meanwhile, countries with low HDI are still suffering the clutches of both infectious and NCDs live cancer and CVDs **(Bray et al., 2015)**.

Lung cancer is the most diagnosed cancer in 36 countries and leading cause of mortality in 93 countries. It is the highest cause of death in men and ranked third in women just below breast cancer and colorectal cancer. In 2020, lung cancer accounted 11.4% of the all the diagnosed cancer cases which is around 2.2 million new cases and 1.8 million deaths. The overall incidence and mortality rates vary extensively in different regions in which the rates are higher in men than in women throughout the world **(Turner, 2020)**.

In 2020, colorectal cancer represented one tenth of all cancer cases and death which accounted for more than 1.9 million new cases and 935,000 deaths. Colorectal cancer has a strong association with oxidative stress-linked DNA damage. It could be a suitable marker for socioeconomic development as incident rates are 4 times higher in transitioned countries making colorectal cancer ranked third in term of incidence and second in mortality rate **(Fidler et al., 2016)**.

The existing cancer treatment like chemotherapy, radiation therapy and surgery are the major defense employed in combating cancers of various kinds **(Androutsopoulo et al., 2008)**. Nevertheless, the negative impacts created by these treatments are a worldwide threat which cries out for an alternative approach especially based on the natural products to better efficacy as well as lesser side effects **(Lee et al., 2011)**. Many bioactive compounds such as triterpenoids, saponins and anthocyanin are known to have antiproliferative and apoptotic activity against lung cancer **(Lu et al., 2017; Li et al., 2015)**. Similarly, bioactive compounds like β-sitosterol, carotenoids, saponin and genistein have prominent anticancer effect against colorectal cancer **(Baskar, 2010; Li et al., 2015; Qi et al., 2011)**.

Ilex paraguariensis fondly known as yerba mate tea (YMT) is a very popular beverages in Argentina, Uruguay and some parts of Brazil. This plant belongs to Aquifoliaceae family, and is found to have anticancer activity against Caco-2 and HT-29 adenocarcinoma cells inhibiting at least 50% of the cancer growth **(de Mejía et al., 2010).** *Ilex laurina,* a plant originated from Columbia from the same species is also known to have astonishing effect against colorectal cancer **(Pérez et al., 2014)**. The anticancer activity of *Ilex* species is credited to the presence of caffeoyl derivatives, quercetin, kaempferol and rutin **(Bastos et al., 2007).** Therefore, *Ilex khasiana* is recruited in this study to find its anticancer properties against A549 (lung cancer) cells and HCT166 (colon cancer) cells.

MATERIALS AND METHODS

Chemicals and reagents

L-Glutamine, phenol red, agarose (low gelling temperature), ethylenediamine tetra-acetic acid (EDTA), Trizma base, Trizma hydrochloride and trichloroacetic acid (TCA) were obtained from Sigma Chemical Co., Bangalore, India. Trypsin-EDTA, Eagle's Minimal Essential Medium (MEM), fetal bovine serum (FBS), 3-(4,5-dimethylthiazole-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT), Folin-ciocalteu's reagent, n-butanol, thiobarbaturic acid (TBA), potassium chloride (KCl), sodium Chloride (NaCl), Triton X-100, acridine orange, sodium bicarbonate, bovine serum albumin (BSA), glutathione (GSH) reduced, nicotinamide adenosine dinucleotide (NADH), nitroblue tetrazolium (NBT), ethidium bromide, phenazine methosulphate (PMS), dimethyl sulphoxide (DMSO), 1-chloro-2,4 dinitrobenzene (CDNB) and 5, 5' dithio 2-nitrobenzoic acid (DTNB) were purchased from HiMedia Laboratories Pvt. Ltd. (Mumbai, India).

Preparation of *I. khasiana* extracts

Ilex khasiana methanol extracts (IKM) and its four fractions were used in this experiment. The crude extracts were prepared for fractionation by dissolving it in 250ml of water using separation funnel method. Solvents of varying polarities namely n-Hexane (0.009), Diethyl ether (0.117), chloroform (0.259) and n-Butanol (0.586) were selected. Firstly, 250ml of n-Hexane was poured into separating funnel containing the same volume of crude extract and the funnel was allowed to rest after vigorous shaking of the mixture. Once settled, the funnel was opened to remove the aqueous solution and the upper layer, the hexane fraction was collected in a clean container. Equal volume of the selected solvent was added repeatedly to the crude extract solution until n-Hexane in the solution gave a clear suspension. This method was applied to all the other solvents having ascending polarities **(Pandey et al., 2014)**. After the process was completed, four distinct fractions IKM Hexane, IKM Diethyl ether, IKM Chloroform and IKM

Butanol were obtained and used for further analysis. The crude extract that remained after fractionation is called residual aqueous fraction (RAF) (**Sasidharan et al., 2011)**.

Cell lines and Culture

The *I. khasiana* extracts were subjected against Type II human lung adenocarcinoma cell line, A549 cells and human colon cancer cell line, HCT116 cells to determine its anticancer activity. The cell lines were obtained from the National Centre for Cell Sciences (NCCS), Pune, India and maintained in 25 cm^2 culture flask containing MEM supplemented with 10% FBS, 1% L-Glutamine and 50 µg/mL gentamicin in a humidified incubator containing 5% CO_2 at 37 ^{O}C (Eppendorf AG, Germany).

Cytotoxicity assay

The different concentration of *I. khasiana* extracts were tested for its cytotoxic effect against A549 and HCT116 cell lines using 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide (MTT) reduction assay **(Mossman., 1983).** Usually, 10^4 cells were seeded into 96 well plates in 100 µl MEM. The cells were incubated at 37 ^{O}C in a CO_2 incubator in an atmosphere of 5% CO_2 in 95 % humidified air. After allowing 24 hours of cell adherence, the cells were treated with different dosage (50, 100, 200, 300 and 500 µg/ml) of IKM and its fraction IKM Dee, IKM BU, IKM Hex and IKM C for 24, 48 and 72 hours respectively with a control sample. The treatment was ceased at each time point by washing the cell with FBS free media. The purple formazan crystals were formed after the addition of 10 µl of MTT (5 mg/ml) and incubated at 37ºC in a CO_2 incubator for 2 hours. At the end of incubation, the crystal was dissolved by the addition of 100 µl of DMSO and incubated again for 30 minutes. the experiment was performed in triplicate and the absorbance were measured at 560 nm using a microplate reader (Spectramax m2e, Molecular Devices).

Cytotoxicity was expressed as inhibition (%) which was calculated by the formula given below:

% inhibition = Control-Treatment/Control X 100.

The most effective extract and the best treatment time was observed and employed for further experiments.

Apoptotic Assay

The morphological changes that occurred on both A549 and HCT116 cell lines due to apoptosis induced by IKM Dee was identified using dual acridine orange/ethidium bromide (AO/EB) fluorescent staining. Different concentration of IKM Dee (25, 50 and 50µg/ml) were used to treat 1×10^5 A549 and HCT116 cells that were cultures in media containing six-well plates. Culture devoid of treatment was considered as control. After 24 hours of treatment, 1X PBS was used to wash the cell before detachment of adhering cells with 1X trypsin EDTA. Cell suspension was obtained by adding 100 µl of FBS free media to the cell pellet. Dual staining was performed by the addition of 2.5 µl each of acridine orange (100 µg/ml) and ethidium bromide (100 µg/ml) in a ratio of 1:1in 25 µl cell suspension for 2 minutes. The cells were placed on a clean slide and observed under a fluorescent microscope (Thermo Fisher Scientific, EVOS^R Fluorescence Imaging, AMEP-4615). Acridine orange penetrates both dead and live cells insinuating the DNA being nucleic acid fluorescent dye giving green coloration to the nuclei. On the contrary, ethidium bromide was absorbed when there is interruption in the cell membrane of a dead cells giving yellowish orange color under fluorescent microscope. Subsequently, the dead cells (apoptotic cells) gave fragmented orange chromatin whereas the cells with green nuclei are designated as live cells. Necrotic cells have similar nuclear morphology with that of viable cells in spite of having the orange stain **(Kasibhatla et al., 2006).**

Apoptotic index was calculated by scoring minimum of 300 cells by using this formula:

Apoptotic index (%) = Number of apoptotic cells scored × 100/Total number of cells counted.

Clonogenic Assay

The colony formation potential of A549 and HCT116 cell lines was assessed using clonogenic assay **(Franken et al., 2006).** The extract IKM Dee was known to have the most potent cytotoxic effect from the above MTT assay. Cells (200 cells approximately) were seeded in a petri dish containing 5ml of media after harvesting the stock culture by trypsinization. After 24 hours of incubation, the cells were treated with three different dosages of IKM Dee (25, 50 and 100µg/ml) for 24 hours. Once the treatment was completed, the cells were washed with 1X PBS and cultured for the next 11 days in a new media. The petri dishes were treated stained with 1% crystal violet in methanol (w/v) for 30 minutes and inverted microscope was used to count colony forming cells.

Plating efficiency (PE) and surviving fraction (SF) of A549 cells were calculated by the following formula:

PE = (Number of colonies counted × 100) / (Number of cells seeded)

SF = (Number of colonies counted) / (Number of cells seeded) × (Mean plating efficiency)

Statistical analysis

One-way ANOVA followed by Tukey post hoc test was employed to determine variation between different treatment groups using Graphpad Prism 8.0.2. All data were expressed as mean ± standard error of mean. All data were considered significant at $P < 0.05$.

RESULTS

Cytotoxicity of *I. khasiana* extracts on A549 cells and HCT116

Type II human lung adenocarcinoma cell line A549 cells were treated with different doses of *I. khasiana* extracts (50, 100, 200, 300 and 500 µg/ml) for 24, 48 and 72 hours. Cytotoxic effect of the extracts was estimated by calculating the percentage inhibition of A549 cells. The results obtained were plotted against the log-doses for the calculation of IC_{50} **(Figure 4.1 A, B and C)**. All the fractions except IKM Hex showed better cytotoxicity compared to IKM in all the observed time points **(Figure 4.2).** Among the treatment groups, IKM Dee exhibited lowest IC_{50} (**26.90±6.08)** at 24 hours **(Table 2.1)**.

Similarly, cytotoxicity of *I. khasiana* extracts (50, 100, 200, 300 and 500 µg/ml) were investigated at three different time point. The percentage inhibition was plotted against its log-doses **(Figure 4.3).** IKM Dee was the most active treatment giving the highest cytotoxic effect at 24 hours **(Figure4.4)** with an IC_{50} of **22.77±4.68 (Table 2.2).** The above results concluded that, IKM Dee has the highest cytotoxicity among all the other treatment groups at 24 hours treatment time. Therefore, IKM Dee was selected and used in all the subsequent experiment with 24 hours as the best treatment time point.

Apoptotic assay

The apoptotic associated cell morphological changes of A549 and HCT116 cells were observed using AO/Etbr Dual staining assay. After treating the cells with IKM Dee (25, 50 and 100µg/ml) for 24 hours, the changes were observed in a fluorescent microscope. The observed images distinctly illustrate the changes in cell morphology such as blebbing, nuclear condensation and fragmentation **(Figure 4.5 and Figure 4.7).** The apoptotic index revealed dose dependent activity on both the cell lines **(Figure 4.6 and Figure 4.8)** in which IKM Dee 50 and IKM Dee 100 did not show any statistical variation. The cell death percentage were found to be 33.50%, 55.18% and 57.62% for

A549 cell and 32.47%, 54.29%and 62.55% for HCT166 cells at 25, 50 and 100µg/ml respectively.

Clonogenic assay

The clonogenicity of A549 and HCT116 cell were determined upon treatment with different dose of IKM Dee (25, 50 and 100µg/ml). After 11 days of treatment, both the cells portrayed concentration dependent growth inhibition **(Figure 4.9 and Figure 4.11).** In A549 cells, 25 and 50µg/ml doses did not show any statistical differences which is similar in HCT116 cell line as well while 100µg/ml of IKM Dee was statistically significant when compared with control and all other treatment **(Figure: 4.10 and Figure 4.12).** The A549 cells exhibited surviving fraction of 52.16, 47.56 and 21.77% and 52.56, 48.12 and 8.42% in HCT116 cell upon treating with different dose of IKM Dee **(Table:4.4).** Here, the highest inhibition 8.42% was found at 100µg/ml in HCT116 cells.

DISCUSSION

Internationally, the incident rates of lung cancer increased with tobacco consumption mainly in the form of smoking. In the same manner, 14% of the cases recorded were attributed to outdoor ambient $PM_{2.5}$ (known as *fine particulate matter*) air pollution in 2017 **(Turner, 2020)**. Regular assessment using low-dose computed tomography (CT) for high-risk individuals helped in early diagnosis and led to drastic decline in the last 10 years with 24% in men and 33% in women compared with no screening **(Pastorino, 2019)**.

The rise in new cases might be directly proportional to the changes in lifestyle such as increased consumption of alcohol, animal-based foods, red meat, lesser physical activity and overweight **(Siegel et al., 2020)**. Some high incidence countries experienced gradual decline in incident rates due to improved lifestyle, regular colonoscopy screening and elimination of precursor contusion **(Keum et al., 2019)**.

Latest report on *Ilex* species stated that 38 of 669 species known have been reported to have various medicinal properties including anticancer activity **(Noureddine et al., 2018).** Many species including *I. paraguariensis* are common beverages known as Yerba mate tea and nine other *Ilex* species namely, *I. argentina* Lillo, *I. brasiliensis* (Spreng.) Loes., *I. brevicuspis* (Spreng.) Loes., *I. dumosa* Reissek, *I. integerrima* Reissek, *I. microdonta* Reissek, *I. pseudobuxus* Reissek, *I. taubertiana* Loes., and *I. theezans* are also marketed as Kudingcha tea **(Maiocchi et al., 2018)**. Interestingly, these non-alcoholic drinks are reported to have therapeutic properties like antibacterial, antiviral, antitumor and antimutagenic properties **(Yao et al., 2022).**

Among the beverages, *I. paraguariensis* is found to be loaded with antioxidant compounds mainly caffeoyl derivatives (caffeic acid, chlorogenic acid, 3,4-dicaffeoylquinic acid, 3,5- dicaffeoylquinic acid and 4,5-dicaffeoylquinic acid), amino acids, flavonoids (quercetin, kaempferol and rutin), minerals (P, Fe and Ca), vitamins C, B1, and B2 **(Bastos et al., 2007).** Thus, *I. paraguariensis* showed cytotoxic effect

against human colon adenocarcinoma cells namely HT-29 and CaCo-2 cells **(de Mejía et al., 2010)** besides their cytotoxic effects on T24 (Urinary bladder Cancer), OE-33 (Caucasian esophageal carcinoma), A549 (Adenocarcinomic human alveolar basal epithelial cells) **(Amigo-Benavent et al., 2017)**.

I. vomitoria a Yaupon holly plant also exhibited anticancer activity against HT-29 human colon adenocarcinoma cell and chemoprotective effect induced by synergistic effect exerted by combination of polyphenols like 3 mono- and di-caffeoylquinic acids, quercetin and kaempferol glycosides on non-cancer colonic myofibroblast CCD-18Co cells by attenuating ROS production **(Noratto et al., 2011)**. *I. chinensis* also showed hepatoprotective activity against N-acetyl-p-aminophenol (APAP)-induced HepG2 cell injury **(Si-Yuan et al., 2021).**

Additionally, *I. kudingcha* has *in vitro* anticancer activities in MCF-7 human breast adenocarcinoma by following the apoptotic pathway. The anticancer mechanism revealed the rise of apoptotic bodies, regulation of Bax and Bcl-2 expression, downregulating iNOS and COX-2 gene expression. Attenuation of COX-2 and iNOS gene expression is reported to have a significant role in reducing colon carcinogenesis **(Zhao et al., 2013).**

There is no report of *Ilex khasiana* on its anticancer activity unlike the other species of *Ilex.* Endemic to north eastern part of India, the plant needs immediate attention and exploration. Form the above studies fractionation of IKM showed drastic increase in its cytotoxicity and IKM Dee is suspected to be the fraction containing most of the anticancer possessing compounds. The cytotoxic activity of IKM Dee on both A549 cells and HCT116 might be attributed to the phenolic and phytosterols content. In both the cell lines the extract was found to induced apoptosis in dose-dependent manner.

SUMMARY

The anticancer property of IKM Dee was subjected against HCT116 (human colorectal carcinoma cell line) and A549 (adenocarcinomic human alveolar basal epithelial cells). Among the three time points chosen (24, 48 and 72 hours) for all the extracts including IKM, IKM Hex, IKM Dee, IKM Bu and IKM C, IKM Dee showed the best cytotoxic effect of **26.90±6.08** and **22.77±4.68** on HCT116 and A549 cells in MTT assay at 24 hours. Therefore, 24 hours was considered the best time point for all the other tests undertaken. IKM Dee exhibited dose-dependent apoptotic activity in which 100 µg/ml concentration gave apoptotic index of **57.62±2.22** in A549 cells and **62.55±2.16** in HCT116 cells, which was comparatively higher when compared to lower doses of 25 and 50 µg/ml. IKM Dee has inhibited the colony formation ability of cancer cells. Clonogenic assay was used to the study the ability of a single cell to form a colony and upon treatment the colony declined drastically compared to the untreated control **(87.93±1.37)** by **21.77±0.78** and **8.42±0.12** for A549 and HCT116 cells at the highest dose of 100 µg/ml. Undoubtedly, the anticancer potency of *Ilex khasiana* was displayed and chemical characterization of the extract will unveil the bioactive compound(s) responsible.

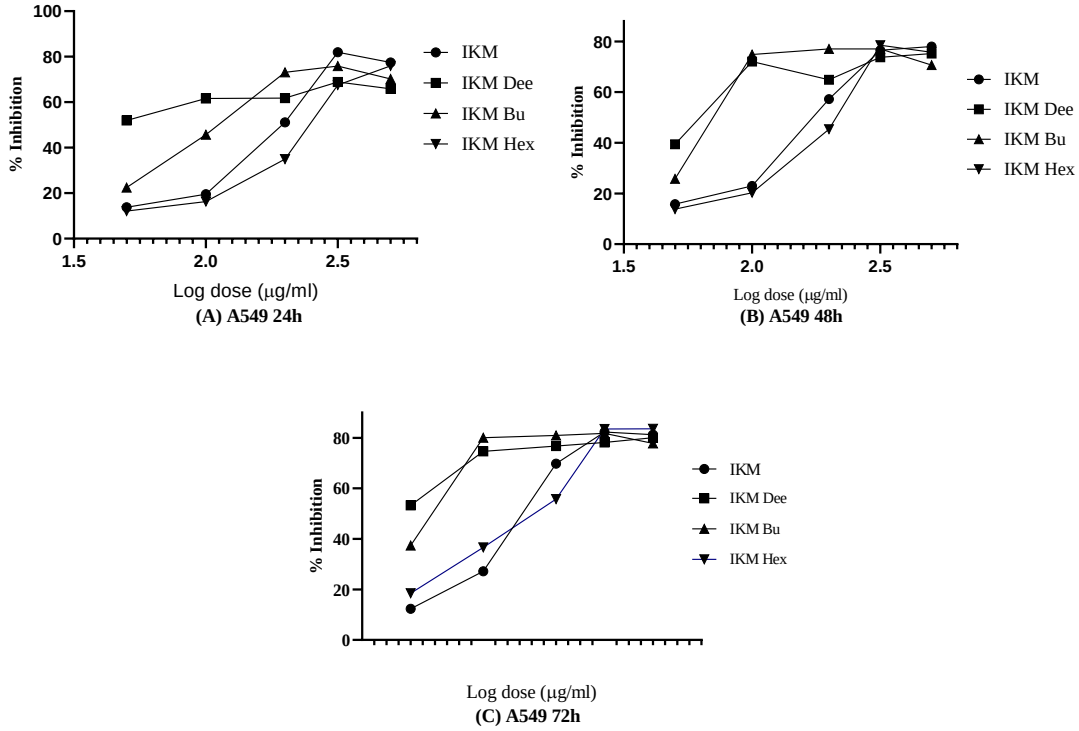


Figure4.1: Inhibitory effect of selected *I. khasiana* extracts on A549 cell lines against its log-doses after 24(A), 48(B) and 72(C) hours. IKM: *I. khasiana* methanol extract, IKM Dee: IKM Diethyl ether fraction, IKM Bu: IKM Butanol fraction, IKM Hex: IKM Hexane fraction.

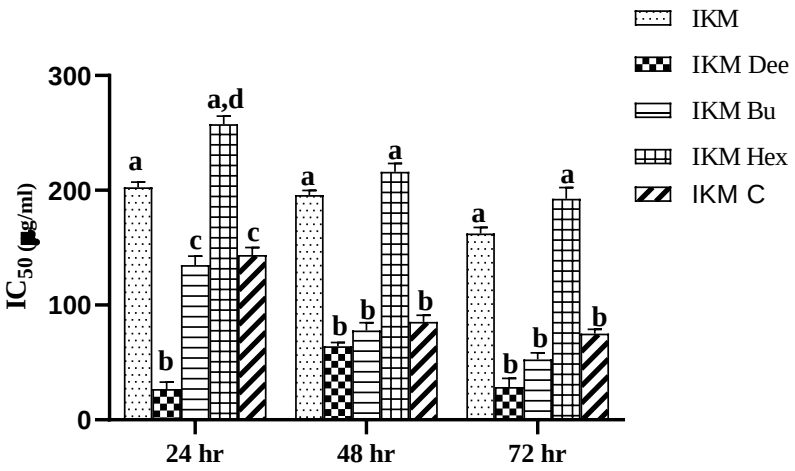


Figure 4.2: IC_{50} of selected *I. khasiana* extracts on A549 cells after 24, 48 and 72 hr treatment. IKM: *I. khasiana* methanol extract, IKM Dee: IKM Diethyl ether fraction, IKM Bu: IKM Butanol fraction, IKM Hex: IKM Hexane fraction. Values are expressed as Mean ± SEM. Different letters indicate significant variation between extracts at each treatment duration.

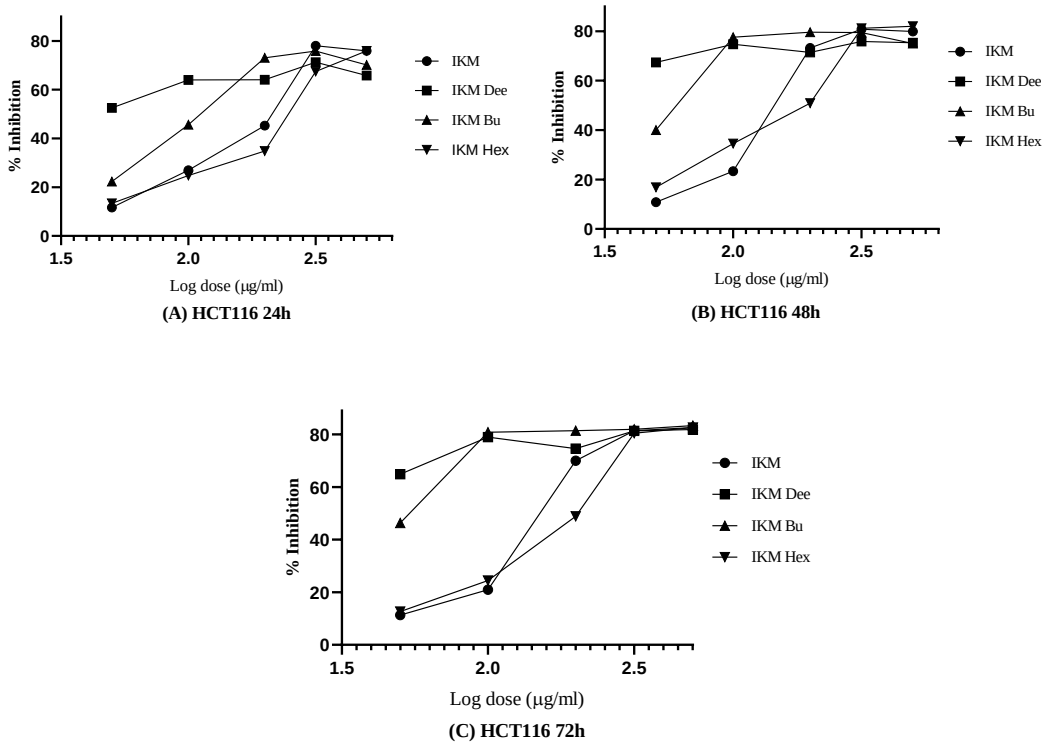


Figure 4.3: Inhibitory effect of selected *I. khasiana* extracts on HCT116 cell lines against its log-doses after 24 (A), 48 (B) and 72 (C) hours. IKM: *I. khasiana* methanol extract, IKM Dee: IKM Diethyl ether fraction, IKM Bu: IKM Butanol fraction, IKM Hex: IKM Hexane fraction.

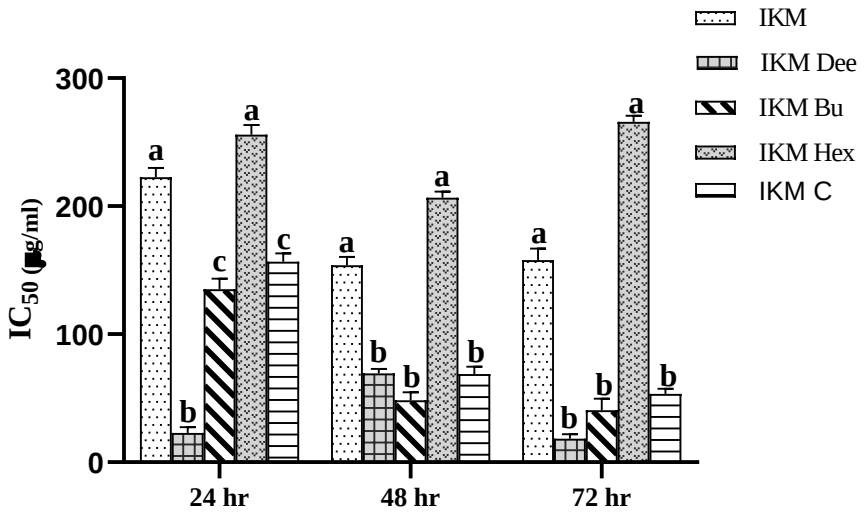


Figure 4.4: IC_{50} of selected *I. khasiana* extracts on HCT116 cells after 24, 48 and 72 hours treatment. IKM: *I. khasiana* methanol extract, IKM Dee: IKM Diethyl ether fraction, IKM Bu: IKM Butanol fraction, IKM Hex: IKM Hexane fraction. Values are expressed as Mean ± SEM. Different letters indicate significant variation between extracts at each treatment duration.

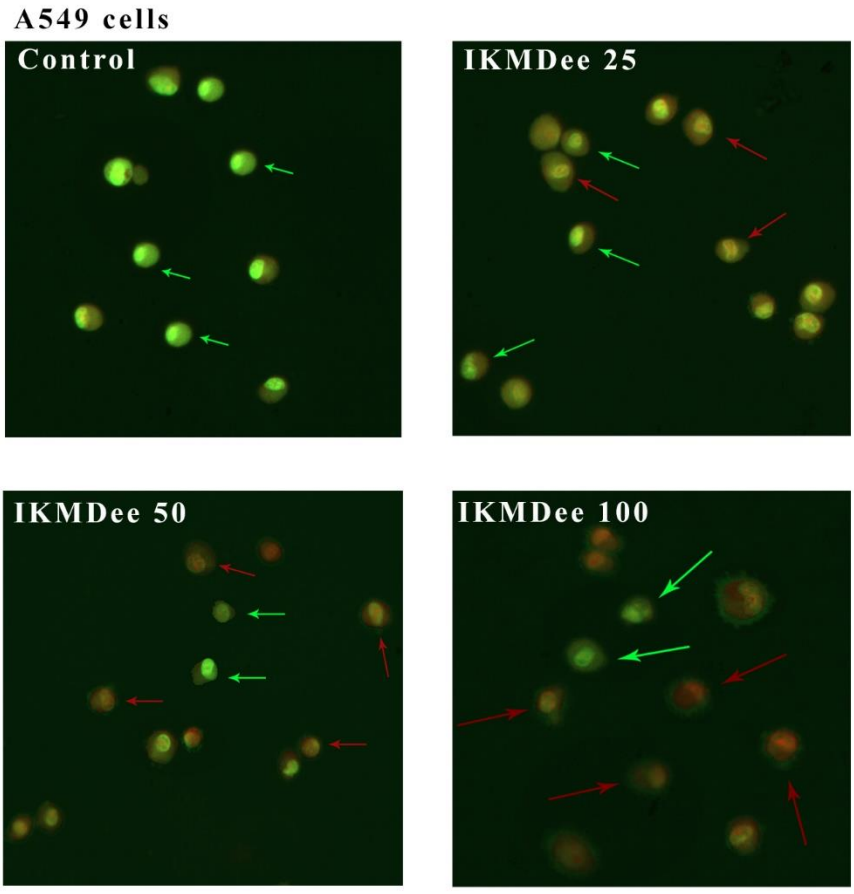


Figure 4.5: AO/EtBr dual staining of A549 cells after treatment with different doses of IKM Dee for 24hr (live cells are indicated by the green arrow and apoptotic cells are shown by the red arrows). IKM Dee: IKM Diethyl ether fraction.

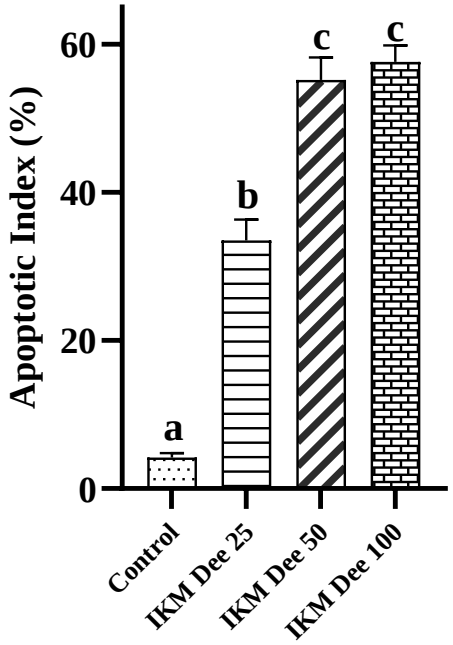


Figure 4.6: Percentage of apoptotic A549 cells after 24 hr treatment with IKM Dee in comparison with Control (A549 cells without any treatment). Values are expressed as Mean ± SEM. Different letters indicate significant variation. IKM Dee: IKM Diethyl ether fraction.

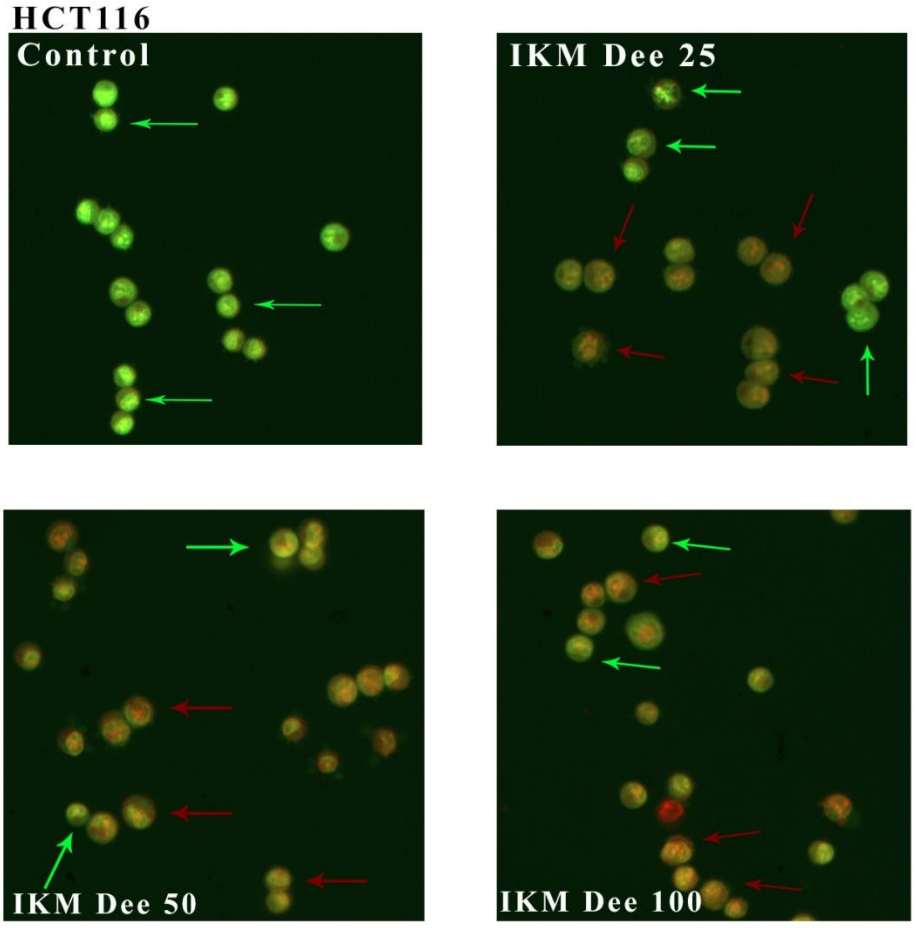


Figure 4.7: AO/EtBr dual staining of HCT116 cells after treatment with different doses of IKM Dee for 24hr (live cells are indicated by the green arrow and apoptotic cells are shown by the red arrows). IKM Dee: IKM Diethyl ether fraction.

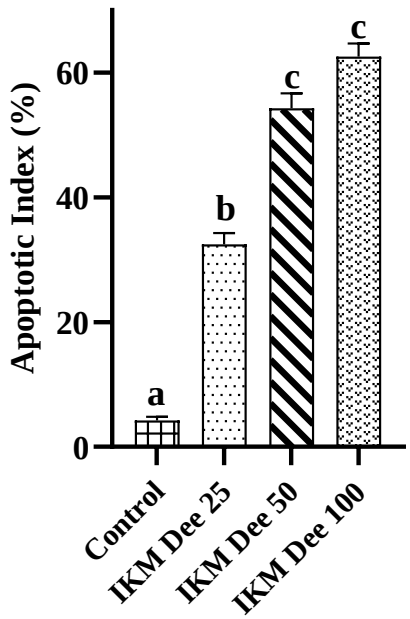


Figure 4.8: Percentage of apoptotic HCT116 cells after 24 hours treatment with IKM Dee in comparison with Control (HCT116 cells without any treatment. Values are expressed as Mean ± SEM. Different letters indicate significant variation. IKM Dee IKM Diethyl ether fraction.

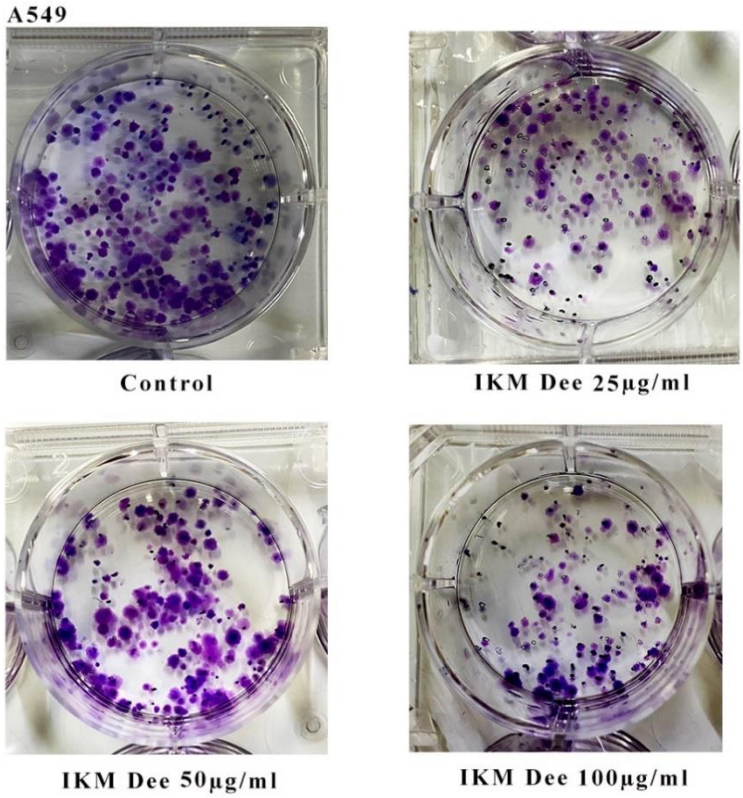


Figure 4.9: Inhibitory effect of different dosage of IKM Dee on colony formation of A549 cells. IKM Dee: IKM Diethyl ether fraction

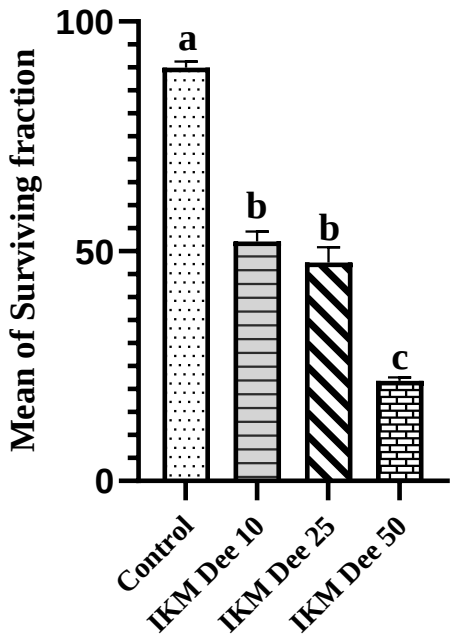


Figure 4.10: Inhibition of proliferation potency of A549 cells by different concentration (10, 25 and 50 µg/ml) of IKM Dee after 24 hours of treatment expressed as surviving fraction. Control group received no treatment. Values are expressed as Mean ± SEM. Different letters indicate significant variation. IKM Dee IKM Diethyl ether fraction.

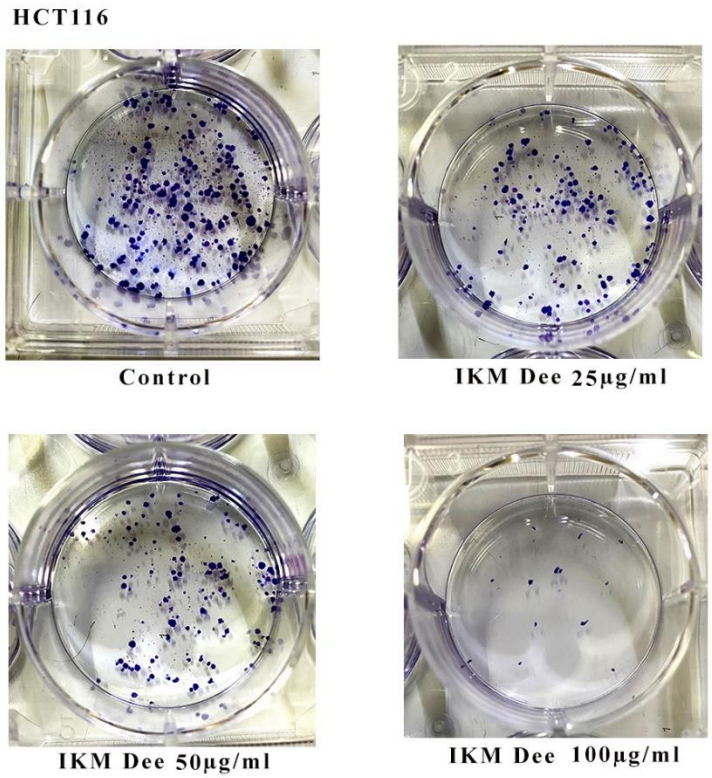


Figure 4.11: Inhibitory effect of different dosage of IKM Dee on colony formation of HCT116 cells. IKM Dee: IKM Diethyl ether fraction

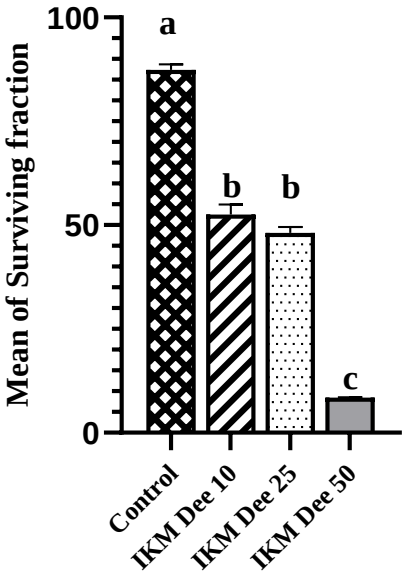


Figure 4.12: Inhibition of proliferation potency of HCT116 cells by different concentration (10, 25 and 50 µg/ml) of IKM Dee after 24 hours of treatment expressed as surviving fraction. Control group received no treatment. Values are expressed as Mean ± SEM. Different letters indicate significant variation. IKM Dee: IKM Diethyl ether fraction.

Table 4.1: IC_{50} of different *I. khasiana* extracts at three time points against A549 cell lines.

Treatment (time)	Extract	Mean ± SEM
24	**IKM**	202.50±4.53
	IKM Dee	**26.90±6.08***
	IKM Bu	134.90±7.64
	IKM Hex	257.70±6.98
	IKM C	143.66±6.54
48	**IKM**	195.77±4.15
	IKM Dee	64.04±3.27
	IKM Bu	77.98±6.55
	IKM Hex	216.13±7.32
	IKM C	85.44±5.76
72	**IKM**	162.27±5.30
	IKM Dee	28.61±7.44
	IKM Bu	52.74±5.66
	IKM Hex	192.50±9.67
	IKM C	75.00±3.99

IKM: *I. khasiana* methanol extract, IKM Dee: IKM Diethyl ether fraction, IKM Bu: IKM Butanol fraction, IKM Hex: IKM Hexane fraction. *indicated the extract with lowest IC_{50} and the most effective treatment duration.

Table 4.2: IC_{50} of different *I. khasiana* extracts at three time points against HCT116 cell lines.

Treatment (time)	Extract	Mean ± SEM
24	**IKM**	222.73±7.14
	IKM Dee	**22.77±4.68***
	IKM Bu	135.10±8.34
	IKM Hex	255.90±7.45
	IKM C	156.56±6.54
48	**IKM**	153.90±6.50
	IKM Dee	69.41±3.49
	IKM Bu	48.28±6.25
	IKM Hex	206.53±4.76
	IKM C	68.90±5.76
72	**IKM**	157.87±8.90
	IKM Dee	18.37±3.53
	IKM Bu	40.51±9.21
	IKM Hex	265.87±4.75
	IKM C	53.287±3.99

IKM: *I. khasiana* methanol extract, IKM Dee: IKM Diethyl ether fraction, IKM Bu: IKM Butanol fraction, IKM Hex: IKM Hexane fraction. *indicated the extract with lowest IC_{50} and the most effective treatment duration.

Table 4.3: Apoptotic index of IKM Dee on A549 and HCT116 cells.

Cell Type	Treatment (µg/ml)	Mean±SEM
	IKM Dee 25	33.50±2.80
	IKM Dee 50	55.18±3.03
A549	IKM Dee 100	**57.62±2.22***
	IKM Dee 25	32.47±1.83
	IKM Dee 50	54.29±2.43
HCT116	IKM Dee 100	**62.55±2.16***

IKM Dee = IKM extract Diethyl ether fraction. * indicated the treatment group having the highest apoptotic index

Table 4.4: Surviving fraction of A549 and HCT116 against IKM Dee.

Cell Type	Treatment (µg/ml)	Mean±SEM
	Control	89.93±1.37
	IKM Dee 25	52.56±3.15
	IKM Dee 50	47.14±4.3
A549	IKM Dee 100	21.77±0.78*
	Control	87.39±1.33
	IKM Dee 25	52.58±2.38
	IKM Dee 50	48.12±3.30
HCT116	IKM Dee 100	8.42±0.12*

IKM Dee = IKM Diethyl ether fraction. *indicated the treatment group having the highest proliferative inhibition.

REFERENCES

Amigo-Benavent, M., Wang, S., Mateos, R., Sarriá, B., & Bravo, L. (2017). Antiproliferative and cytotoxic effects of green coffee and yerba mate extracts, their main hydroxycinnamic acids, methylxanthine and metabolites in different human cell lines. Food and chemical toxicology, 106, 125-138.

Androutsopoulos, V., Arroo, R. R., Hall, J. F., Surichan, S., & Potter, G. A. (2008). Antiproliferative and cytostatic effects of the natural product eupatorin on MDA-MB-468 human breast cancer cells due to CYP1-mediated metabolism. Breast Cancer Research, 10(3), 1-12.

Baskar, A. A., Ignacimuthu, S., Paulraj, G. M., & Al Numair, K. S. (2010). Chemopreventive potential of β-sitosterol in experimental colon cancer model-an in vitro and in vivo study. BMC complementary and alternative medicine, 10(1), 1-10.

Bastos, D. H., Saldanha, L. A., Catharino, R. R., Sawaya, A., Cunha, I. B., Carvalho, P. O., & Eberlin, M. N. (2007). Phenolic antioxidants identified by ESI-MS from yerba mate (*Ilex paraguariensis*) and green tea (*Camelia sinensis*) extracts. Molecules, 12(3), 423-432.

Bray, F., & Soerjomataram, I. (2015). The changing global burden of cancer: transitions in human development and implications for cancer prevention and control. Cancer: disease control priorities, 3, 23-44.

Bray, F., Laversanne, M., Weiderpass, E., & Soerjomataram, I. (2021). The ever-increasing importance of cancer as a leading cause of premature death worldwide. Cancer, 127(16), 3029-3030.

de Mejía, E. G., Song, Y. S., Heck, C. I., & Ramírez-Mares, M. (2010). Yerba mate tea (Ilex paraguariensis): Phenolics, antioxidant capacity and *in vitro* inhibition of colon cancer cell proliferation. Journal of Functional Foods, 2(1), 23-34.

Fidler, M. M., Soerjomataram, I., & Bray, F. (2016). A global view on cancer incidence and national levels of the human development index. International journal of cancer, 139(11), 2436-2446.

Franken, N. A., Rodermond, H. M., Stap, J., Haveman, J., & Van Bree, C. (2006). Clonogenic assay of cells in vitro. Nature protocols, 1(5), 2315-2319.

Kasibhatla, S., Amarante-Mendes, G. P., Finucane, D., Brunner, T., Bossy-Wetzel, E., & Green, D. R. (2006). Acridine orange/ethidium bromide (AO/EB) staining to detect apoptosis. Cold Spring Harbor Protocols, 2006(3), pdb-prot4493.

Lee, S. H., Jaganath, I. B., Wang, S. M., & Sekaran, S. D. (2011). Antimetastatic effects of *Phyllanthus* on human lung (A549) and breast (MCF-7) cancer cell lines. PloS one, 6(6), e20994.

Li, T., Pan, H., Feng, Y., Li, H., & Zhao, Y. (2015). Bioactivity-guided isolation of anticancer constituents from *Hedera nepalensis* K. Koch. South African Journal of Botany, 100, 87-93.

Lu, J. N., Panchanathan, R., Lee, W. S., Kim, H. J., Kim, D. H., Choi, Y. H., & Hong, S. C. (2017). Anthocyanins from the fruit of vitis coignetiae pulliat inhibit tnf-augmented cancer proliferation, migration, and invasion in A549 cells. Asian Pacific journal of cancer prevention: APJCP, 18(11), 2919.

Maiocchi, M., Corrales, M. L., Cardoso Schiavi, P., Serrano, N., Petenatti, E. M., Marchevsky, E. J., & Del Vitto, L. Á. (2018). Nutritional parameters of mate cocido with milk prepared from two species of Ilex and their commercial mixture for school age population.

Mosmann T. (1983). Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J Immunol Methods, 16;65(1-2):55-63.

Noratto, G. D., Kim, Y., Talcott, S. T., & Mertens-Talcott, S. U. (2011). Flavonol-rich fractions of yaupon holly leaves (*Ilex vomitoria*, Aquifoliaceae) induce microRNA-146a and have anti-inflammatory and chemopreventive effects in intestinal myofribroblast CCD-18Co cells. Fitoterapia, 82(4), 557-569.

Noureddine, T., El Husseini, Z., Nehme, A., & Massih, R. A. (2018). Antibacterial activity of *Ilex paraguariensis* (Yerba Mate) against Gram-positive and Gram-negative bacteria. The Journal of Infection in Developing Countries, 12(09), 712-719.

Pandey, A., & Tripathi, S. (2014). Concept of standardization, extraction and pre phytochemical screening strategies for herbal drug. Journal of Pharmacognosy and Phytochemistry, 2(5).

Pastorino, U., Silva, M., Sestini, S., Sabia, F., Boeri, M., Cantarutti, A., & Marchianò, A. (2019). Prolonged lung cancer screening reduced 10-year mortality in the MILD trial: new confirmation of lung cancer screening efficacy. Annals of Oncology, 30(7), 1162-1169.

Pérez, J. M., Maldonado, M. E., Rojano, B. A., Alzate, F., Sáez, J., & Cardona, W. (2014). Comparative antioxidant, antiproliferative and apoptotic effects of *Ilex laurina* and *Ilex paraguariensis* on colon cancer cells. Tropical Journal of Pharmaceutical Research, 13(8), 1279-1286.

Qi, W., Weber, C. R., Wasland, K., & Savkovic, S. D. (2011). Genistein inhibits proliferation of colon cancer cells by attenuating a negative effect of epidermal growth factor on tumor suppressor FOXO3 activity. BMC cancer, 11(1), 1-9.

Sasidharan, S., Chen, Y., Saravanan, D., Sundram, K. M., & Latha, L. Y. (2011). Extraction, isolation and characterization of bioactive compounds from plants' extracts. African journal of traditional, complementary and alternative medicines, 8(1).

Siegel, R. L., Miller, K. D., Goding Sauer, A., Fedewa, S. A., Butterly, L. F., Anderson, J. C., & Jemal, A. (2020). Colorectal cancer statistics, 2020. CA: a cancer journal for clinicians, 70(3), 145-164.

Si-Yuan, S. H. A. O., Ruo-Fei, L. I., Hua, S. U. N., & Shuai, L. I. (2021). New triterpenoid saponins from the leaves of *Ilex chinensis* and their hepatoprotective activity. Chinese Journal of Natural Medicines, 19(5), 376-384.

Turner, M. C., Andersen, Z. J., Baccarelli, A., Diver, W. R., Gapstur, S. M., Pope III, C. A., ... & Cohen, A. (2020). Outdoor air pollution and cancer: An overview of the current evidence and public health recommendations. CA: a cancer journal for clinicians, 70(6), 460-479.

World Health Organization. (2020). Global Health Estimates 2020: Deaths by Cause, Age, Sex, by Country and by region. World Health Organization, 2000-2019.

Yao, X., Zhang, F., & Corlett, R. T. (2022). Utilization of the Hollies (*Ilex* L. spp.): A Review. Forests, 13(1), 94.

Zhao, X., Wang, Q., Qian, Y., & Song, J. L. (2013). *Ilex kudingcha* CJ Tseng (Kudingcha) has in vitro anticancer activities in MCF-7 human breast adenocarcinoma cells and exerts anti-metastatic effects in vivo. Oncology letters, 5(5), 1744-1748.

CHAPTER - 5

INVESTIGATION OF *IN VIVO* ANTICANCER ACTIVITY OF *ILEX KHASIANA* ON DALTON'S LYMPHOMA BEARING MICE.

INTRODUCTION

Cancer is multifactorial disease marked by uncontrolled cell division of somatic cell that once performed normal DNA replication and proliferation. This uninhibited growth could invade the adjacent cells in a process called metastasis. The comprehensive blueprint of cancer traits was laid out including the enriching information on the self-sufficiency of cancer cells, circumventing anti-growth signal, dodging apoptosis, unlimited replication potency, angiogenesis, invading neighboring cell-metastasis, alteration of energy metabolism and avoiding immune action that upregulated the genome instability and tumor inducing inflammation **(Fouad and Aanei, 2017; Hanahan and Weinberg, 2011).** The development in the management of strokes and heart attack led to the decrease in mortality rate related to this cause and ultimately made cancer the leading cause of death in most of the countries worldwide **(Bray et al., 2021)**. A case studies from 185 countries showed awful incidence of 19.3 million new cancer case and approximately, 10 million death was recorded related to 38 different cancer sites in the year 2020 **(Ferlay et al., 2021).**

Lymphomas are immune system related tumor in which non-Hodgkin lymphoma accounted for 90% of all the case and 10% of lymphoma is related to Hodgkin's lymphoma. Diagnosis of Non-Hodgkin lymphoma is a difficult task due to its wide clinical and histological variation of which more than 85% of the cases are related to B lymphocytes and the rest emerged from T lymphocytes or NK lymphocytes with a subtypes like diffuse large B cell lymphoma (DLBCL), follicular lymphoma (FL), and Burkitt's lymphoma (BL). **(Armitage et al., 2017)**. According to Global

Cancer Statistics (2020), 544,352 new cases of non-Hodgkin lymphoma was recorded which made up the 2.8% of all the cancer cases recorded with 259,793 new deaths which was accounted for 2.6% of all the cancer related death **(Sung et al., 2021)**.

Dalton's lymphoma is a Non-Hodgkin transplantable T-cell lymphoma in thymus of murine, used widely for evaluation of known and novel drug of both natural and synthetic origin. Thus, many behaviors of a cancer cells like its progression, signaling process, proliferation and growth can be observed in DLA cells **(Das and Vinayak, 2014)**. The most recommended therapy for Non-Hodgkin lymphoma include anti-CD20 immunotherapy – namely rituximab or Obinutuzumab along with chemotherapy agents. Moreover, many therapeutics drugs are on their final stage of clinical trials for the treatment of Non-Hodgkin lymphoma like Blinatumomab, Polatuzumab vedotin, Enzastaurin, Nivolumab, Aveluma and a few more. However, in relapsed or refractory cases, therapy became for challenging **(Chaudhari et al., 2019)**.

There are 669 reported *Ilex* species, from which only 38 species are well documented (6% of all the reported species) regarding its medicinal values and importance. Many *Ilex* species namely *I. vomitoria* leaves, *I. kudingcha*, and *I. paraguariensis* exhibited cytotoxic effect against breast cancer, oral cancer, nasopharyngeal carcinoma, and colon adenocarcinoma cells. The mode of mechanism includes inhibition of cell viability, anti-proliferative, anti-metastatic and apoptotic action against cancer cells **(Gómez-Juaristi et al., 2018).** Chlorogenic acid, caffeoylquinic acids were the prominent compounds that activate the pro-apoptotic factors caspase-3 and caspase-9 in TCA8113 squamous cancer cells and caspase-8 and caspase-3 in HT-29 human colon cancer cells. This led to the decline in inflammatory mediator NF-κB, which regulates cell proliferation, anti-apoptosis, and cell metastasis **(Puangpraphant et al., 2011).** Many *Ilex* species exhibited positive impact on antioxidant enzymes. *I. paraguariensis* was reported to increase ferric-reducing antioxidant potential in dyslipidemic subjects as well as glutathione (GSH) concentration and decreased serum lipid hydroperoxides (LOOH) levels in type 2

diabetic mellitus subjects **(Boaventura et al., 2013).** Consumption of yerba tea also remarkably increase catalase, superoxide dismutase and glutathione peroxidase by 28.7%, 21.3% and 9.6% respectively **(Boaventura et al., 2015).**

Ilex brasiliensis is known to be effective against BW 5147 lymphoma cell line, the HPLC analysis detected chlorogenic acid and caffeic acid in the extract which might the compounds associated with the plant's antiproliferative action against lymphoma cell line **(Filip et al., 2008).** Likewise, *I. affinis* also has antiproliferative effect against EL4, a T lymphoblast that was established from a lymphoma induced in a C57BL mouse by 9,10-dimethyl-1,2-benzanthracene **(Cogoi et al., 2014).** Thus, the phenolic compounds, chlorogenic acids like mono- and dicaffeoylquinic acids and flavonols were the contributing factor for *Ilex* species medicinal potency **(Barg et al., 2014)**.

A species of holly, Ilex khasiana Purk. (family Aquifoliaceae) is one of a few critically endangered medicinal plants in India. It is endemic to Khasi Hills, Meghalaya, and Aizawl, Mizoram. In the traditional medicine of Khasi and Mizo people, the leaves are used a panacea for all sorts of infections **(Lalnunfela et al., 2020)**. There are no specific reports on its chemical constituents and its potency against cancer cell. So, this experiment will lead to a ground breaking record on *I. khasiana*'s anticancer activity.

MATERIALS AND METHODS

Chemicals and Reagents

Dimethyl sulfoxide (DMSO), 1- chloro-2,4-dinitrobenzene (CDNB), 5, 5'dithio 2-nitrobenzoic acid (DTNB), ethylenediamine tetra-acetic acid (EDTA), reduced glutathione (GSH), nitroblue tetrazolium (NBT), phenazine methosulphate (PMS), 1, ethidium bromide, acridine orange, sodium pyruvate, thiobarbituric acid (TBA), and cytochalasin B were obtained from Sigma Aldrich Chemical Co. (Kolkata, India). Coomassie brilliant blue, reduced nicotinamide adenine dinucleotide (NADH) was purchased from HiMedia, Mumbai, India. Potassium chloride (KCl), hydrogen peroxide

(H_2O_2) and trichloroacetic acid (TCA), were procured from SD Fine-Chem Ltd., Mumbai, India., Disodium biphosphate (Na_2HPO_4), hydrochloric acid (HCl), sodium chloride (NaCl), sodium hydroxide (NaOH), sulphuric acid (H_2SO_4), n-butanol, Tris (hydroxymethyl) aminomethane (Tris buffer) and ammonium oxalate were supplied by Merck India Limited, Mumbai. Doxorubicin was requisitioned from Getwell Pharmaceuticals, Gurgaon, India.

Housing and Handling of the Animals.

Swiss albino mice weighing 20 -25g of both sexes were used for the *in vivo* anticancer studies of *Ilex khasiana.* The animals were placed in polyvinyl cages under a controlled atmospheric condition with a temperature of 24-25ºC, 50% humidity and light and dark (12 h each) cycle in Institutional Animal House, RIPANS. The animals were given commercially available food pellet and water *ad libitum.* The whole experiment is approved and carried out according to guidelines given by the RIPANS Institutional Animal Ethics Committee with approval number: **IAEC/RIPANS/26,** Aizawl, Mizoram.

Acute Toxicity

The acute toxicity study of different extracts of *Ilex khasiana* was performed according to Organization for Economic Co-operation and Development (OECD) guidelines. The animals of both sexes were used for the study (n=6). The animals were fasted 3-4 hours but water *ad libitum* before giving different extract of *Ilex khasiana*. The animals were observed for 14 days for any behavioral or physiological changes. If mortality was observed on 2-3 animals, then the treatment was considered as toxic dose.

Methods

The therapeutic effect of *I. khasiana* mice was determined using weight change and survival time on DLA bearing mice. Animal were divided into five groups, each containing six animals each. Group - I received normal saline and served as vehicle control. Group – II received doxorubicin (0.5 mg/kg b.w) intraperitoneally. The last

three groups received IKM 100, 250 and 500 (mg/kg b.w). All the animals received 1× 10^6 DLA cells in 0.25 ml, the day of transplant was considered as day '0'. Each group was given the assigned treatment for seven days after 48 hours of tumor transplantation.

Antioxidant assay, cytotoxicity, lipid peroxidation and serum enzyme activities were estimated in a new group of animals having the same treatment as above.

Survivability and weight change

The DLA bearing mice were monitored closely for weight change and survival record. After the treatment, animals from each group were weighed every 3 days up to 20 days individually and the change in weights were documented. The median survival time (MST) and average survival time (AST) were determined and % increase in median life span (IMLS) and % increase in average life span (IALS) were calculated **(Gupta et al., 2000).** According to National Cancer Institute Protocols, the treatment that exerted T/C value ≥ 120% is considered active against the target.

T/C= MST of the treated group/ MSL of the control group.

Preparation for biochemical assays

Liver and DLA fluid were collected after the completion of treatment by ketamine overdose **(Al-Batran et al., 2013).** Glass homogenizer was used to prepare 5% (w/v) liver homogenate in buffer (5 mM EDTA, 0.15 M NaCl, pH 7.4) and centrifuge for 30 min at 13,000 rpm at 4 °C. The supernatant was stored in an ice-cold condition for further analysis. The DLA fluid obtained was washed with NH_4Cl and 1X PBS two times and 5% (w/v) homogenate was obtained by sonicating the cells in buffer (5mM EDTA, 0.15M NaCl, pH 7.4) using sonicator (PCi Analytics). The supernatant was collected after centrifugation at10,000 rpm for 30 min at 4 °C for further biochemical assay.

Antioxidant status

Tissue and liver homogenates were subjected to biochemical assays. Protein contents were calculated against the standard BSA by using standard protocol **(Lowry et al., 1951).** Ellman's reaction – the reaction of a sample with DTNB was measured at 412 nm to measure glutathione level **(Moron et al., 1979).** Incubation of 0.5 ml of 0.1 M phosphate buffer (pH 6.5), 0.1 ml of 20 mM CDNB, and 8.8 ml distilled for 10 min followed by addition of 0.5 ml of 20 mM GSH and 0.1 ml of cell homogenate was used to determine GST level **(Beutler, 1984)**. The sample was measured at 340 nm using UV-VIS double beam spectrophotometer at 1 min intervals for 6 minutes.

GST activity = Absorbance of sample – Absorbance of blank × 1000/9.6 × Volume of sample

Where, 9.6 is the molar extinction coefficient for GST.

Superoxide Dismutase Assay

NBT reduction method was used for the estimation of SOD activity **(Fried et al., 1975).** The reaction time of 90 min was given at 30°C for 100 µl of 186 µM phenazene methosulfate, 300 µl of 3.0 mM nitroblue tetrazolium,200 µl of 780 µM NADH and 100 µl of cell homogenate.After termination of the reaction by adding 1000 µl of acetic acid followed by the addition of 4 ml n-butanol, the absorbance of the mixture was taken at 560nm using UV/VIS double beam spectrophotometer. The percent inhibition was calculated by measuring the absorbance of blank without SOD enzyme. The SOD activity was calculated using the formula:

(Blank-Sample)/Blank X 100.

The enzyme activity was expressed in unit (1 unit=50% inhibition of NBT reduction/mg protein).

Lipid peroxidation assay

Malondialdehyde (MDA) was formed from the oxidation of polyunsaturated fatty acids and was used as the index to measure peroxidation reaction of lipid. MDA gives red fluorescent upon reaction with thiobarbituric acid (**Beuege and Aust, 1978).** The absorbance of the sample was measured at 535nm and the concentration of MDA was calculated using the extinction coefficient of $1.56\times 10^6\ M^{-1}\ cm^{-1}$.

Determination of Liver and Kidney function

The animals were treated with the same dose mentioned above. After seven days of treatment, the blood was collected at different time intervals from the retro orbital sinuses using a capillary tube after anesthetizing the mice with ketamine. The samples were stored at 4ºC and centrifuges at 1000 rpm for 5 min and the serum was used to estimate AST, ALT and Creatinine using commercially available kits (Coral Clinical Systems, Uttarakhand, India).

DNA damage determination using Comet Assay

Comet assay was employed to measure strand breakage of DNA in DLA Cell using standard protocol with slight modification **(Singh et al., 1988).** Damages in DNA upon treatment with different dose of IKM (100, 250 and 500 mg/kg b.w) extracts are observed using single cell electrophoresis. Subsequently, the DLA cells were aspirated from both the control and DLA treated groups, the cells were washed with NH_4Cl and 1X PBS. Later, 2×10^4 DLA cells from different groups are mixed with 0.5% low-melting point agarose (LMPA) prepared in 1X PBS and 75 µl of the mixture was set on a frosted 1% normal-melting point agarose (NMPA) precoated slides, cover slip was laid on top of it. The third layer of 90 µl 0.5% LMPA was added upon gentle removal of the cover slip on the solidified slide incubated at 4^OC. The slides were incubated in lysing solution (2.5 M NaCl, 100 mM Na_2EDTA, 10 mM Trizma base, 1% Triton X-100 and 10% DMSO, pH 10) for 2 hrs. The Electrophoresis tank was filled with freshly prepared alkaline electrophoresis buffer (300 mM NaOH, 1 mM Na_2EDTA, pH13) and the

prepared slides were placed horizontally to allow unwinding of DNA for 20 min. The slides were washed for 5 min in neutralization buffer (0.4 M Tris-HCl, pH 7.5) after running the electrophoresis at 24 V and 300 mA for 30 min. The slides were washed with distilled H_2O and stained for 5 min with ethidium bromide (EtBr) solution (2 μg/mL). All the treatment slides were prepared in triplicate and were observed under a fluorescent microscope (Thermo Fisher Scientific, EVOS^R Fluorescence Imaging, AMEP-4615) with a magnification of 200x. Randomly selected cells were captured and analyzed for tail length and olive moment using Image J software.

Statistical analysis

Data are expressed as mean ± standard error of the mean. The significance for survival analysis was determined by Kaplan Meier test, One-way analysis of variance (ANOVA) was performed to test significant variations on serum enzyme activities of treatment groups followed by Tukey multiple comparison of means and IC_{50} was calculated using GraphPad Prism software ver. 8.0.2. A p value <0.05 was considered statistically significant.

RESULTS

Acute toxicity

The animals were observed up to 24 hours after oral administration of IKM 2000 mg/kg b.w, no sing of discomfort and lethality was observed. Thus, the LD_{50} of IKM is more than the given dose of 2000 mg/kg body weight.

Survivability and weight change

The *in vivo* anticancer activity of *I. khasiana* was determined by giving three different doses 100, 250 and 500 mg/kg b.w of IKM on mice induced with DLA. Among all the tested groups, Dox receiving group had the longest survival time, followed by IKM250, IKM500 and IKM100, control group having the shortest survival time **(Figure 5.1)**. The control group that received normal saline died within 25 days with MST and AST of 25.50 and 18.33 days. The longest surviving group i.e., Dox group died within 41 days from the treatment with MST and AST of 41.00 and 28.17, IKM250 was the most effective group with 33.50 and 22.83 (MST and AST). The anticancer efficacy of the treated group was observed by calculating T/C in which Dox exhibited 160.78% followed by 131.37, 127.45 and 109.80% for IKM250, IKM500 and IKM100 respectively. Among the extracts, the highest dose did not exhibit the best anticancer activity when compared to IKM250 for which the reason remains unknown. The anticancer activity of *I. khasiana* on the survivability of the DLA induced mice was given in **Table 5.1.** Besides survival time, the changes in weight among the individuals of different groups were measured every three days. The tumor proliferation increased gradually in all the treatment groups as time increases, in which vehicle control group that received normal saline has the highest weight gain. Groups that received Dox has the lowest weight change. Among the extracts, IKM250 has the highest efficacy in inhibiting the weight gain followed by IKM500, IKM100 has the lowest effect **(Figure 5.2).** Thus, like the other species of *Ilex*, *I. khasiana* also exhibited a remarkable cancer proliferation inhibition.

Antioxidant status

The animals were divided into five groups and after seven days of treatment the antioxidant potential of *I. khasiana* was investigated by determining the level of different antioxidants. The GSH level was significantly increase in groups that received Dox and IKM250, 18.95±1.12 and 20.34±1.40 μmol/mg protein respectively **(Table 5.2).** Likewise, the GST level was also higher in Dox and IKM 250 receiving group **(Table 5.2).** Interestingly, the IKM extract did not show dose dependent nature as the GSH and GST level in untreated, IKM100 and IKM500 groups **(Figure 5.3 and Figure 5.4)**. SOD level was highest in animals that received Dox, followed by IKM250. The extract may exert hepato-protective potential as the higher dose of IKM increased the level SOD in the liver sample as shown in **Figure 5.5.**

Serum enzyme assays

Serum enzymes were investigated in DLA tumor bearing Mice after 7 days of treatment to determine the kidney and hepato- protective potency of *I. khasiana.* The two liver enzymes ALT and AST were significantly ($p<0.05$) elevated in animal that received normal saline **(Figure 5.9: A and B)**. There was a slight decline in both ALT and AST level in animals the received Dox (20 mg/kg b.w) and IKM (100, 250, 500 mg/kg b.w). Among the extract doses, IKM 250 mg/kg b.w was found to be the most effective treatment even when compared with the standard reference **(Table 5.5)**. The kidney function test was performed by estimation of creatinine level and there were no significant ($p<0.05$) changes in all the other groups other than IKM250 and IKM500 in which there were significant decline in the enzyme level **(Figure 5.9: C)**.

Comet Assay

After seven days of treatment, ascites were taken from the DLA bearing mice and the DNA damage level was determined using Comet assay. The tail length and olive moment of the damaged DLA cells were observed in which IKM250 induced the highest DNA damage more than the standard drug Dox **(Table)** and were significantly different

at $p<0.05$. The tail length and the olive moment of different treatment group was displayed in **Figure 5.8,** in which IKM250 exhibited tail length and olive moment of 11.89±0.60 and 2.66±0.08 which was comparatively much higher than 6.52±0.34 and 0.96±0.16 respectively.

DISCUSSION

Cancer being a multifactorial disease require complexity even in the treatment, and the pursuit of cancer treatment is ever increasing. There is an emergency in the improvement of drug discovery as well as upgrading the potency of the present drugs. Natural products from plants, microbes and other organisms are the main reservoir of the much-needed therapeutic drugs. Thus, 25% of drugs discovered between 1981 and 2019 are all the natural related products **(Newman and Cragg, 2020).** Historically, natural products played an important role in drug discovery such as irinotecan, vincristine, etoposide and paclitaxel from plants, actinomycin D and mitomycin C from bacteria as well as marine-derived bleomycin among these many of the compounds are still the bulwark of cancer therapy. Both camptothecin and taxol are the most renowned compound ever derived naturally till date since 1960s from the maneuver initiated by National Cancer Institute (NCI) to discover therapeutic values of natural products **(Wall, 1998).**

The remarkable advancement in the field of drug discovery is the antibody drug conjugates (ADCs), in which the drug can reach the targeted tumor using the tissue specific binding nature of monoclonal antibody (mAb) which will leave the normal cell unaffected from the cytotoxic effect of the drug. So far, 60 ADCs are under clinical trial and 11 ADCs are widely used since 2011, Brentuximab vedotin is the ADC used for the treatment of Hodgkin lymphoma and anaplastic large cell lymphoma **(Abdollahpour-Alitappeh et al., 2019).**

Ilex species have well-documented phytochemical constituents that contributed widely as a natural remedy. *I. paraguariensis,* a famous yerba tea has been known for its phenolic and xanthine compounds like chlorogenic acid and caffeine respectively that prevents red blood cells lysis induced by hydrogen peroxide **(Peralta et al., 2013).** *I. latifolia* and *I. kudingcha* is also known to possess important antioxidant called caffeoylquinic acid and other major phenolic compounds mono- and dicaffeoylquinic acids were also isolated from *I. kudingcha* and *I. cornuta* **(Pirker and Goodman, 2010).**

This dicaffeoylquinic acids induced apoptosis by activation of caspase-8 and caspase-3 in human colon cancer cells **(Puangpraphant et al., 2011).** Flavonoid derived from *I. vomitoria* exhibited chemopreventive effects in intestinal myofibroblast CCD-18Co cells **(Noratto et al., 2011).**

The antioxidants assay determined the level of GSH and GST to estimate damage caused by oxidation in the targeted tissue. In both DLA cells and live of DLA bearing mice the antioxidant level decreased gradually but upon treatment with Dox and IKM showed increased level of GSH and GST especially in IKM250. The transduction and transcriptional pathways got activated under this stress and cytoplasmic death domain of TNFR1 formed complex 1, which activates NF-κβ, JNK and P53 cascade to propagate inflammation and survival signaling when TNF-α to TNFR1. The treatment with IKM indicated the reversal of toxicity induced by cancerous cells **(Dutta et al., 2018)**. The SOD level also increased significantly in in both Dox and IKM250 which further confirmed the anticancer potency of IKM. Surprisingly, MDA the mutagenic product of lipid peroxidation increases significantly in Dox treated samples both in DLA fluid and liver tissue which might be the excessive production of superoxide anion and reduction in detoxifying hydroperoxide, as a result of DOX treatment **(Lalmuansangi et al., 2020).**

Ascetic fluid is the source of nutrition for tumor cells, thus the rapid increase in ascites volume is required for tumor growth and the increase in survivability marked the cytotoxic effect of the plant extract **(Haldar et al.,2010)**. There was a significant decrease in body weight among the groups treated with IKM and Dox compared to the vehicle control group **(Figure 5.2)** and the lifespan of DLA bearing mice was increased upon treatment with IKM **(Figure 5.1).**

Liver enzymes (ALT and AST) were estimated to evaluate tissue damage and the alteration in these enzymes indicated the effect of DLA on normal tissue and the IKM250 was found to have hepatoprotective effect by lowering the enzyme level when compared with all the other treatment group **(Figure 5.7 A & B).** Unstable liver enzymes

leads to liver disease and these changes in the levels could be initiated by lowering of liver metabolic and catabolic capacity, tissue necrosis and structural alteration of normal hepatocytes **(Middleton, 1998)**. The kidney function test was determined by measuring the creatinine level. The enzyme level drastically elevates in untreated group, Dox and IKM100, but mice that received IKM250 and IKM500 seemed to lower the enzyme level **(Figure 5.7 C)**. The nephroprotective action of IKM might be associated with the antioxidant compound present in it **(Sarker and Nahar, 2020).**

DNA damage of DLA cell caused by Dox and IKM treatment was determined by Comet assay, a single-cell gel electrophoresis. The increase DNA damage was supported by increase in comet tail length. Running the gel at higher pH can also show DNA damage like image as the DNA uncoiled itself under the high pH and gave tail-like appearance when extending towards anode **(Colin, 2004)**. Comet assay showed that IKM250 has the longest tail length even compared to Dox which showed the toxicity of IKM extract.

Thus, the medicinal values of *I. khasiana* is based on its phytochemical constituents, the anticancer properties of IKM were undeniable and its hepato and nephro-protective could make the plant stand out as the future drug model to replace synthetic drugs that have indisputable side effects.

SUMMARY

The present work constitutes the first report ever made on the anticancer potency of *I. khasiana* extract. DLA is one of the best tools to study the anticancer activity of the selected plant on murine model. IKM250 has the highest antiproliferative effect against DLA cell by giving 33.55 and 22.83 MST and AST, Dox having the highest anticancer potency. The T/C (%) showed the effectiveness of the treatment in which Dox exhibited 160.78% and IKM250, 131.37%. The tumor growth was estimated by determining the weight change, Dox treated group has the lowest weight gain, however among the extract IKM250 stayed close to Dox followed by IKM500 and IKM100. The antioxidant enzymes level gave the activity of the extract and Dox against ROS when compared to the control group. GSH, GST and SOD increase in all the treatment group. IKM250 gave the highest level of antioxidant enzymes even when compared with the standard drug. The result ensures the hepatoprotective nature of IKM. Surprisingly, MDA level increased significantly in Dox receiving group induced by excessive production of superoxide anion and reduction in detoxifying hydroperoxide, as a result of DOX treatment. Biochemical assay was performed to determine the liver and kidney function test. IKM250 remained the best dose with the lowest dose of 16.61±1.54 (ALT), 108.66±2.31(AST) and 0.05±0.07 (Creatinine) which is comparatively lower when compared to the control group 26.30±1.52 (ALT), 139.12±1.76 (AST) and 0.9±0.06 (Creatinine). Thus, the hepatoprotective and nephroprotective potential of IKM cannot be underestimated. The IKM has a strong cytotoxic effect on DLA cells when observed through the Comet assay. IKM250 exhibited greater DNA damage (11.89± 0.57) than Dox (10.7±0.6) in terms of tail length. Therefore, in IKM extract the best dose is IKM250 for all the observed parameter and the deviation from dose-dependency is still unknown.

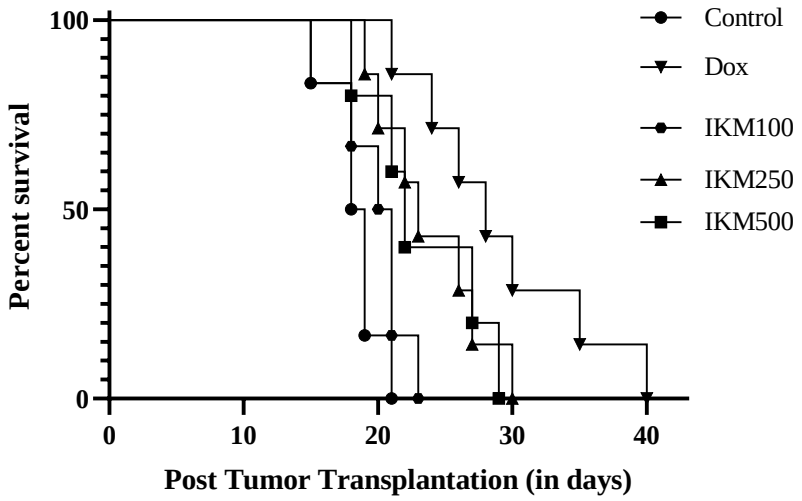


Figure 5.1: A Kaplan Meier's estimate of survival time of DLA bearing mice up to 40 days from day of transplant. Control: group receiving normal saline; DOX: DLA bearing mice treated with doxorubicin (20 mg/kg b.w); IKM100, IKM250, IKM500: Methanolic extract of *I. khasiana* at the dose of 100, 250, 500 mg/kg b.w, respectively.

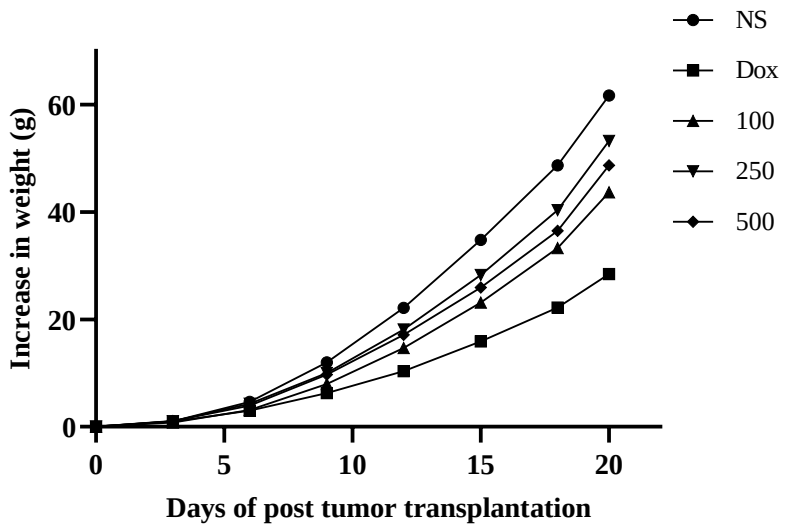


Figure 5.2: Average increase in body weight of DLA mice treated with different doses of IKM in three days intervals. Control: group receiving normal saline; DOX: DLA bearing mice treated with doxorubicin (20 mg/kg b.w); IKM100, IKM250, IKM500: Methanolic extract of *I. khasiana* at the dose of 100, 250, 500 mg/kg b.w, respectively.

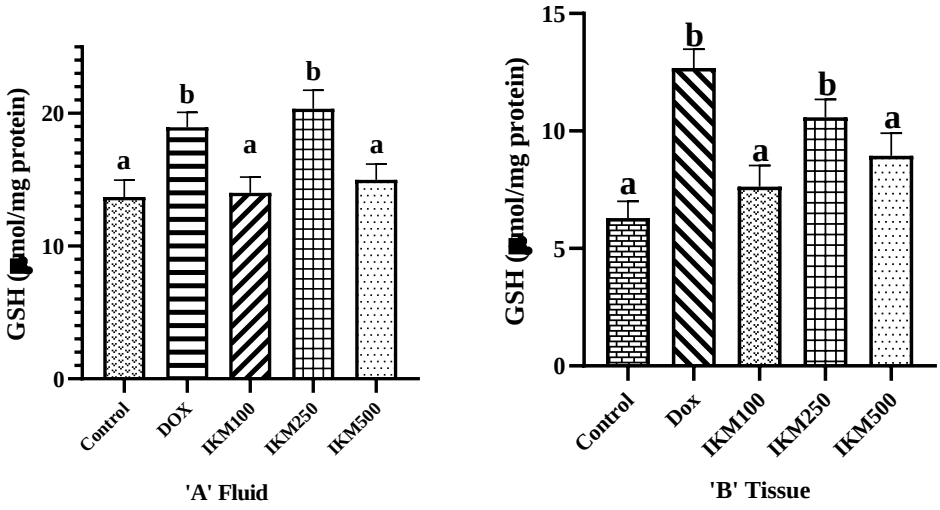


Figure 5.3: Effects IKM on Glutathione level (GSH) on DLA fluid (A) and liver (B) of DLA bearing mice. Means not sharing the same letter are significantly different at $p<0.05$. Control: group receiving normal saline; DOX: DLA bearing mice treated with doxorubicin (20 mg/kg b.w); IKM100, IKM250, IKM500: Methanolic extract of *I. khasiana* at the dose of 100, 250, 500 mg/kg b.w, respectively.

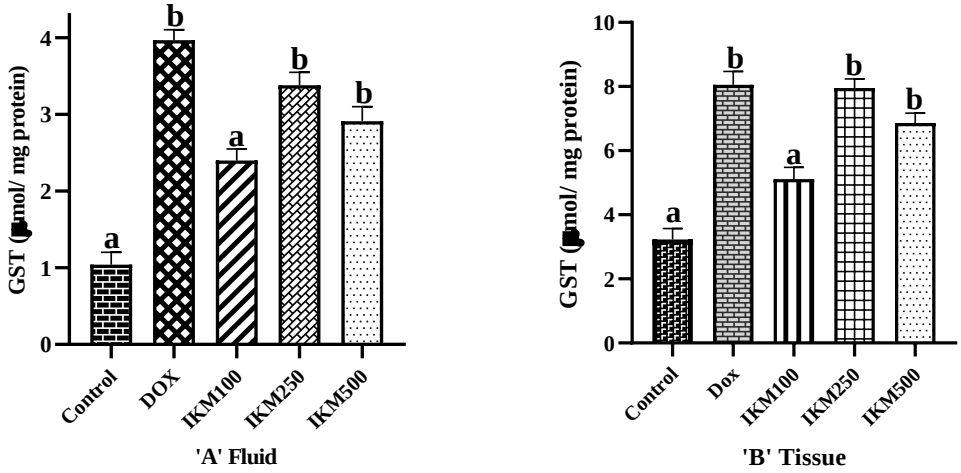


Figure 5.4: Effects IKM on Glutathione-s-transferase activity (GST) on DLA fluid (A) and liver (B) of DLA bearing mice. Means not sharing the same letter are significantly different at $p<0.05$. Control: group receiving normal saline; DOX: DLA bearing mice treated with doxorubicin (20 mg/kg b.w); IKM100, IKM250, IKM500: Methanolic extract of *I. khasiana* at the dose of 100, 250, 500 mg/kg b.w, respectively.

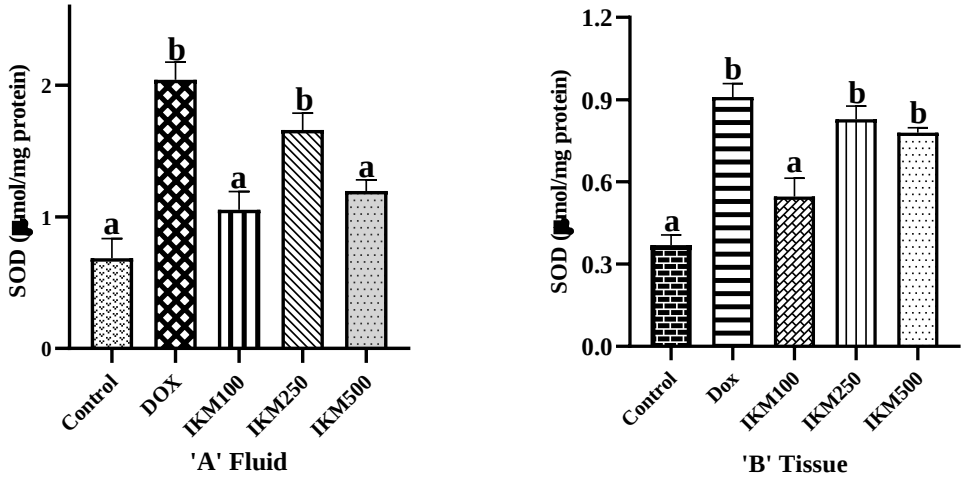


Figure 5.5: Effects IKM on superoxide dismutase activity (SOD) on DLA fluid (A) and liver (B) of DLA bearing mice. Means not sharing the same letter are significantly different at $p<0.05$. Control: group receiving normal saline; DOX: DLA bearing mice treated with doxorubicin (20 mg/kg b.w); IKM100, IKM250, IKM500: Methanolic extract of *I. khasiana* at the dose of 100, 250, 500 mg/kg b.w, respectively.

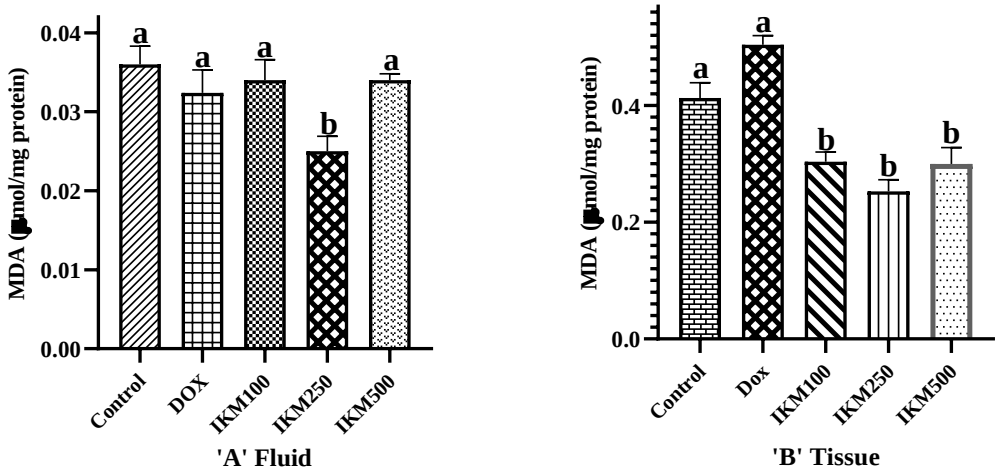


Figure 5.6: Effects IKM on lipid peroxidation expressed in malondialdehyde (µmol/mg protein) in DLA fluid (A) and liver (B) of DLA bearing mice. Means not sharing the same letter are significantly different at $p<0.05$. Control: group receiving normal saline; DOX: DLA bearing mice treated with doxorubicin (20 mg/kg b.w); IKM100, IKM250, IKM500: Methanolic extract of *I. khasiana* at the dose of 100, 250, 500 mg/kg b.w, respectively.

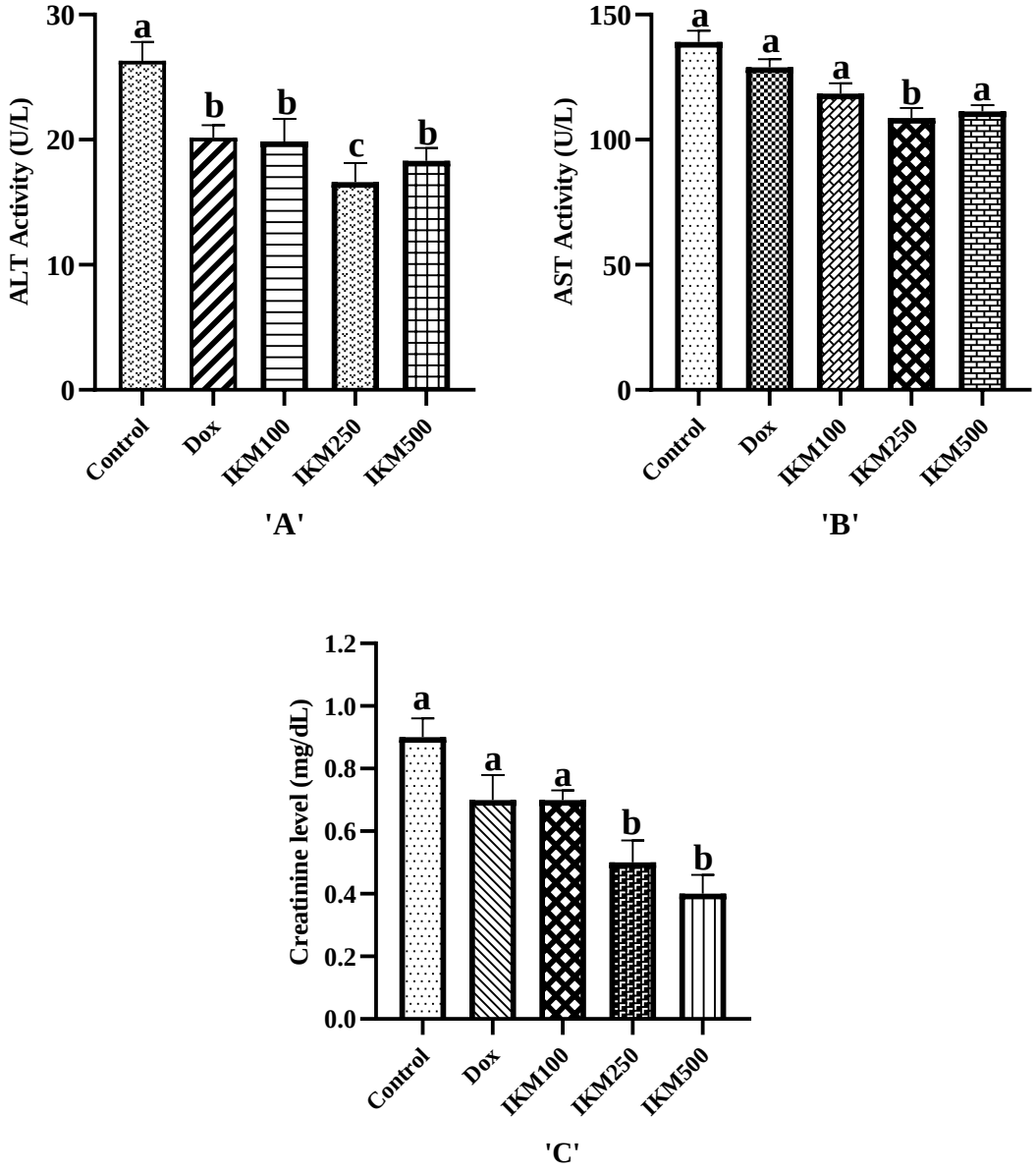


Figure 5.7: Effect of IKM on the liver enzymes (A) ALT, (B) AST and kidney function (C) Creatinine on Dalton's lymphoma ascites bearing mice. Means not sharing the same letter are significantly different at $p<0.05$. Control: group receiving normal saline; DOX: DLA bearing mice treated with doxorubicin (20 mg/kg b.w); IKM100, IKM250, IKM500: Methanolic extract of *I. khasiana* at the dose of 100, 250, 500 mg/kg b.w, respectively.

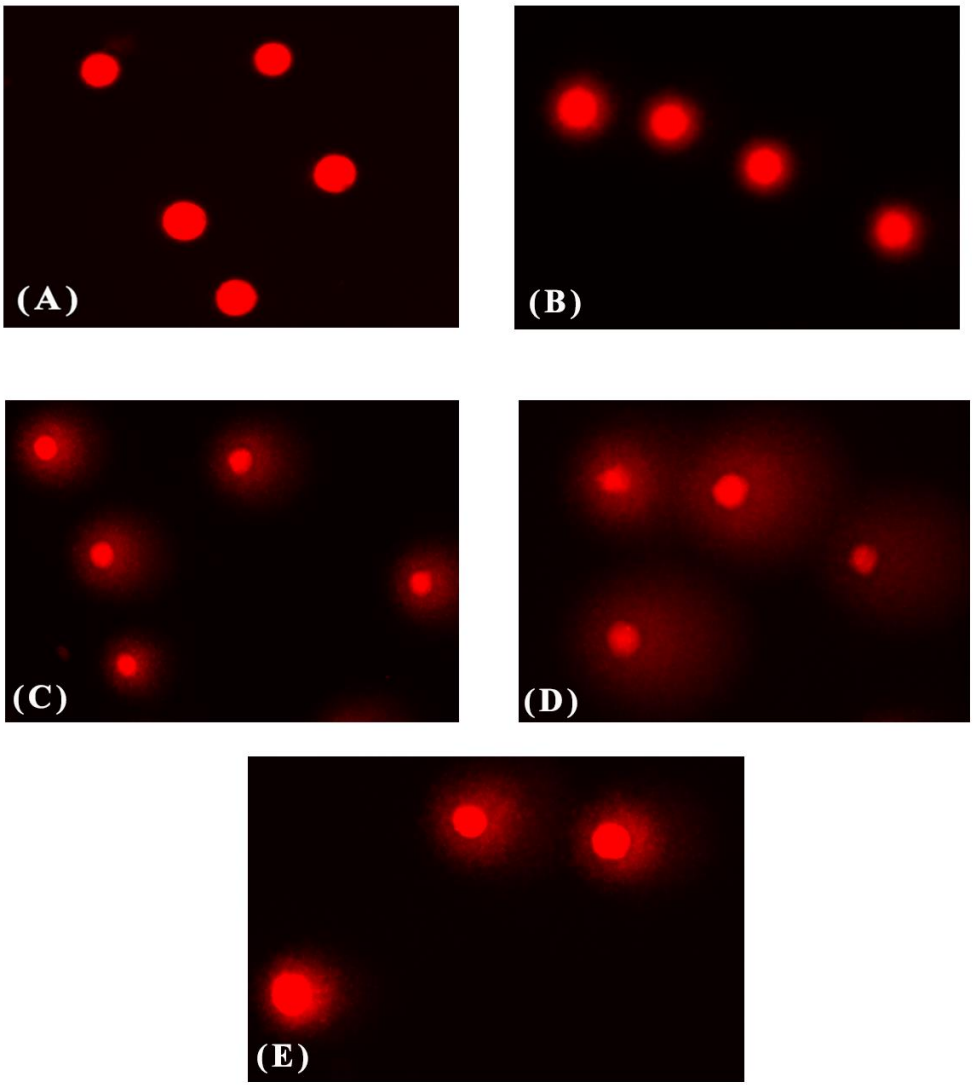


Figure 5.8: Fluorescence images of Comets observed in (A) Control: group receiving normal saline; (B) DOX: DLA bearing mice treated with doxorubicin (20 mg/kg b.w); (C) IKM100, (D) IKM250, (E) IKM500: Methanolic extract of *I. khasiana* at the dose of 100, 250, 500 mg/kg b.w, respectively.

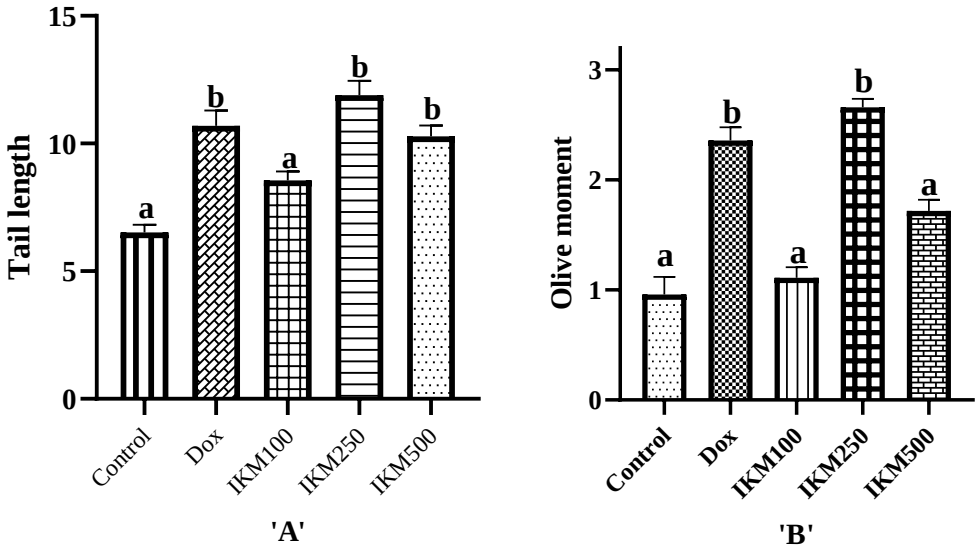


Figure 5.9: The extent of DNA damage expressed in terms of Tail length (A) and Olive moment (B). Means not sharing the same letter are significantly different at $p<0.05$. Control: group receiving normal saline; DOX: DLA bearing mice treated with doxorubicin (20 mg/kg b.w); IKM100, IKM250, IKM500: Methanolic extract of *I. khasiana* at the dose of 100, 250, 500 mg/kg b.w, respectively.

Table 5.1: Effect of different doses of IKM and DOX treatment on DLA bearing mice on the tumor response assessment based on MST, AST, % IMLS, % IALS and T/C ratio.

Group (mg/kg b.w)	MST	AST	IMLS	IALS	T/C	T/C*100
NS	25.50	18.33	-	-	-	-
$Dox_{0.5}$	41.00	28.17	60.78	53.68	01.61	160.78
IKM100	28.00	19.67	09.80	7.311	01.09	109.80
IKM250	33.50	22.83	31.37	24.55	01.32	131.37
IKM 500	32.50	23.16	27.45	26.39	01.27	127.45

MST: Mean survival time; AST: Average survival time; IMLS: Increase in mean life span; IALS: Increase in average life span.

Table 5.2: Alterations in the glutathione (GSH) level in mice bearing Dalton's lymphoma treated with Dox and IKM.

Treatment (mg/kg b.w)	GSH (µmol/mg protein)	
	DLA Fluid	Liver
	Mean ± SEM	Mean ± SEM
Control	13.71±1.26[a]	6.30±0.70[a]
$Dox_{0.5}$	18.95±1.12[b]	12.68±0.80[b]
IKM100	14.01±1.19[a]	7.63±0.90[a]
IKM250	20.34±1.40[b]	10.58±0.76[b]
IKM500	14.98±1.20[a]	08.95±0.96[a]

Means not sharing the same letter are significantly different at $p<0.05$. Control: group receiving normal saline; DOX: DLA bearing mice treated with doxorubicin (20 mg/kg b.w); IKM100, IKM250, IKM500: Methanolic extract of *I. khasiana* at the dose of 100, 250, 500 mg/kg b.w, respectively.

Table 5.3: Alterations in the Glutathione-S-transferase (GST) level in mice bearing Dalton's lymphoma treated with Dox and IKM.

Treatment (mg/kg b.w)	GST (µmol/mg protein)	
	DLA Fluid	Liver
	Mean ± SEM	Mean ± SEM
Control	1.04±0.16 [a]	3.23±0.34 [a]
$Dox_{0.5}$	3.97±0.13 [b]	8.05±0.42 [b]
IKM_{100}	2.40±0.15 [a]	5.11±0.37 [a]
IKM_{250}	3.38±0.17 [b]	7.95±0.29 [b]
IKM_{500}	2.91±0.19 [b]	6.86±0.31 [b]

Means not sharing the same letter are significantly different at $p<0.05$. Control: group receiving normal saline; DOX: DLA bearing mice treated with doxorubicin (20 mg/kg b.w); IKM100, IKM250, IKM500: Methanolic extract of *I. khasiana* at the dose of 100, 250, 500 mg/kg b.w, respectively.

Table 5.4: Alterations in the Superoxide Dismutase (SOD) level in mice bearing Dalton's lymphoma treated with Dox and IKM.

Treatment (mg/kg b.w)	SOD (µmol/mg protein)	
	DLA Fluid	Liver
	Mean ± SEM	Mean ± SEM
Control	0.69±0.15 [a]	0.37±0.04 [a]
$Dox_{0.5}$	2.04±0.13 [b]	0.91±0.05 [b]
IKM_{100}	1.05±0.14 [a]	0.55±0.07 [a]
IKM_{250}	1.66±0.13 [b]	0.83±0.05 [b]
IKM_{500}	1.20±0.08 [a]	0.78±0.02 [b]

Means not sharing the same letter are significantly different at $p<0.05$. Control: group receiving normal saline; DOX: DLA bearing mice treated with doxorubicin (20 mg/kg b.w); IKM100, IKM250, IKM500: Methanolic extract of *I. khasiana* at the dose of 100, 250, 500 mg/kg b.w, respectively.

Table 5.5: Alterations in the Lipid peroxidation level in mice bearing Dalton's lymphoma treated with Dox and IKM.

Treatment (mg/kg b.w)	MDA (µmol/mg protein)	
	DLA Fluid Mean ± SEM	Liver Mean ± SEM
Control	0.036± 0.0023[a]	0.413±0.026 [a]
Dox$_{0.5}$	0.032±0.0029 [a]	0.504±0.016 [a]
IKM$_{100}$	0.034±0.0026 [a]	0.304±0.016 [b]
IKM$_{250}$	0.025±0.0019 [b]	0.253±0.020 [b]
IKM$_{500}$	0.025±0.0019 [a]	0.300±0.028 [b]

Means not sharing the same letter are significantly different at $p<0.05$. Control: group receiving normal saline; DOX: DLA bearing mice treated with doxorubicin (20 mg/kg b.w); IKM100, IKM250, IKM500: Methanolic extract of *I. khasiana* at the dose of 100, 250, 500 mg/kg b.w, respectively.

Table 5.6: Effects of IKM and DOX activities of serum enzymes in DLA bearing mice.

Treatment (mg/kg b.w)	Serum enzymes		
	ALT (U/L) Mean ± SEM	AST (U/L) Mean ± SEM	CRE (mg/dL) Mean ± SEM
Control	26.30±1.52 [a]	139.12±1.76 [a]	0.9±0.06 [a]
Dox$_{0.5}$	20.17±0.98 [b]	128.88±2.32 [a]	0.07±0.08 [a]
IKM$_{100}$	19.85±1.80 [b]	110.49±2.99 [a]	0.07±0.03 [a]
IKM$_{250}$	16.61±1.54 [c]	108.66±2.31 [b]	0.05±0.07 [b]
IKM$_{500}$	18.32±1.02 [b]	111.30±2.54 [a]	0.04±0.06 [b]

Means not sharing the same letter are significantly different at $p<0.05$. Control: group receiving normal saline; DOX: DLA bearing mice treated with doxorubicin (20 mg/kg b.w); IKM100, IKM250, IKM500: Methanolic extract of *I. khasiana* at the dose of 100, 250, 500 mg/kg b.w, respectively.

Table 5.7: DNA damage induced by IKM on DLA cells in Comet assay.

Treatment (mg/kg b.w)	Tail length	Olive moment
Control	6.52±0.30 [a]	0.96±0.16 [a]
$Dox_{0.5}$	10.7±0.60 [b]	2.36±0.12 [b]
IKM_{100}	8.56±0.34 [a]	1.11±0.097 [a]
IKM_{250}	11.89± 0.57 [b]	2.66±0.076 [b]
IKM_{500}	10.28±0.43 [b]	1.72±0.10 [a]

Means not sharing the same letter are significantly different at $p<0.05$. Control: group receiving normal saline; DOX: DLA bearing mice treated with doxorubicin (20 mg/kg b.w); IKM100, IKM250, IKM500: Methanolic extract of *I. khasiana* at the dose of 100, 250, 500 mg/kg b.w, respectively.

REFERENCES

Abdollahpour-Alitappeh, M., Lotfinia, M., Gharibi, T., Mardaneh, J., Farhadihosseinabadi, B., Larki, P., ... & Bagheri, N. (2019). Antibody–drug conjugates (ADCs) for cancer therapy: Strategies, challenges, and successes. Journal of cellular physiology, 234(5), 5628-5642.

Al Batran, R., Al-Bayaty, F., Jamil Al-Obaidi, M. M., Abdualkader, A. M., Hadi, H. A., Ali, H. M., & Abdulla, M. A. (2013). *In vivo* antioxidant and antiulcer activity of Parkia speciosa ethanolic leaf extract against ethanol-induced gastric ulcer in rats. PloS one, 8(5), e64751.

Armitage, J. O., Gascoyne, R. D., Lunning, M. A., & Cavalli, F. (2017). Non-hodgkin lymphoma. The Lancet, 390(10091), 298-310.

Barg, M., Rezin, G. T., Leffa, D. D., Balbinot, F., Gomes, L. M., Carvalho-Silva, M., ... & Andrade, V. M. (2014). Evaluation of the protective effect of *Ilex paraguariensis* and *Camellia sinensis* extracts on the prevention of oxidative damage caused by ultraviolet radiation. Environmental toxicology and pharmacology, 37(1), 195-201.

Beutler, E. (1984). Red cell metabolism: A manual of biochemical methods (3rd ed., p. 188). Grune and Stratton Inc.

Boaventura, B. C. B., da Silva, E. L., Liu, R. H., Prudêncio, E. S., Di Pietro, P. F., Becker, A. M., & Amboni, R. D. D. M. C. (2015). Effect of yerba mate (*Ilex paraguariensis* A. St. Hil.) infusion obtained by freeze concentration technology on antioxidant status of healthy individuals. LWT-Food Science and Technology, 62(2), 948-954.

Boaventura, B. C. B., Di Pietro, P. F., Klein, G. A., Stefanuto, A., de Morais, E. C., de Andrade, F., ... & da Silva, E. L. (2013). Antioxidant potential of mate tea (*Ilex*

paraguariensis) in type 2 diabetic mellitus and pre-diabetic individuals. Journal of Functional Foods, 5(3), 1057-1064.

Bray, F., Laversanne, M., Weiderpass, E., & Soerjomataram, I. (2021). The ever-increasing importance of cancer as a leading cause of premature death worldwide. Cancer, 127(16), 3029-3030.

Buege, J. A., & Aust, S. D. (1978). Microsomal lipid peroxidation. In Methods in enzymology (Vol. 52, pp. 302-310). Academic press.

Chaudhari, K., Rizvi, S., & Syed, B. A. (2019). Non-Hodgkin lymphoma therapy landscape. Nature Reviews Drug Discovery, 18(9), 663-665.

Cogoi, L. C., Martino, R. F., Giberti, G. C., Anesini, C. A., & Filip, R. (2014). Study of the Potential Value of *Ilex affinis* (Aquifoliaceae) as a Novel Source for the Food and Pharmaceutical Industries.

Collins, A. R. (2004). The comet assay for DNA damage and repair. Molecular biotechnology, 26(3), 249-261.

Das, L., & Vinayak, M. (2014). Long term effect of curcumin in regulation of glycolytic pathway and angiogenesis via modulation of stress activated genes in prevention of cancer. PloS one, 9(6), e99583.

Dutta, S., Chakraborty, A. K., Dey, P., Kar, P., Guha, P., Sen, S., ... & Chaudhuri, T. K. (2018). Amelioration of CCl4 induced liver injury in swiss albino mice by antioxidant rich leaf extract of *Croton bonplandianus* Baill. PloS one, 13(4), e0196411.

Ferlay, J., Colombet, M., Soerjomataram, I., Parkin, D. M., Piñeros, M., Znaor, A., & Bray, F. (2021). Cancer statistics for the year 2020: An overview. International Journal of Cancer, 149(4), 778-789.

Filip, R., Ferraro, G., Manuele, M. G., & Anesini, C. (2008). *Ilex Brasiliensis*: phytochemical composition and mechanism of action against the proliferation of a lymphoma cell line. Journal of food biochemistry, 32(6), 752-765.

Fouad, Y. A., & Aanei, C. (2017). Revisiting the hallmarks of cancer. American journal of cancer research, 7(5), 1016.

Fried, R. (1975). Enzymatic and non-enzymatic assay of superoxide dismutase. Biochimie, 57(5), 657-660.

Gómez-Juaristi, M., Martínez-López, S., Sarria, B., Bravo, L., & Mateos, R. (2018). Absorption and metabolism of yerba mate phenolic compounds in humans. Food chemistry, 240, 1028-1038.

Gupta, S., Zhang, D., Yi, J., & Shao, J. (2004). Anticancer activities of *Oldenlandia diffusa*. Journal of Herbal Pharmacotherapy, 4(1), 21-33.

Haldar, P. K., Kar, B., Bala, A., Bhattacharya, S., & Mazumder, U. K. (2010). Antitumor activity of *Sansevieria roxburghiana* rhizome against Ehrlich ascites carcinoma in mice. Pharmaceutical Biology, 48(12), 1337-1343.

Hanahan, D., & Weinberg, R. A. (2011). Hallmarks of cancer: the next generation. cell, 144(5), 646-674.

Huang, M., Lu, J. J., & Ding, J. (2021). Natural products in cancer therapy: past, present and future. Natural Products and Bioprospecting, 11(1), 5-13.

Lalmuansangi, C., Zosangzuali, M., Lalremruati, M., Tochhawng, L., & Siama, Z. (2020). Evaluation of the protective effects of *Ganoderma applanatum* against doxorubicin-induced toxicity in Dalton's Lymphoma Ascites (DLA) bearing mice. Drug and Chemical Toxicology, 1-11.

Lalnunfela, C., Lalthanpuii, P. B., Lalhriatpuii, T. C., & Lalchhandama, K. (2020). An endangered medicinal plant, *Ilex khasiana* exhibits potent antiparasitic activity against intestinal tapeworm. Pharmacognosy Journal, 12(4).

Middleton, E. (1998). Effect of plant flavonoids on immune and inflammatory cell function. Flavonoids in the living system, 175-182.

Moron, M. S., Depierre, J. W., & Mannervik, B. (1979). Levels of glutathione, glutathione reductase and glutathione S-transferase activities in rat lung and liver. Biochimica et biophysica acta (BBA)-general subjects, 582(1), 67-78.

Newman, D. J., & Cragg, G. M. (2020). Natural products as sources of new drugs over the nearly four decades from 01/1981 to 09/2019. Journal of natural products, 83(3), 770-803.

Noratto, G. D., Kim, Y., Talcott, S. T., & Mertens-Talcott, S. U. (2011). Flavonol-rich fractions of yaupon holly leaves (*Ilex vomitoria*, Aquifoliaceae) induce microRNA-146a and have anti-inflammatory and chemopreventive effects in intestinal myofribroblast CCD-18Co cells. Fitoterapia, 82(4), 557-569.

Peralta, I. N., Cogoi, L., Filip, R., & Anesini, C. (2013). Prevention of hydrogen peroxide-induced red blood cells lysis by *Ilex paraguariensis* aqueous extract: participation of phenolic and xanthine compounds. Phytotherapy Research, 27(2), 192-198.

Pirker, K. F., & Goodman, B. A. (2010). Caffeoylquinic acid derived free radicals identified during antioxidant reactions of bitter tea (*Ilex latifolia* and *Ilex kudincha*). Food & Function, 1(3), 262-268.

Puangpraphant, S., Berhow, M. A., Vermillion, K., Potts, G., & Gonzalez de Mejia, E. (2011). Dicaffeoylquinic acids in Yerba mate (*Ilex paraguariensis* St. Hilaire) inhibit NF-κB nucleus translocation in macrophages and induce apoptosis by activating caspases-8 and-3 in human colon cancer cells. Molecular nutrition & food research, 55(10), 1509-1522.

Puangpraphant, S., Berhow, M. A., Vermillion, K., Potts, G., & Gonzalez de Mejia, E. (2011). Dicaffeoylquinic acids in Yerba mate (*Ilex paraguariensis* St. Hilaire)

inhibit NF-κB nucleus translocation in macrophages and induce apoptosis by activating caspases-8 and-3 in human colon cancer cells. Molecular nutrition & food research, 55(10), 1509-1522.

Sarker, S. D., & Nahar, L. (2020). Medicinal Natural Products: A Disease-Focused Approach. Academic Press. Medicinal Natural Products: A Disease-Focused Approach. Academic Press.

Singh, N. P., McCoy, M. T., Tice, R. R., & Schneider, E. L. (1988). A simple technique for quantitation of low levels of DNA damage in individual cells. Experimental cell research, 175(1), 184-191.

Sung, H., Ferlay, J., Siegel, R. L., Laversanne, M., Soerjomataram, I., Jemal, A., & Bray, F. (2021). Global cancer statistics 2020: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. CA: a cancer journal for clinicians, 71(3), 209-249.

Thavamani, B. S., Mathew, M., & Palaniswamy, D. S. (2014). Anticancer activity of *Cocculus hirsutus* against Dalton's lymphoma ascites (DLA) cells in mice. Pharmaceutical biology, 52(7), 867-872.

Wall, M. E. (1998). Camptothecin and taxol: discovery to clinic. Medicinal research reviews, 18(5), 299-314.

Yao, X., Zhang, F., & Corlett, R. T. (2022). Utilization of the Hollies (Ilex L. spp.): A Review. Forests, 13(1), 94.

CHAPTER - 6

SCREENING OF BIOACTIVE COMPOUNDS AND QUANTIFICATION USING HIGH PERFORMANCE THIN LAYER CHROMATOGRAPHY AND GAS CHROMATOGRAPHY – MASS SPECTROSCOPY.

INTRODUCTION

Ilex khasiana is an evergreen tree of Aquifoliaceae family endemic to Khasi Hills in Meghalaya, and grown only in Aizawl, Mizoram, India. There is a vast history of the genus *Ilex* on their medicinal uses in different traditional practices such as for the treatments of coronary heart diseases, hypertension, hyperlipemia and hepatitis to name a few **(Lalnunfela et al., 2019)**. *I. khasiana* leaf extract effectively killed the tapeworm *R. tetragona*. It caused structural damages on the tegument of the tapeworm, distortion of the microtriches, clumping of the spines, erosion of the microtriches and extensive contraction of the tegument indicated the characteristic antiparasitic effects under scanning electron microscopy **(Lalnunfela et al., 2020)**.

Chromatography technique is employed to separate, identify and purify the components or compounds present in the mixture both in qualitative and quantitative analysis. The unknown sample in the stationary phase separate from each other with the help of mobile phase. The separation principle is based on adsorption, molecular weight, partition or difference molecular characteristics of the compound **(Coskun, 2016).**

Thin-layer chromatography is marked by adsorption of solid-liquid. The adsorbent substances like silica gel or cellulose are coated on solid substances like glass or aluminium plate. The liquid solvent that allows the substance to travel through the stationary phase in the form of capillary action is called mobile phase. The analytes separation and rate depend on the polarity of the solvent **(Sherma and Fried, 2003)**.

The distance travelled by the substance and the solvent is used for calculation of relative mobility called R_f value **(Donald et al., 2006)**. Many useful methods for determination of important compounds like quercetin, isovitexin, orientin, vitexin and many more is developed that helps in precise, specific and accurate detection of compound(s) from unknown samples. HPTLC fingerprinting analysis is a well-suited technique to unveil the unexplored bioactive compounds for drug discovery and novel compound detection **(Attimarad et al., 2011)**.

Gas chromatography is another simple yet multifarious, sensitive and rapid technique that vaporized the sample and allowed the compound to separate in the gaseous mobile phase. Gas chromatography coupled with Mass spectroscopy is widely used for direct analysis of components especially in the field of phytochemistry to detect the unknown bioactive compounds as the technique plays an important part in analyzing non polar and other volatile substances **(Sermakkani and Thangapandian, 2012).**

Quercetin, a polyphenol substance is found abundantly in plants and is a renowned natural product because of its medicinal contributions. The uses of quercetin can be justified by its its antioxidant, anti-inflammatory, antiplatelet, antiapoptotic, nepho-, gastro-, angio-, cardio- and chondroprotective properties **(Shebeko et al., 2018)**. It is a pentahydroxy flavone having hydroxyl group at the 3-, 3'-, 4'-, 5- and 7-positions, and the most abundant dietary flavonoids found in many plants and its related product such as *Allium fistulosum, Allium sativum, Allium cepa, Sambucus nigra, Solanum nigrum, Centella asiatica, Hypericum hircinum, Hypericum perforatum, Apium graveolens, Brassica oleracea* to name a few. Among its notable properties the one that stands out is its synergistic properties with other drugs or bioactive compounds. It enhanced the efficacy of well-known drugs like amoxicillin, epigallocathechin gallate, menadione, trichostatin A and ceftazidime. Besides its effectiveness in chemotherapy, it displayed inhibitory effect against the negative effects of idarubicin, cisplatin, daunorubicin, and tert-butyl hydroperoxide **(Yusuf et al., 2020).**

Beta-sitosterol or β-sitosterol is also given multiple names like (3β)-stigmast-5-en-3-ol, 22:23-dihydro stigmasterol, α-dihydro fucosterol, cinchol, cupreol, rhamnol, quebrachol, and sitosterin. It is an important phytosterols sharing structural similarity with cholesterol present throughout different parts of the plants such as rhizomes, fruits and leaves **(Ulbricht, 2016).** β-sitosterol's antioxidant activity is a remarkable factor attributing in its medicinal properties, modulating the antioxidant enzymes and human estrogen receptor besides its role in angiogenesis and wound healing **(Moon et al., 1999)**. β-sitosterol inhibits cholesterol adsorption helping in the treatment of hyperlipidemia and also prevent different cardiovascular diseases **(Yeshurun and Gotto, 1976).** The anti-inflammatory activity of β-sitosterol may be achieved by increasing anti-inflammatory cytokines and down regulating the secretion of pro-inflammatory cytokines, TNF-α as well as edema **(Nirmal et al., 2012)**. It has been reported that β-sitosterol induced apoptosis in breast and colon cancer cell lines **(Tasyriq et al., 2012; Awad et al., 1996)**.

Therefore, screening of *I. khasiana* for its bioactive compounds is the key to unravel the plant from uncertain identity and its medicinal values. The HPTLC and GC-MS technique delivered the exact assay required for this particular requirement.

MATERIALS AND METHODS

Chemicals and Reagents

Asiatic acid, Boswellic acid, Chebulagic acid, Ellagic acid, Ferulic acid, Gallic acid, Quercetin and β-Sitosterol were obtained from Natural Remedies Private Limited, Bangalore, India. Methanol HPLC grade 99.7 % from Merck, Absolute Ethanol from Merck, TLC Silica gel 60 F_{254} plate, CAMAG Linomat 5, CAMAG TLC Scanner and CAMAG Smart DIGI which was programmed through winCATS planar chromatography manager software version 1.4.5, Gas Chromatography-Mass spectrometry (GC-MS) (Thermo Scientific TRACE™ 1300 ISQ™ LT).

HPTLC Method for Quantification of Quercetin

Preparation of *I. khasiana* extracts

Ilex khasiana petroleum ether extract (IKP), chloroform extract (IKC), methanol extracts (IKM) and its four fractions were used in this experiment. IKM fractions were obtained by dissolving IKM in 250ml of water and mixed with namely n-Hexane (0.009 polarity index), Diethyl ether (0.117), chloroform (0.259) and n-Butanol (0.586) after vigorously shaking the extract with the selected solvent one at a time. The two layers formed were separated using separating funnel **(Pandey et al., 2014)**. After this, IKP, IKC, IKM and its four distinct fractions IKM Hexane, IKM Diethyl ether, IKM Chloroform and IKM Butanol were obtained. Screening of chemical constituents of *Ilex khasiana* was performed using these extracts.

Preparation of the reference standard

1mg of Asiatic acid, Boswellic acid, Chebulagic acid, Ellagic acid, Ferulic acid, Gallic acid, Quercetin and β-Sitosterol were dissolved in 1000µl of methanol to get the stock solution of 1mg/ml each.

Standard preparation

Stock solution of reference standard (1mg/ml) was diluted in methanol to obtain working concentration of 50µg/ml.

Screening of extracts

The samples IKP, IKC, IKM and four IKM fractions namely IKMBu (IKM Butanol fraction), IKM DEE (Diethyl ether fraction), IKM Hex (Hexane fraction) and IKM C (Chloroform fraction) were subjected to screening against all the reference standards using the appropriate mobile phase. In 20x10 precoated TLC plate, 5µl of all the extracts were spotted on TLC plate using CAMAG Linomat 5 applicator and the plate was developed using the appropriate mobile phase in CAMAG twin trough developing chamber. The plate was removed and allowed to air-dry when the solvent

front reached 7 cm. The plate was subjected to CAMAG TLC Scanner for scanning at the appropriate wavelength using WIN CATS software to obtain the extract fingerprint in the form of chromatogram.

Calibration and Quantification of Quercetin

The obtained working standard solutions were then applied at 2, 4, 6, 8, 10, 12, and 14 µl on TLC Silica gel 60 F_{254} plate to obtain seven-point linear calibration curves. 4µl of IKM C (50 mg/ml) was taken and applied on the TLC plate respectively at the speed of 150 nl/sec using CAMAG Linomat 5 applicator. CAMAG twin trough chamber was pre-saturated with this developing solvent for 15 minutes and the TLC plate was developed up to a height of 7 cm in Toluene: Ethyl Acetate: Formic Acid (5:4:0.2, v/v/v) **(Laila et al., 2014)**. The plate was removed after development and the spots were visualized at 254nm using CAMAG Smart DIGI. Quantitative evaluation of the plate was performed using CAMAG TLC Scanner in the absorbance mode at 254 nm, with the following conditions: slit width 5 x 0.45 mm, scanning speed 40 mm/s and data resolution 50µm/step using deuterium lamp.

Calibration and Quantification of β-Sitosterol

winCATS planar chromatography manager software version 1.4.5 was employed to apply 10 standard levels containing 100 ng to 550 ng of β-Sitosterol. IKM Hex and IKM Dee (50mg/ml) extracts were chosen from the screening results for quantification and were spotted in triplicate. The application rate of CAMAG Linomat 5 was 150nl/sec using nitrogen as the spray gas. In a solvent saturated developing chamber (20x10) containing 20ml of Toluene: methanol (16:2) mobile phase **(Shilajan and Swar, 2013).** When the solvent front reached 70% of the plate's height, the plate was removed and allowed to dry at room temperature. The plate derivatization was performed by spraying the TLC plate with Anisaldehyde sulphuric acid reagent and placed in the oven at 100^{o}C until prominent bands appeared. The plate was then scanned using TLC scanner under absorbance mode using the following condition: wavelength

at 366nm, slit dimension 5 x 0.45mm, scanning speed 40mm/s and data resolution 100 µm/step.

Validation of HPTLC method

The methods that were used for validation were performed following the International Conference on Harmonization (ICH) guidelines **(Shailajan et al., 2012).**

Linearity

Fourteen (14) different concentrations of standard were prepared for linearity studies(n=14). Peak area was measured and taken as a response. The plate was then developed by using mobile phase. The peak area was plotted against concentrations for the calibration curve.

Limit of detection and limit of quantification

For the evaluation limit of detection (LOD) and limit of quantification (LOQ) different concentrations of the standard stock solutions were applied to determine the following equation.

$$\text{LOD} = \frac{3.3 \times \text{Standard Deviation of the y-intercept}}{\text{Slope of the calibration curve}}$$

$$\text{LOQ} = \frac{10 \times \text{ Standard Deviation of the y-intercept}}{\text{Slope of the calibration curve}}$$

Recovery

For percent recovery, known concentrations of standards were added to a pre analyzed sample. The spiked samples were then analyzed by proposed HPTLC method.

Gas chromatography – Mass spectroscopy

The screening of IKP, IKC, IKM, IKM Hex, IKM Dee, IKM BU and IKM C were performed by dissolving these samples in methanol to obtain 50mg/ml of working concentration using Gas Chromatography-Mass spectrometry (GC-MS) (Thermo Scientific TRACE™ 1300 ISQ™ LT). Sample was loaded with 5 plunger strokes to obtain 1 µl of the targeted sample. The stationary phase, TR-5MS (260F142P) has the dimension of 30m x 0.25 mm x 0.25µm with film thickness of 0.25µm. Helium gas was used at 1ml/min as a carrier gas. The temperature as high as 250°C and 220°C was required for the transfer line and ion-source line. The mass spectrometer was run for 54 minutes at a range of 10- 1100 amu. Thermo Scientific™ Xcalibur™ software was used to operate all the set up and libraries of Wiley Registry™ and National Institute of Standards and Technology database provide the compound information based on retention time, chemical formula and molecular weight **(Mihigo et al., 2015)**.

RESULTS

HPTLC analysis of *I. khasiana*

HPTLC technique is a useful to detect analytes at a smaller range of micro and nanogram level within a short period of time and lesser cost **(Kamboj and Saluja, 2017).** HPTLC fingerprinting of *Ilex khasiana* was performed by screening nine well-known bioactive compounds namely Asiatic acid, Boswellic acid, Chebulagic acid, Ellagic acid, Ferulic acid, Gallic acid, Quercetin and β-Sitosterol on IKP, IKC, IKM, IKM Dee, IKM Bu, IKM Hex and IKM C. Among the nine reference standards screened, Quercetin was detected only in IKM C and β-Sitosterol was detected in both IKM Hex and IKM Dee **(Table 6.1).**

Quantification of Quercetin

Based on HPTLC fingerprinting of the selected extracts, quercetin was found to be present in IKM C sharing R_f value of 0.49±1 using Toluene: Ethyl Acetate: Formic Acid (5:4:0.2, v/v/v) as a mobile phase **(Figure 6.1)**. The chromatogram showed the precision of the mobile phase used and the sensitivity of methods adopted in **Figure 6.1** and **Figure 6.2**. The separation and detection of quercetin in IKM C was compared with the reference standard having seven different concentrations when viewed under CAMAG UV-Smart Digi (**Figure 6.3)**. The calibration curve was linear at 100-500 ng for quercetin in which the correlation coefficient was 0.97 between the standard and the sample, which indicated the reliability of the method. The lowest amount at which the compound can be detected, Limit of detection (LOD) was 68.01 µg/spot and the lowest quantifiable amount, limit of quantification was as low as 206.07 µg/spot **(Table 6.2)**. The amount quantified 372.98 ng/spot with an area of 1538.33 as shown in **Table: 6.4.**

Quantification of β-Sitosterol

The quantification and methods validation of β-Sitosterol was performed using a protocol developed by **Shilajan and Swar (2013).** HPTLC fingerprinting result showed the presence of β-Sitosterol in both IKM Hex and IKM Dee and further taken for quantification using HPTLC technique **(Figure 6.2)**. The R_f value 0.43 was obtained after running the applied samples in CAMAG twin trough chamber in Toluene: methanol (16:2) up to 70% of the plate length (10cm). The calibration was found to be linear at 100-550 ng/spot with correlation coefficient of 0.99, the lowest quantifiable mount was estimated to be 143.28 and limit of detection was 42.99 **(Table 6.5)**. The method applied was found to be precise and reliable giving recovery percentage of not less than 97 in all the sample tested **(Table 6.6)**. The lowest quantifiable mount was estimated to be 143.28 and limit of detection was 42.99. The quantification of β-Sitosterol in IKM Hex and IKM Dee was found to be 257.04 and 198.68 respectively **(Table 6.7)**.

Gas chromatography – Mass spectroscopy (GC-MS)

The bioactive compounds of *Ilex khasiana* were determined using GC-MS with their Retention time (RT), chemical formula, Molecular weight (MW) and peak area (in percentage). The screening of IKP showed the presence of 13 active compounds within the range of 17.65 to 31.37 retention time. The most abundant compound was 7,9-Di-tert-butyl-1-oxaspiro (4,5) deca-6,9-diene-2,8-dione (92.56%) at 22.49 retention time followed by 6-Hydroxy-4,4,7a-trimethyl-5,6,7,7a-tetrahydrobenzofuran-2(4H)-one, Phenol, 2,4-bis(1,1-dimethylethyl) and n-Hexadecanoic acid with more than 50% of peak area **(Table 6.8)**.

There are 11 bioactive compounds found in IKC **(Table 6.9)** where 7,9-Di-tert-butyl-1-oxaspiro (4,5) deca-6,9-diene-2,8-dione was found to be the most abundant compound detected at 22.49 retention time. All the compounds were detected between 13 to 30 retention time. Whereas, IKM exhibited 17 active compounds, all within the range of 7.58 to 31.30 retention time **(Table 6.10)**, n-Hexadecanoic acid being the most

abundant compound at 58.85%, followed by Desulphosinigrin, [1,1'-Bicyclopropyl]-2-octanoic acid, 2'-hexyl-, methyl ester, 6-Oxa-bicyclo [3.1.0] hexan-3-one at 48.19, 40.99, 34.26 % respectively.

The Hexane fraction of IKM (IKM Hex) extract was known to possess 18 compounds between the peak area percentage of 11.41 and 72.45. The most prominent compounds are n-Hexadecanoic acid, Phenol, 2,4-bis(1,1-dimethylethyl) and Benzene, (1-methylnonyl) detected at 22.76, 17.88 and 21.27 minutes **(Table 6.11)**. The compounds detected in IKM Bu were slightly lower, 11 compounds were detected and Phenol, 2,4-bis(1,1-dimethylethyl) was the most abundant (70.74%) at 17.89 minutes **(Table 6.12)**. IKM Dee displayed the presence of 19 prominent peaks between 6.32 and 27.68 minutes of the retention time **(Table 6.13)**. Among them, Phenol, 2,4-bis(1,1-dimethylethyl) was the most abundant (70.14%) at 17.88 minutes. Butylated Hydroxytoluene was detected with peak area of 54.82% at 17.94 minutes. The compounds detected in IKM C are the least in number found within the 17.71 to 30.66 minutes of the retention time **(Table 6.14)**. Among the 8 compounds, 7,9-Di-tert-butyl-1-oxaspiro (4,5) deca-6,9-diene-2,8-dione was detected at 22.49 minutes having the peak area of 85.38% was found to be a good antioxidant.

DISCUSSION

Natural products have a long list of significance as a precursor for drug discovery in therapeutic industry for the development of novel drugs for the treatment of cardiovascular disease, anticancer and multiple sclerosis to name a few **(Allard et al., 2017)**. Most of the natural compounds due to its diversity and complex structure have higher molecular mass that consists of a greater number of carbon and oxygen atoms leading to more donator and acceptor of H- bonds. Thus, octanol–water partition coefficients (cLogP values) leading to higher hydrophilicity and compound rigidity when compared with the synthetic compounds **(Rutz et al., 2019)**.

Natural products are basically a pool of bioactive compounds which are enhanced to have higher therapeutic effects biologically as a defense and regulatory moiety. The safety and efficacy of natural products is undeniable supported by the traditional uses of natural products **(Lachance et al., 2012)**. Biological screening of natural products is a crucial and laborious process from the extraction of biological materials to studying of the bioactivity of the extract. Bio-activity guided assay is an important approach in which the extracts are sub fractioned and tested to find out the exact location of the targeted biomolecules. These multiple metabolites are subjected to a process called 'Metabolomics' which will screen the available fraction for its compound contents **(Liu and Locasale, 2017).** It provides accurate information of the metabolites like novel analogues, new structure and prioritize isolation using computational approach generating respective spectra as well as plausible analog structure **(Allard et al., 2018)**. Initial screening of the crude extract and fractions can be performed using both liquid and solid phase TLC and Gas chromatography after which structural elucidation is performed by NMR spectroscopy or high-resolution mass spectrometry (HRMS), or respective combined methods involving upstream liquid chromatography (LC) **(Atanasov et al., 2021).**

Other species under the genus *Ilex* show quite a wide range of medicinal values. *I. pubescens* is used for the treatment of coronary heart diseases, and as anti-

inflammatory and analgesic agent in traditional Chinese medicine **(Hao et al., 2013)**. Its leaves contain phenolics and phenylpropanoids which are responsible for its antipyretic, anti-inflammatory, analgesic, cardiovascular and circulatory activities. The four substituted catechol in the plant may be responsible for the vasodilator and hyperemic effects **(Koo, 1985)**. It is also known to be effective in the treatment of hypertension, hyperlipemia, and hepatitis **(Zhang et al., 1981)**. *I. cornuta* also played an important role in Chinese Traditional medicine in the treatment for dizziness and hypertension **(Wenjuan et al., 1986)**. *I. ficoidea* and *I. centrochinens is* showed potent anti-inflammatory and antioxidant activities. *I. paraguariensis* is shown to have anti-obesity effects in mice **(Li et al., 2011)**.

HPTLC method was employed for the screening and quantification of targeted compounds. This method provided simple, accurate, precise and cost-effective analysis. The methods showed its reliability from the statistical analysis. Among the two compounds quantified, quercetin is a well known flavanols for its antioxidant and anticancer activity being one of the most abundant dietary flavonoids. Quercetin has an antibacterial effect against *B. subtilis, E. coli, S. aureus* and *C. albicans* in which ciprofloxacin-narigenin hybrids were the active compounds, exhibiting strong inhibitory activity on DNA gyrase as well as antibacterial effect on resistant strains **(Xiao et al., 2014).** Quercetin exhibited more than 90% (**90.4 ± 0.6)** antioxidant activity in DPPH radical scavenging activity. Quercetin inhibited clonal expansion of MCF-7 human mammary cells with the inhibition of TrxR activity that led to apoptotic cell death via caspase 3 inhibition. Cytotoxicity test exhibited the anticancer effect of quercetin on A375, HCT-15, BxPC3, MCF-7, MCF-7 ADR, A431, A431/Pt, 2008, and C13 with IC_{50} values of lower than 4 mM (**Alizadeh and Ebrahimzadeh, 2021).**

It has been reported that, quercetin has a long-lasting anti-inflammatory activity both in human and animal models **(Ghosh, 1999).** Inhibitory activity of quercetin against both inflammation enzymes cyclooxygenase (COX) and lipoxygenase (LOX) attributed to the compound's anti-inflammatory potency. It acts mostly on the

intracellular signaling kinases and phosphatases, leukocytes, enzymes and membrane proteins **(Muthian and Bright, 2004).** Quercetin is known to activate AMPK/SIRT1 pathway thus reducing inflammation related to this pathway **(Ayissi et al., 2014)**. It also downregulated iNOS and IFN-γ expression in inflammation induced by high fat diet **(Das et al., 2013)** and by reducing IL-1β, IL-6, TNF-α levels and NF-κB expression it inhibits inflammation **(Lin et al., 2014)**.

β-sitosterol is one of the commonly found phytosterols, having chemical structure that resembles cholesterol. It regulates membrane permeability and is an important structural component of plant cell membrane. β-sitosterol depicted different namely anhydrous, hemihydrate and monohydrate based on number of water molecules attached it **(Sayeed et al., 2016).** The medicinal properties of β-sitosterol include antinociceptive, analgesic, immunomodulatory, antimicrobial, anticancer, anti-inflammatory, protective effect against NAFLD, lipid lowering effect, hepatoprotective, protective effect on respiratory diseases, wound healing effect, antioxidant and anti-diabetic activity **(Babu and Jayaraman, 2020)**. The anti-inflammatory activity of β-sitosterol is marked by reduced secretion of pro-inflammatory cytokines, TNF-α as well as edema and upregulation of anti-inflammatory cytokines **(Valerio and Awad, 2011)**. Regardless of the types of cancer cells, β-sitosterol induced apoptotic cell death **(Bin Sayeed and Ameen, 2015)**. It has been reported that β-sitosterol inhibits the proliferation of a breast cancer cell line MCF-7 and induced apoptosis in MDA-MB-231 human breast cancer cells through the activation of caspases **(Pagliacci et al., 1994; Awad et al., 2003).** It also reduced b-catenin and proliferating cell nuclear antigen (PCNA) expression, that promotes accumulation of transcriptionally active p53 that favors cell survival **(Inamine et al., 2005)**. The downregulation of Bcl-2, degradation of poly-(ADP-ribose) polymerase (PARP), and phospholipase C (PLC)-gamma1 protein, and activation of caspase-3 upregulate apoptosis in human leukemic U937 cells **(Park et al., 2007).** Inhibition of G0/G1 cell cycle arrest and decrease in CDK4 and cyclin D1, and an increase in p21/Cip1and p27/Kip1 protein levels resulted in lung cancer suppression **(Vundru et al., 2013).** β-sitosterol inhibit the growth of SGC-7901

human stomach cancer cells by down regulation of bcl-2/bax ratio and DNA damage **(Zhao et al., 2009).**

The present study revealed the unexplored richness of *I. khasiana*, and fractionation of the crude extracts permitted systematic approach of compound screening. HPTLC served as the best technique to develop fingerprint of an unknown compounds from a selected plant. The fraction ensures compound partitioning of bioactive compound to pursue bioactivity guided assay and to determine the targeted compound. The HPTLC methods employed was found to be precise and accurate from the validation result. GC-MS remains the undisputed tool to easily identify and know the abundance of a compound in a test sample. The MS library gave the whole idea of the medicinal values of the plant and to pin point the desired compound from the crude and fraction of *I. khasiana.*

SUMMARY

HPTLC technique was found to be robust, precise and accurate from the validation result. Among the available reference standards, Quercetin was detected in IKM C fraction at 0.48 R_f value and β-sitosterol was found to be present in IKM Hex and IKM Dee at 0.43 R_f value. Toluene: Ethyl Acetate: Formic Acid (5:4:0.2) was selected as the most appropriate mobile phase for separation of quercetin from the sample while, Toluene: methanol (16:2) was used to separate β-sitosterol from IKM Hex and IKM Dee. Method validation for quercetin quantification showed linear graph at 100-500 ng/spot with a correlation coefficient of 0.97 and the mean recovery is 98.75% and the amount of quercetin detected was 372.98 ng/spot. The limit of detection for quercetin was 68.01 µg/band and the limit of quantification was 206.07 µg/band. β-sitosterol quantification method gave linear graph at 100-550 ng/spot with correlation coefficient of 0.99. The limit of detection was 42.99 and limit of quantification was 143.28 µg/band. The amount of β-sitosterol calculated was 257.04 and 198.68 ng/spot and the average mean recovery was found to be 98.80 and 98.70% in IKM Hex and IKM Dee respectively. GC-MS was employed for screening all the *I. khasiana* extracts namely IKP, IKC, IKM and its fraction – IKM Bu, IKM Hex, IKM Dee and IKM C. Among the compounds detected, Linolenic acid, squalene, 7,9-Di-tert-butyl-1-oxaspiro (4,5)deca-6,9-diene-2,8-, desulphosinigrin, arbutin, resorcinol and lupeol were prominent compound found abundantly. The medicinal values of these compounds were well-known and could be the reason for *I. khasiana*'s wide range of pharmaceutical activity.

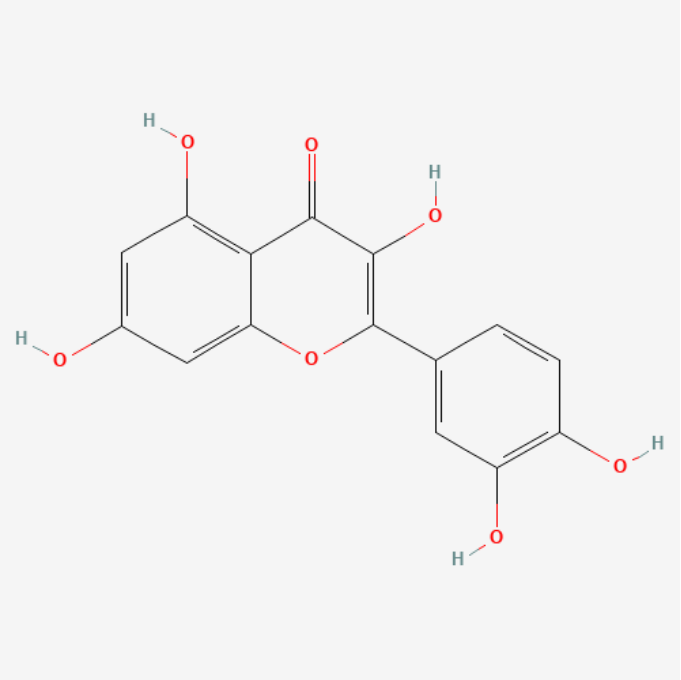


Figure 6.1: Structure of Quercetin.

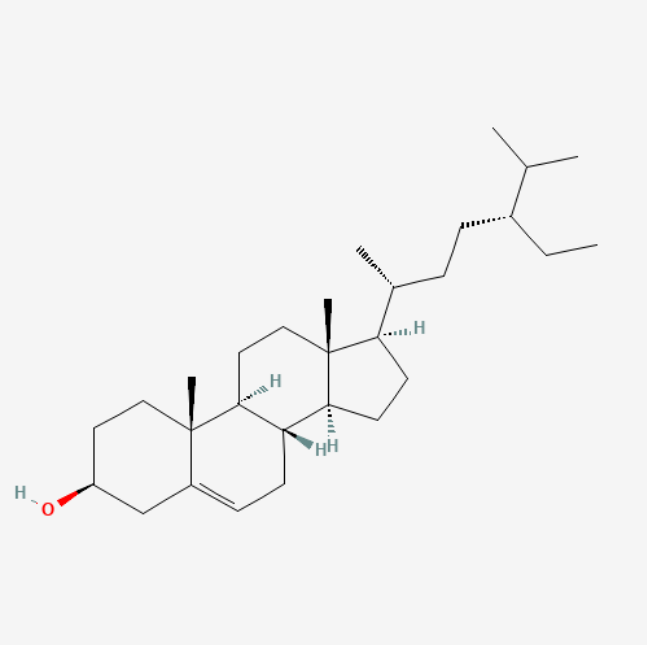
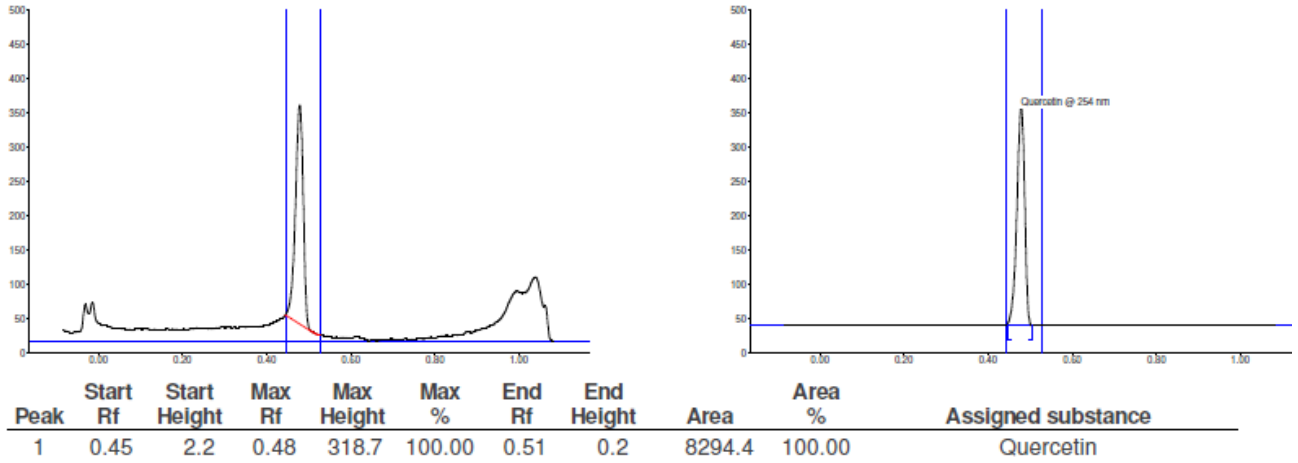
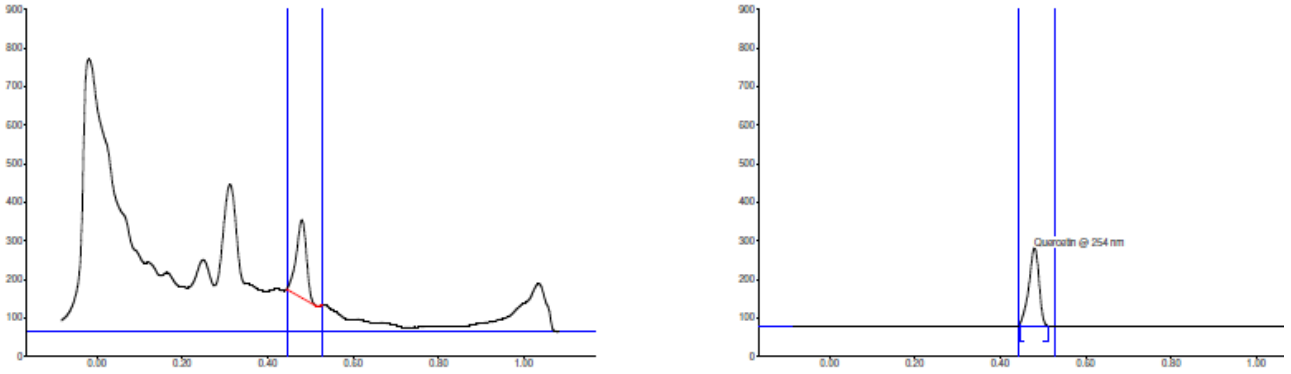


Figure 6.2: Structure of β-sitosterol.



Peak	Start Rf	Start Height	Max Rf	Max Height	Max %	End Rf	End Height	Area	Area %	Assigned substance
1	0.45	2.2	0.48	318.7	100.00	0.51	0.2	8294.4	100.00	Quercetin

Figure 6.3: Chromatogram profile of standard quercetin detected at 254nm and its respective R_f value.



Peak	Start Rf	Start Height	Max Rf	Max Height	Max %	End Rf	End Height	Area	Area %	Assigned substance
1	0.45	1.9	0.48	202.1	100.00	0.51	0.1	6698.9	100.00	Quercetin

Figure 6.4: Chromatogram profile of quercetin detected in IKM C at 254nm and its respective R_f value. (IKM C = IKM Chloroform).

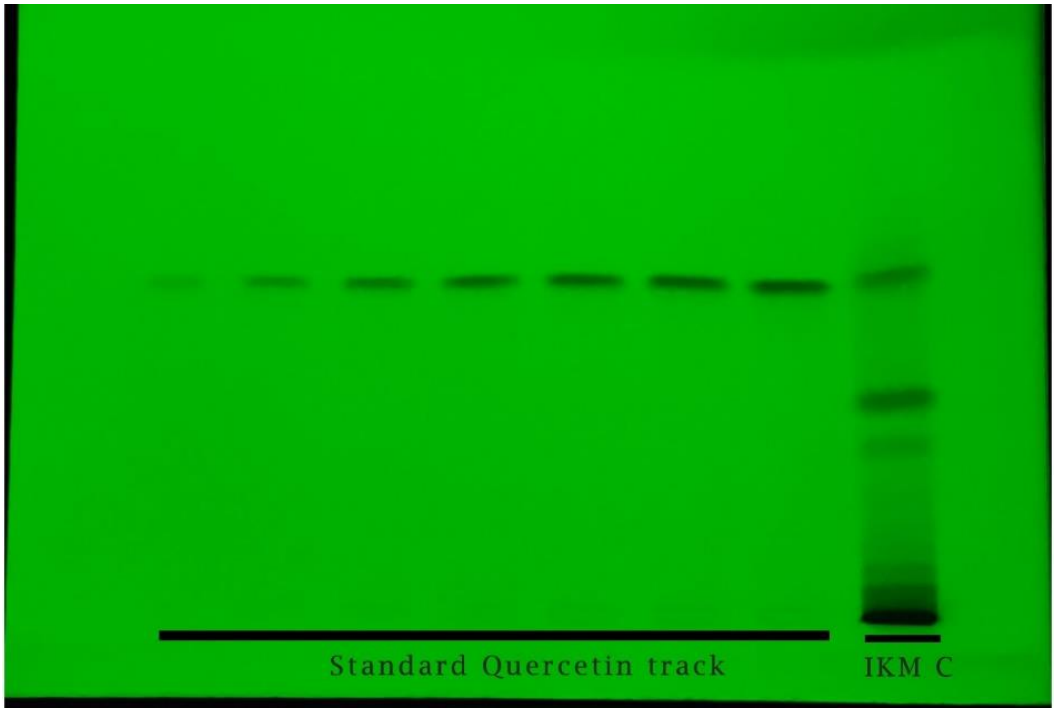
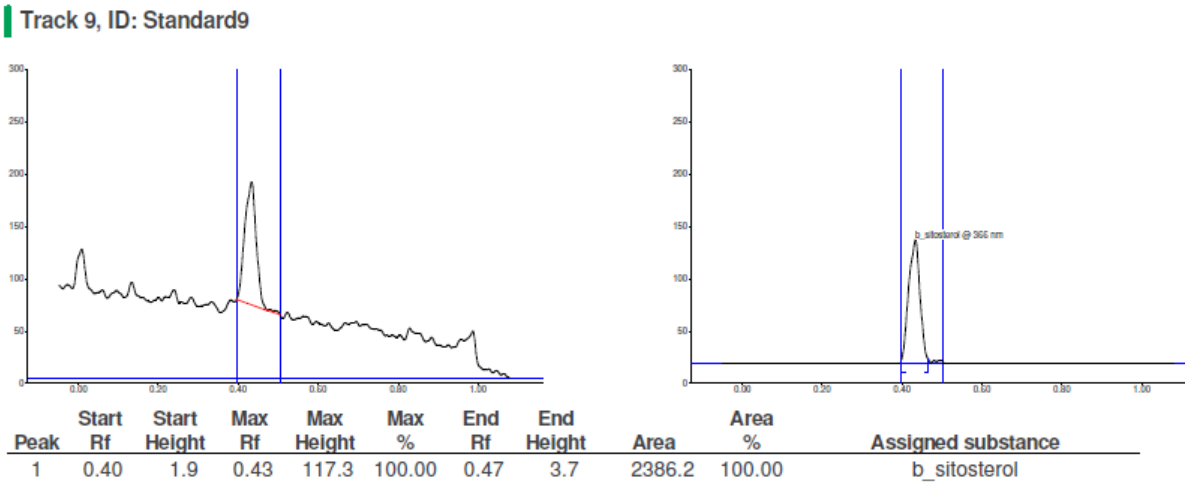
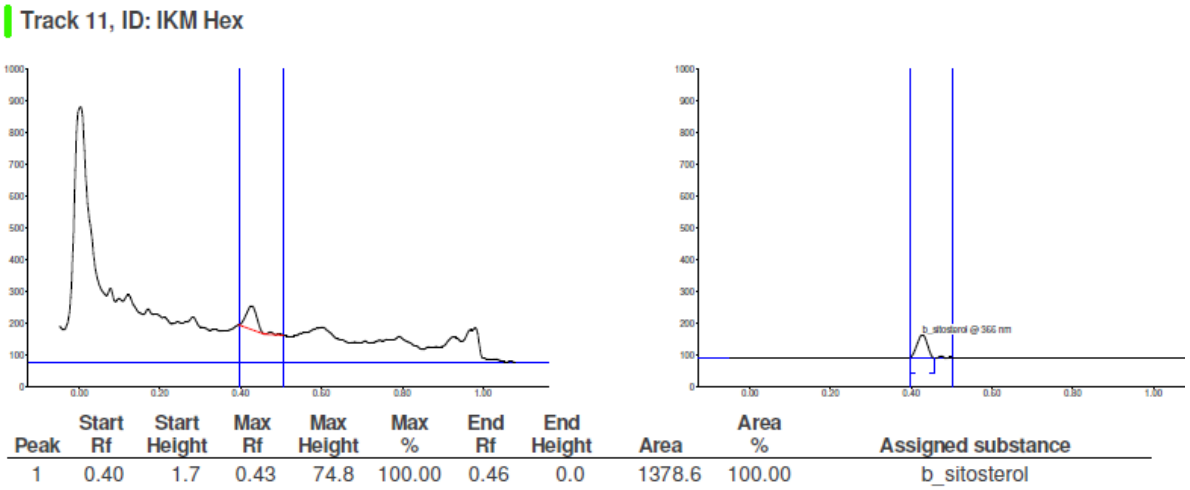


Figure 6.5: TLC plate showing the separation of IKM C with seven levels concentration of Quercetin standard. (IKM C = IKM Chloroform fraction).



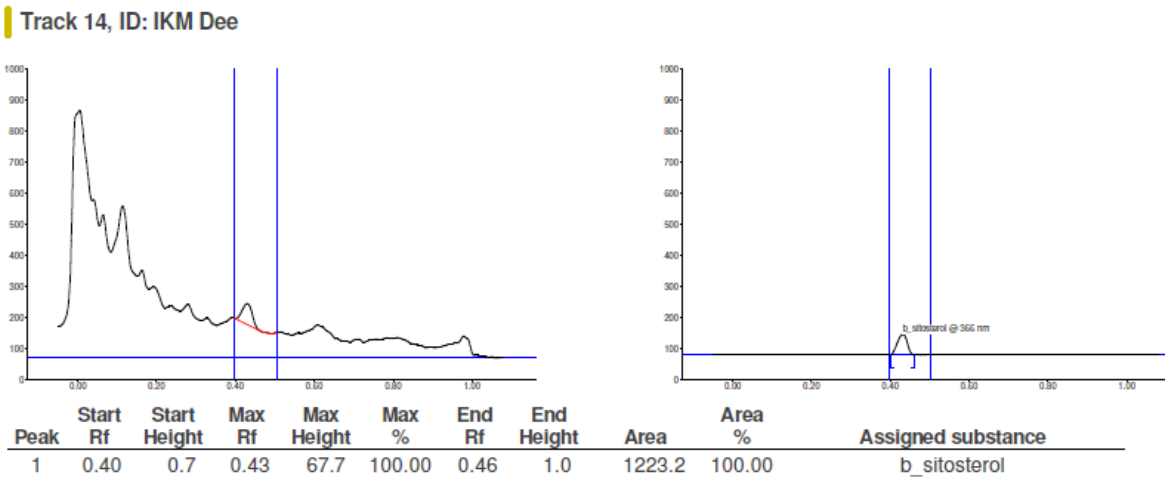
Peak	Start Rf	Start Height	Max Rf	Max Height	Max %	End Rf	End Height	Area	Area %	Assigned substance
1	0.40	1.9	0.43	117.3	100.00	0.47	3.7	2386.2	100.00	b_sitosterol

Figure 6.6: Chromatogram profile of standard β- sitosterol detected at 366 nm and its respective R_f value.



Peak	Start Rf	Start Height	Max Rf	Max Height	Max %	End Rf	End Height	Area	Area %	Assigned substance
1	0.40	1.7	0.43	74.8	100.00	0.46	0.0	1378.6	100.00	b_sitosterol

Figure 6.7: Chromatogram profile of β- sitosterol detected in IKM Hex at 254nm and its respective R_f value. (IKM Hex = IKM Hexane fraction).



Peak	Start Rf	Start Height	Max Rf	Max Height	Max %	End Rf	End Height	Area	Area %	Assigned substance
1	0.40	0.7	0.43	67.7	100.00	0.46	1.0	1223.2	100.00	b_sitosterol

Figure 6.8: Chromatogram profile of β- sitosterol detected in IKM Hex at 254nm and its respective R_f value. (IKM Dee = IKM Diethyl ether fraction).

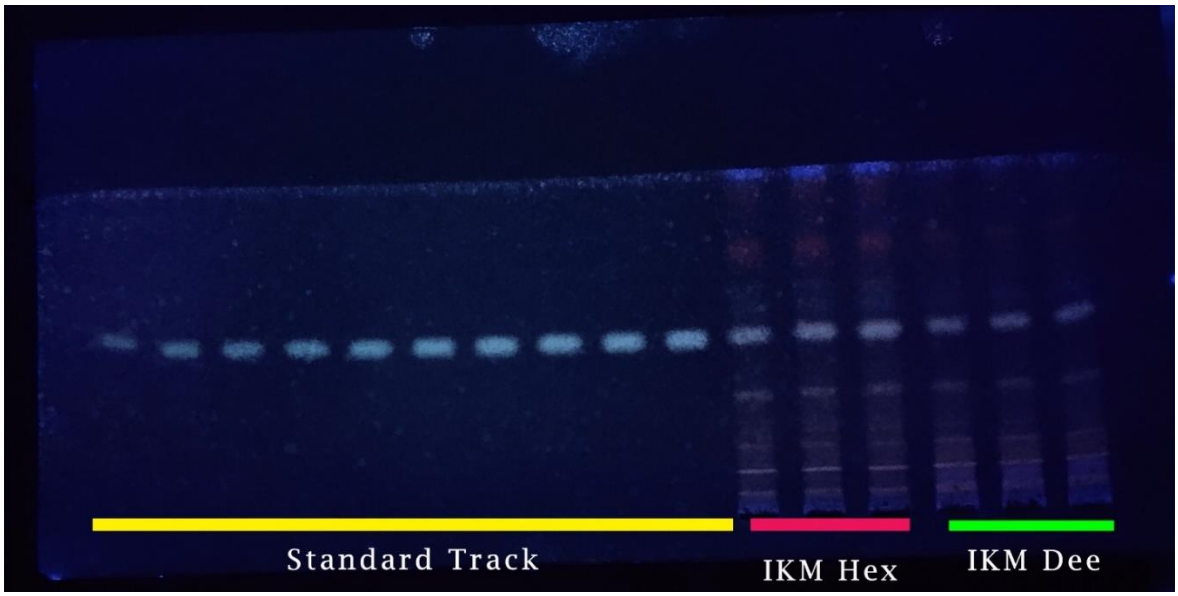


Figure 6.9: TLC plate showing the separation of IKM Hex and IKM Dee with ten levels concentration of β- sitosterol standard. (IKM Hex = IKM Hexane fraction; IKM Dee = IKM Diethyl ether fraction).

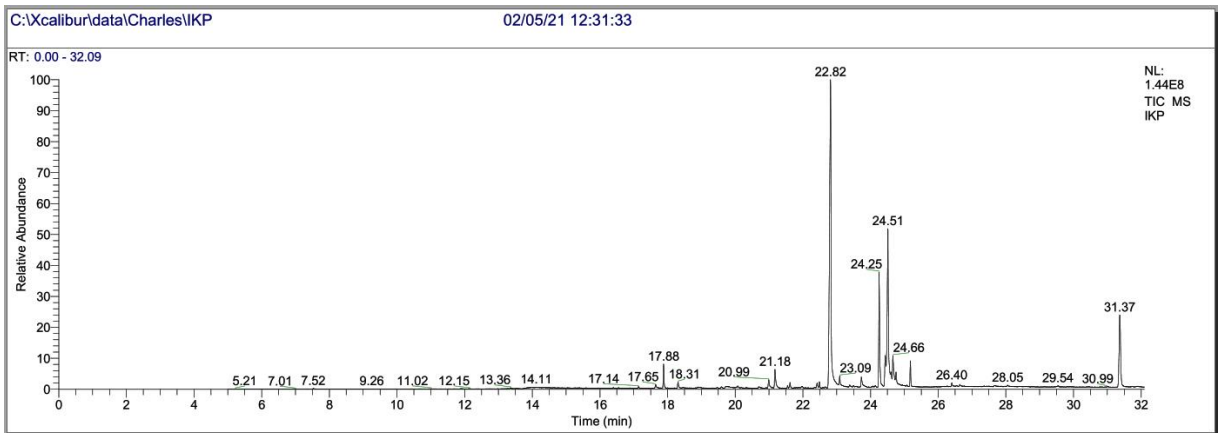


Figure 6.10: Chromatogram of compounds detected in IKP by Gas chromatography-Mass Spectrometry (GC-MS). (IKP= *Ilex khasiana* petroleum ether extract).

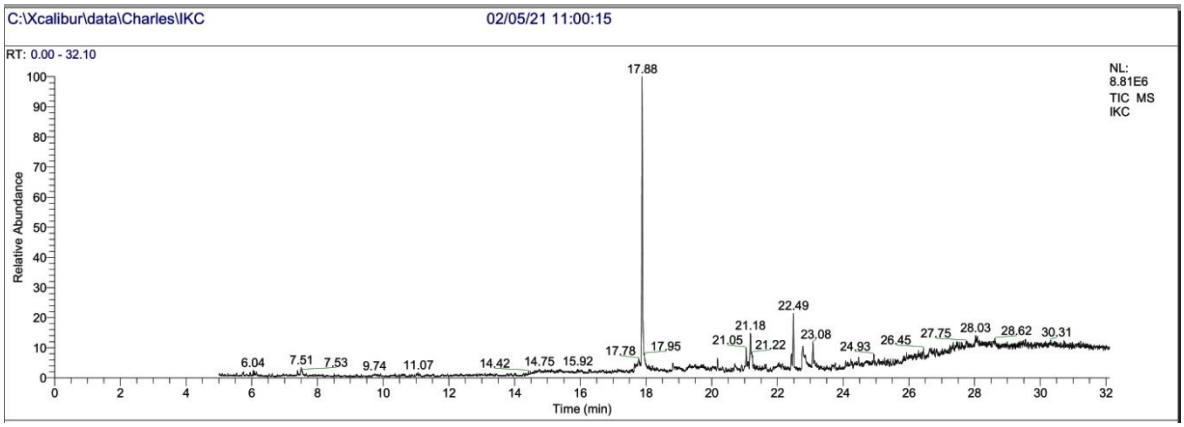


Figure 6.11: Chromatogram of compounds detected in IKC by Gas chromatography-Mass Spectrometry (GC-MS). (IKC= *Ilex khasiana* chloroform extract).

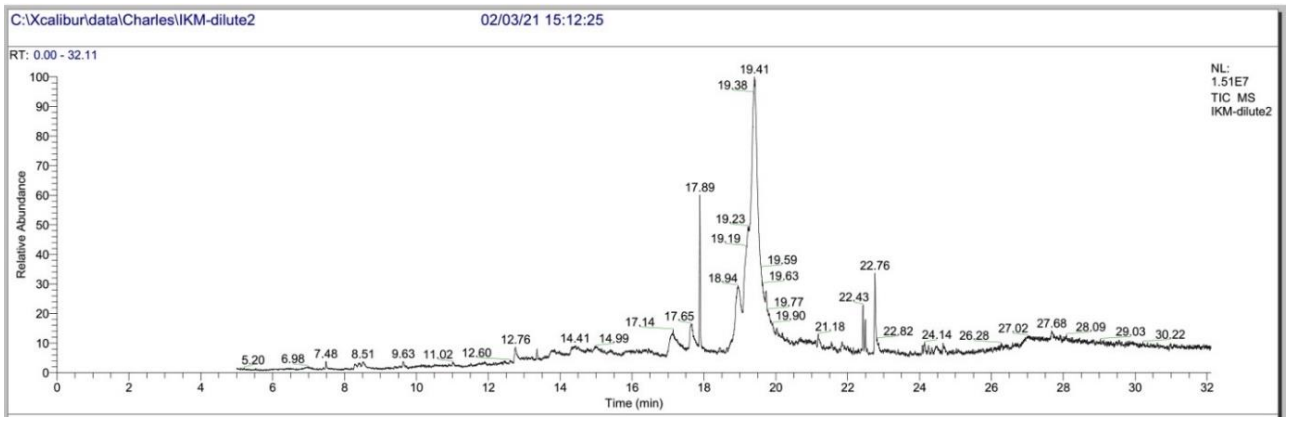


Figure 6.12: Chromatogram of compounds detected in IKM by Gas chromatography-Mass Spectrometry (GC-MS). (IKM = *Ilex khasiana* methanol extract).

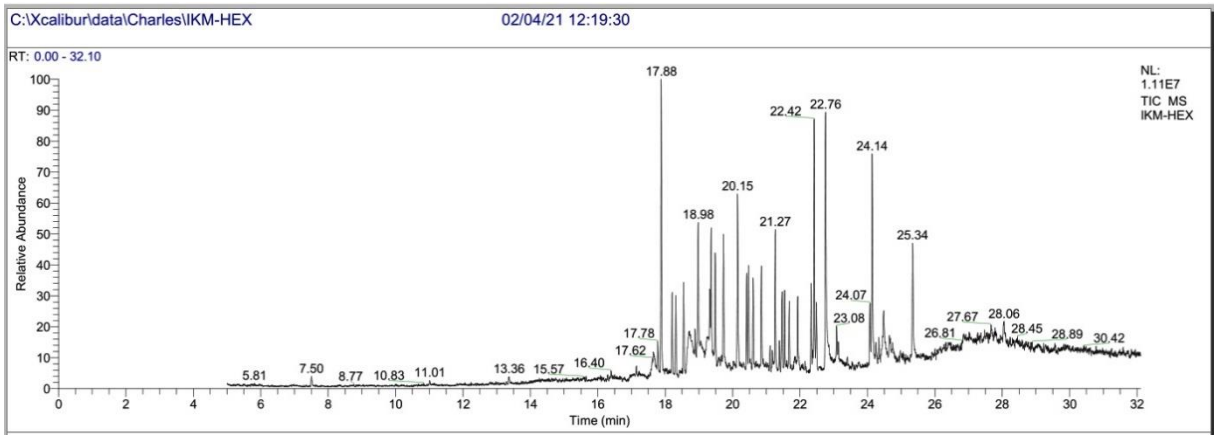


Figure 6.13: Chromatogram of compounds detected in IKM Hex by Gas chromatography- Mass Spectrometry (GC-MS). (IKM Hex = IKM Hexane).

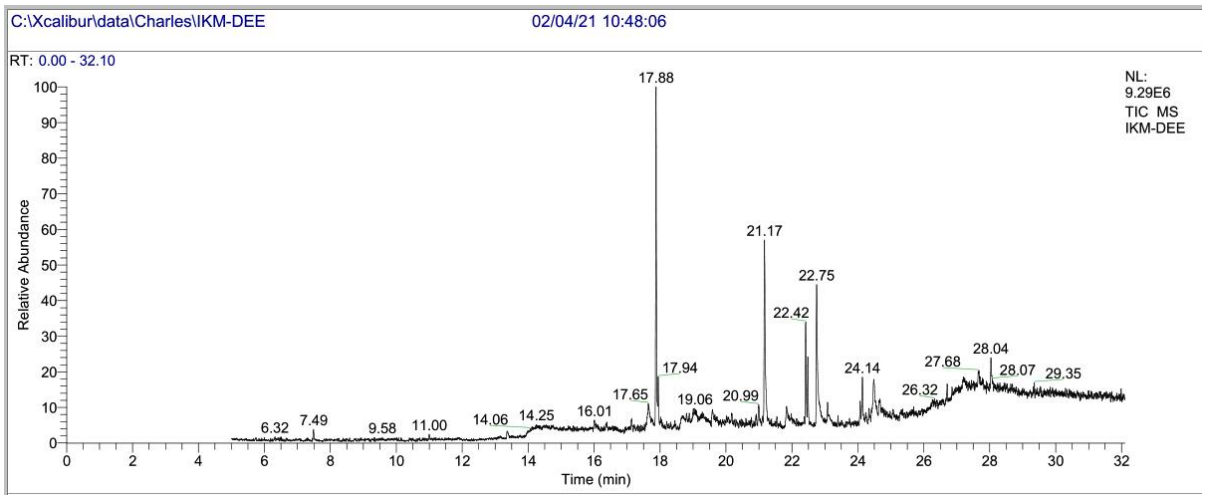


Figure 6.14: Chromatogram of compounds detected in IKM Dee by Gas chromatography- Mass Spectrometry (GC-MS). (IKM Dee = IKM Diethyl ether fraction)

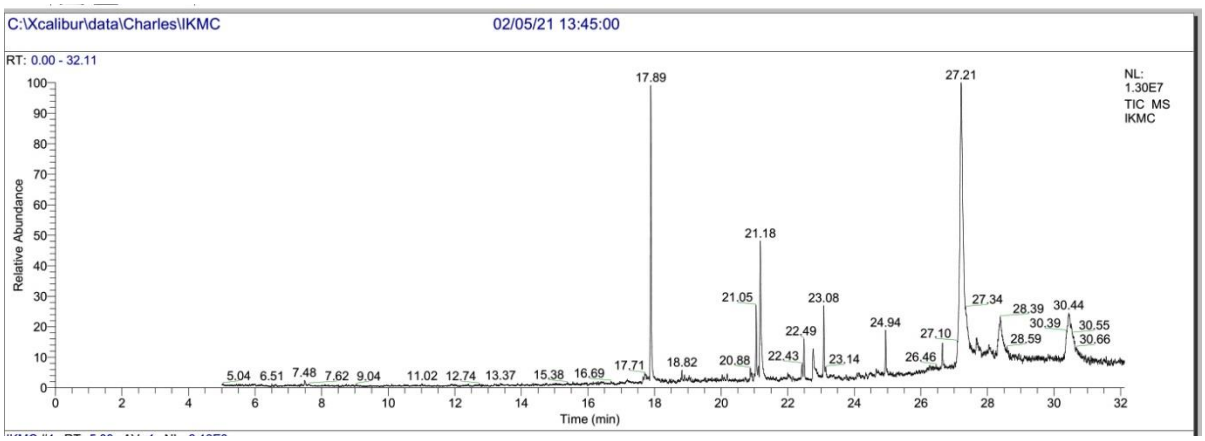


Figure 6.15: Chromatogram of compounds detected in IKM C by Gas chromatography-Mass Spectrometry (GC-MS). (IKM C = IKM Chloroform fraction)

Table 6.1: Lists of compounds used in screening of *I. khasiana* extracts.

Sl.no.	Types of Extract	Compound Screened	Mobile Phase	Extract in which compound is detected
1	IKM, IKM Hex, IKM Dee, IKM Bu, IKM C	Asiatic acid	Toluene: Ethyl Acetate: Formic Acid (5:5:1)	**ND**
2	IKM, IKM Hex, IKM Dee, IKM Bu, IKM C	Boswellic acid	Acetic Acid: Hexane:Ethyl Acetate: Toluene (0.3:1:8:2)	**ND**
3	IKM, IKM Hex, IKM Dee, IKM Bu, IKM C	Chebulagic acid	Ethyl Acetate: Toluene: Formic Acid: Methanol (6:1:1:2)	**ND**
4	IKM, IKM Hex, IKM Dee, IKM Bu, IKM C	Chlorogenic acid	Ethyl Acetate: Dichloromethane: formic Acid: Acetic Acid:Water (10:2.5:1:1:1.1)	**ND**
5	IKM, IKM Hex, IKM Dee, IKM Bu, IKM C	Ellagic acid	Toluene: Ethyl Acetate: Methanol: Formic Acid (5:4.5:3:2.5)	**ND**
6	IKM, IKM Hex, IKM Dee, IKM Bu, IKM C	Ferulic acid	Toluene: Ethyl Acetate: Formic Acid (5:5:0.2)	**ND**
7	IKM, IKM Hex, IKM Dee, IKM Bu, IKM C	Gallic acid	Toluene: Ethyl Acetate: Methanol: Formic Acid (5:4.5:3:2.5)	**ND**
8	IKM, IKM Hex, IKM Dee, IKM Bu, IKM C	Quercetin	Toluene: Ethyl Acetate: Formic Acid (5:4:0.2).	**IKM C**
9	IKM, IKM Hex, IKM Dee, IKM Bu, IKM C	β-Sitosterol	Toluene: methanol (16:2)	**IKM Hex & IKM Dee**

ND = Not detected

Table 6.2: Lists of method validation parameter on Quercetin quantification.

Sl.no	Parameter	Result
1	Linearity (ng/band)	100-500 ng/spot
2	R_f value	0.48±1
3	Correlation Coefficient	0.97
4	Slope	18.01
5	Intercept	65.34
6	LOD (µg/band)	68.01
7	LOQ (µg/band)	206.07

Table 6.3: Percentage recovery of quercetin in validation of the method.

Amount of Sample (in µl)	Amount of Standard (ng/spot)	Expected Area	Area Obtained	Mean Recovery (%)	RSD %
4	200	11372.13	11230.47	98.75	0.52
4	300	11721.88	11458.57	97.75	0.46
4	400	12952.83	12798.83	98.81	0.27

Table 6.4: Quantification result of Quercetin in IKM C.

Sl No.	Sample	R_f Value	Mean Area (n=1)	Amount Calculated (in ng)	Regression
1	IKM C	0.48±1	1538.33	372.98	Linear

Table 6.5: Lists of method validation parameter on β- sitosterol quantification.

Sl.no	Parameter	Result
1	Linearity (ng/band)	100-550 ng/spot
2	Rf value	0.43±1
3	Correlation Coefficient	0.99
4	Slope	3.05
5	Intercept	209.87
6	LOD (µg/band)	42.99
7	LOQ (µg/band)	143.28

Table 6.6: Percentage recovery of β- sitosterol in validation of the method.

Amount of Sample (in µl)	Amount of Standard (ng/spot)	Expected Area	Area Obtained	Mean Recovery (%)	RSD %
IKM Hex					
4	200	2867.54	2801.07	97.68	0.31
4	300	3288.94	3197.32	97.21	0.41
4	400	3429.34	3388.34	98.80	0.33
IKM Dee					
4	200	2509.91	2467.11	98.29	0.29
4	300	2877.31	2806.60	97.54	0.27
4	400	3398.21	3218.12	98.70	0.32

Table 6.7: Quantification result of β- sitosterol in IKM Hex and IKM Dee.

Sl. No.	Sample	R_f Value	Mean Area (n=3)	Amount Calculated (in ng)	Regression
1	IKM Hex	0.43	1558.12	257.04	Linear
2	IKM Dee	0.43	1326.10	198.68	Linear

Table 6.8: Compounds identified from IKP by GC-MS.

Sl. no	RT	PA (%)	Compound	CF	MW
1	17.65	20.59	2-Buten-1-one, 1-(2,6,6-trimethyl-1-cyclohexen-1-yl)	$C_{13}H_{20}O$	192
2	17.88	69.77	Phenol, 2,4-bis(1,1-dimethylethyl)	$C_{14}H_{22}O$	206
3	18.31	38.32	2(4H)-Benzofuranone, 5,6,7,7a-tetrahydro-4,4,7a-trimethyl	$C_{11}H_{16}O_2$	180
4	20.99	83.83	6-Hydroxy-4,4,7a-trimethyl-5,6,7,7a-tetrahydrobenzofuran-2(4H)-one	$C_{11}H_{16}O_3$	196
5	21.18	41.25	5,5,8a-Trimethyl-3,5,6,7,8,8a-hexahydro-2H-chromene	$C_{12}H_{20}O$	180
6	22.49	92.56	7,9-Di-tert-butyl-1-oxaspiro (4,5)deca-6,9-diene-2,8-dione	$C_{17}H_{24}O_3$	276
7	22.82	63.19	n-Hexadecanoic acid	$C_{16}H_{32}O_2$	256
8	23.09	44.87	Hexadecanoic acid, ethyl ester	$C_{16}H_{36}O_2$	284
9	24.25	38.34	3,7,11,15-Tetramethyl-2-hexadecen-1-ol	$C_{16}H_{40}O$	296
10	24.51	40.68	9,12,15-Octadecatrienoic acid	$C_{18}H_{30}O_2$	278
11	24.66	47.39	Octadecanoic acid	$C_{18}H_{36}O_2$	284
12	25.18	23.99	3,7,11,15-Tetramethyl-2-hexadecen-1-ol	$C_{20}H_{40}O$	296
13	31.37	42.38	Squalene	$C_{30}H_{50}$	410

Table 6.9: Compounds identified from IKC by GC-MS.

Sl. no	RT	PA (%)	Compound	CF	MW
1	13.10	18.19	Docosahexaenoic acid, 1,2,3-propanetriyl ester	$C_{69}H_{98}O_6$	1022
2	14.75	15.49	Benzoxazol, 2,3-dihydro-2-thioxo-3-diallylaminomethyl	$C_{14}H_{16}N_{2O}S$	260
3	17.14	13.20	Morphinan-4,5-epoxy-3,6-di-ol, 6-[7-nitrobenzofurazan-4-yl]amino	$C_{26}H_{27}N_5O_6$	505
4	17.88	73.26	Phenol, 2,4-bis(1,1-dimethylethyl)	$C_{14}H_{22}O$	206
5	21.18	19.35	-4-Hydroxy-3,5,5-trimethyl-4-(3-oxobut-1-en-1-yl)cyclohex-2-enone	$C_{13}H_{18}O_3$	222
6	22.42	16.28	Oxiraneundecanoic acid	$C_{19}H_{36}O_3$	312
7	22.49	86.41	7,9-Di-tert-butyl-1-oxaspiro(4,5)deca-6,9-diene-2,8-dione	$C_{17}H_{24}O_3$	276
8	24.74	15.65	Octadecane, 3-ethyl-5-(2-ethylbutyl)	$C_{26}H_{54}$	366
9	26.45	11.25	9-Octadecenoic acid, (2-phenyl-1,3-dioxolan-4-yl)methyl ester	$C_{28}H_{44}O_4$	444
10	29.55	11.76	Arsenous acid, tris(trimethylsilyl) ester	$C_9H_{27}AsO_3Si_3$	342
11	30.72	12.35	Tris(tert-butyldimethylsilyloxy)arsane	$C_{18}H_{45}AsO_3Si_3$	468

Table 6.10: Compounds identified from IKM by GC-MS.

Sl. no	RT	PA (%)	Compound	CF	MW
1	7.58	25.44	Dimethyl(chloromethyl) silyloxymethylbenzene	$C_{10}H_{15}ClOSi$	214
2	8.51	34.26	6-Oxa-bicyclo [3.1.0] hexan-3-one	$C_5H_6O_2$	98
3	11.02	10.28	1,4-Benzenediol, 2,6-bis(1,1-dimethylethyl)	$C_{14}H_{22}O_2$	222
4	12.60	14.81	Melezitose	$C_{18}H_{32}O_{16}$	504
5	12.76	18.82	4H-Pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl	$C_6H_8O_4$	144
6	16.46	48.19	Desulphosinigrin	$C_{10}H_{17}NO_6S$	279
7	17.14	22.03	1,4-Diacetyl-3-acetoxymethyl-2,5-methylene-l-rhamnitol	$C_{14}H_{22}O_8$	318
8	17.89	72.89	Phenol, 2,4-bis(1,1-dimethylethyl)	$C_{14}H_{22}O$	206
9	18.94	12.86	D-Glucopyranoside, methyl	$C_7H_{14}O_6$	194
10	19.41	24.28	Ethyl à-d-glucopyranoside	$C_8H_{16}O_6$	208
11	19.74	32.12	Desulphosinigrin	$C_{10}H_{17}NO_6S$	279
12	11.78	21.18	9-Octadecenoic acid, (2-phenyl-1,3-dioxolan-4-yl) methyl ester	$C_{28}H_{44}O_4$	444
13	22.43	11.19	Pentadecanoic acid, 14-methyl-, methyl ester	$C_{17}H_{34}O_2$	270
14	22.76	58.85	n-Hexadecanoic acid	$C_{16}H_{32}O_2$	256
15	24.14	40.99	[1,1'-Bicyclopropyl]-2-octanoic acid, 2'-hexyl-, methyl ester	$C_{21}H_{38}O_2$	322
16	27.68	31.68	2-Bromotetradecanoic acid	$C_{14}H_{27}BrO_2$	306
17	31.30	14.90	Androstane-11,17-dione, 3-[(trimethylsilyl)oxy]-, 17-[O-(phenylmethyl)oxime	$C_{29}H_{43}NO_3Si$	481

Table 6.11: Compounds identified from IKM Hex by GC-MS.

Sl. no	RT	PA (%)	Compound	CF	MW
1	16.40	11.42	2-Myristynoyl pantetheine	$C_{25}H_{44}N_2O_5S$	484
2	17.15	13.46	1,4-Diaminonaphthalene	$C_{10}H_{10}N_2$	158
3	17.88	72.45	Phenol, 2,4-bis(1,1-dimethylethyl)	$C_{14}H_{22}O$	206
4	18.55	64.05	Benzene, (1-ethyloctyl)	$C_{16}H_{26}$	218
5	18.98	52.66	Benzene, (1-methylnonyl)	$C_{16}H_{26}$	218
6	20.15	42.42	Benzene, (1-methyldecyl)	$C_{17}H_{28}$	232
7	20.47	59.80	Benzene, (1-butyloctyl)	$C_{18}H_{30}$	246
8	21.27	43.58	Benzene, (1-methylundecyl)	$C_{18}H_{30}$	246
9	22.23	30.36	Benzene, (1-methyldodecyl)	$C_{19}H_{32}$	260
10	22.42	41.92	Hexadecanoic acid, methyl ester	$C_{17}H_{34}O_2$	270
11	22.76	63.95	n-Hexadecanoic acid	$C_{16}H_{32}O_2$	256
12	24.14	14.75	9,12,15-Octadecatrienoic acid, methyl ester	$C_{19}H_{32}O_2$	292
13	24.48	16.84	Cyclopropaneoctanoic acid, 2-[[2-[(2-ethylcyclopropyl)methyl]cyclopropyl]methyl]	$C_{22}H_{38}O_2$	334
14	25.34	23.20	2,3-Dimethyl-1,4,4a,9a-tetrahydroanthracene-9,10-dione	$C_{16}H_{16}O_2$	240
15	26.87	20.55	Ethyl iso-allocholate	$C_{26}H_{44}O_5$	436
16	27.67	18.76	9-Octadecenoic acid, (2-phenyl-1,3-dioxolan-4-yl)methyl ester	$C_{28}H_{44}O_4$	444
17	28.45	16.32	1,25-Dihydroxyvitamin D_3	$C_{30}H_{52}O_3Si$	488
18	28.70	15.69	Androstane-11,17-dione, 3-[(trimethylsilyl)oxy]-, 17-[O-(phenylmethyl)oxime]	$C_{29}H_{43}NO_3Si$	481

Table 6.12: Compounds identified from IKM Bu by GC-MS.

Sl. no	RT	PA (%)	Compound	CF	MW
1	13.94	36.33	Cyclohexan-1,4,5-triol-3-one-1-carboxylic acid	$C_7H_{10}O_6$	190
2	16.47	34.49	1-Methyl-1-n-octyloxy-1-silacyclobutane	$C_{12}H_{26}OS$	214
3	17.89	70.74	Phenol, 2,4-bis(1,1-dimethylethyl)	$C_{14}H_{22}O$	206
4	20.18	25.23	Melezitose	$C_{18}H_{32}O_{16}$	504
5	22.49	81.27	7,9-Di-tert-butyl-1-oxaspiro(4,5)deca-6,9-diene-2,8-dione	$C_{17}H_{24}O_3$	276
6	22.76	64.02	n-Hexadecanoic acid	$C_{16}H_{32}O_2$	256
7	24.65	11.32	2-Bromotetradecanoic acid	$C_{14}H_{27}BrO_2$	306
8	26.67	16.50	9-Octadecenoic acid, (2-phenyl-1,3-dioxolan-4-yl)methyl ester	$C_{28}H_{44}O_4$	444
9	26.91	71.96	Arbutin	$C_{12}H_{16}O_7$	272
10	27.67	37.05	Hexadecanoic acid, 1-(hydroxymethyl)-1,2-ethanediyl ester	$C_{35}H_{68}O_5$	568
11	29.56	11.81	Androstane-11,17-dione, 3-[(trimethylsilyl)oxy]-, 17-[O-(phenylmethyl)oxime]	$C_{29}H_{43}NO_3Si$	481

Table 6.13: Compounds identified from IKM Dee by GC-MS.

Sl. no	RT	PA (%)	Compound	CF	MW
1	6.32	14.56	Docosahexaenoic acid, 1,2,3-propanetriyl ester	$C_{69}H_{98}O_6$	1022
2	7.49	25.66	Dimethyl(chloromethyl)silyloxymethylbenzene	$C_{10}H_{15}ClOSi$	214
3	8.94	14.09	8,11-Octadecadiynoic acid, methyl ester	$C_{19}H_{30}O_2$	290
4	11.0	18.50	1,4-Benzenediol, 2,6-bis(1,1-dimethylethyl)	$C_{14}H_{22}O_2$	222
5	13.15	15.63	5,7-Dodecadiyn-1,12-diol	$C_{12}H_{18}O_2$	194
6	14.12	20.63	Resorcinol	$C_6H_6O_2$	110
7	17.14	26.26	5,6,7,8,9,10-hexahydro-9-phenyl-spiro[2H-1,3-benzoxazine-4,1'-cyclohexane]-2-thione	$C_{19}H_{25}NOS$	315
8	17.65	16.03	Hexadecane, 1,1-bis(dodecyloxy)	$C_{40}H_{82}O_2$	594
9	17.88	70.14	Phenol, 2,4-bis(1,1-dimethylethyl)	$C_{14}H_{22}O$	206
10	17.94	54.82	Butylated Hydroxytoluene	$C_{15}H_{24}O$	220
11	20.99	14.34	[1,1'-Bicyclopropyl]-2-octanoic acid, 2'-hexyl-, methyl ester	$C_{21}H_{38}O_2$	322
12	21.84	18.88	Caffeine	$C_8H_{10}N_4O_2$	194
13	22.42	30.46	Hexadecanoic acid, methyl ester	$C_{17}H_{34}O_2$	270
14	22.79	60.89	n-Hexadecanoic acid	$C_{17}H_{32}O_2$	256
15	24.07	10.30	1,2-15,16-Diepoxyhexadecane	$C_{16}H_{30}O_2$	254
16	24.14	12.45	9,12,15-Octadecatrienoic acid, 2,3-dihydroxypropyl ester	$C_{21}H_{36}O_4$	352
17	24.66	15.05	12-Methyl-E,E-2,13-octadecadien-1-ol	$C_{19}H_{36}O$	280
18	26.32	13.63	2-Bromotetradecanoic acid	$C_{14}H_{27}BrO_2$	306
19	27.68	14.59	9-Octadecenoic acid, (2-phenyl-1,3-dioxolan-4-yl)methyl ester	$C_{28}H_{44}O_4$	444

Table 6.14: Compounds identified from IKM C by GC-MS.

Sl. no	RT	PA (%)	Compound	CF	MW
1	17.71	36.23	Phenol, 2,4-bis(1,1-dimethylethyl)	$C_{14}H_{22}O$	206
2	20.88	17.42	Fumaric acid, 2-(2-methoxyethyl)hexyl 2,3-dichlorophenyl ester	$C_{19}H_{24}Cl_2O_5$	402
3	21.18	41.88	5,5,8a-Trimethyl-3,5,6,7,8,8a-hexahydro-2H-chromene	$C_{12}H_{20}O$	180
4	22.49	85.38	7,9-Di-tert-butyl-1-oxaspiro(4,5)deca-6,9-diene-2,8-dione	$C_{17}H_{24}O_3$	276
5	24.66	23.06	2-Bromotetradecanoic acid	$C_{14}H_{27}BrO_2$	306
6	28.39	16.43	Lupeol	$C_{30}H_{50}O$	426
7	30.44	16.59	1-Heptatriacotanol	$C_{37}H_{76}O$	536
8	30.66	12.46	Androstane-11,17-dione, 3-[(trimethylsilyl)oxy]-, 17-[O-(phenylmethyl)oxime]	$C_{29}H_{43}NO_3Si$	481

REFERENCES

Alizadeh, S. R., & Ebrahimzadeh, M. A. (2021). Quercetin derivatives: Drug design, development, and biological activities, a review. European journal of medicinal chemistry, 114068.

Allard, P. M., Bisson, J., Azzollini, A., Pauli, G. F., Cordell, G. A., & Wolfender, J. L. (2018). Pharmacognosy in the digital era: shifting to contextualized metabolomics. Current opinion in biotechnology, 54, 57-64.

Allard, P. M., Genta-Jouve, G., & Wolfender, J. L. (2017). Deep metabolome annotation in natural products research: towards a virtuous cycle in metabolite identification. Current Opinion in Chemical Biology, 36, 40-49.

Atanasov, A. G., Zotchev, S. B., Dirsch, V. M., & Supuran, C. T. (2021). Natural products in drug discovery: advances and opportunities. Nature Reviews Drug Discovery, 20(3), 200-216.

Attimarad, M., Ahmed, K. M., Aldhubaib, B. E., & Harsha, S. (2011). High-performance thin layer chromatography: A powerful analytical technique in pharmaceutical drug discovery. Pharmaceutical methods, 2(2), 71-75.

Awad, A. B., Chen, Y. C., Fink, C. S., & Hennessey, T. (1996). beta-Sitosterol inhibits HT-29 human colon cancer cell growth and alters membrane lipids. Anticancer research, 16(5A), 2797-2804.

Awad, A. B., Roy, R., & Fink, C. S. (2003). β-sitosterol, a plant sterol, induces apoptosis and activates key caspases in MDA-MB-231 human breast cancer cells. Oncology reports, 10(2), 497-500.

Ayissi, V. B. O., Ebrahimi, A., & Schluesenner, H. (2014). Epigenetic effects of natural polyphenols: A focus on SIRT1-mediated mechanisms. Molecular nutrition & food research, 58(1), 22-32.

Babu, S., & Jayaraman, S. (2020). An update on β-sitosterol: A potential herbal nutraceutical for diabetic management. Biomedicine & Pharmacotherapy, 131, 110702.

Bach, A., Bender–Sigel, J., Schrenk, D., Flügel, D., & Kietzmann, T. (2010). The antioxidant quercetin inhibits cellular proliferation via HIF-1-dependent induction of p21WAF. Antioxidants & Redox Signaling, 13(4), 437-448.

Bin Sayeed, M. S., & Ameen, S. S. (2015). Beta-sitosterol: a promising but orphan nutraceutical to fight against cancer. Nutrition and cancer, 67(8), 1216-1222.

Bouic, P. J., & Lamprecht, J. H. (1999). Plant sterols and sterolins: a review of their immune-modulating properties. Altern Med Rev, 4(3), 170-7.

Coskun, O. (2016). Separation techniques: chromatography. Northern clinics of Istanbul, 3(2), 156.

Das, N., Sikder, K., Bhattacharjee, S., Majumdar, S. B., Ghosh, S., Majumdar, S., & Dey, S. (2013). Quercetin alleviates inflammation after short-term treatment in high-fat-fed mice. Food & function, 4(6), 889-898.

Donald, P. L., Lampman, G. M., Kritz, G. S., & Engel, R. G. (2006). Introduction to organic laboratory techniques. Thomson Brooks/Cole, 797-817.

Ghosh, B. (1999). Quercetin inhibits LPS-induced nitric oxide and tumor necrosis factor-α production in murine macrophages. International journal of immunopharmacology, 21(7), 435-443.

Hao, D., Gu, X., Xiao, P., Liang, Z., Xu, L., & Peng, Y. (2013). Research progress in the phytochemistry and biology of *Ilex* pharmaceutical resources. Acta Pharmaceutica Sinica B, 3(1), 8-19.

Inamine, M., Suzui, M., Morioka, T., Kinjo, T., Kaneshiro, T., Sugishita, T., & Yoshimi, N. (2005). Inhibitory effect of dietary monoglucosylceramide 1-O-β-glucosyl-N-2′-hydroxyarachidoyl-4, 8-sphingadienine on two different categories of colon

preneoplastic lesions induced by 1, 2-dimethylhydrazine in F344 rats. Cancer science, 96(12), 876-881.

Kamboj, A., & Saluja, A. K. (2017). Development of validated HPTLC method for quantification of stigmasterol from leaf and stem of *Bryophyllum pinnatum*. Arabian journal of Chemistry, 10, S2644-S2650.

Koo, A. (1985). Microvascular techniques for in vivo assay of vasoactive agents from Chinese medicinal herbs. ChangH. M., YeungH. W., TsoW. W., KooA., eds, Advances in Chinese Medicinal Materials Research. World Scientific, Singapore, 559-80.

Lachance, H., Wetzel, S., Kumar, K., & Waldmann, H. (2012). Charting, navigating, and populating natural product chemical space for drug discovery. Journal of Medicinal Chemistry, 55(13), 5989-6001.

Laila, O., Murtaza, I., Abdin, M. Z., Ahmad, S., Ganai, N. A., & Jehangir, M. (2014). Development and validation of HPTLC method for simultaneous estimation of diosgenin and quercetin in fenugreek seeds (Trigonella foenum-graceum). International Scholarly Research Notices, 2014.

Lalnunfela, C., Lalremsanga, H. T., Lalhriatpuii, T. C., Lalnunzira, D., & Lalchhandama, K. (2019). Unveiling the Unexplored and Critically Endangered *Ilex khasiana* for its Antioxidant Properties. Journal of Natural Remedies, 19(4), 214-220.

Lalnunfela, C., Lalthanpuii, P. B., Lalhriatpuii, T. C., & Lalchhandama, K. (2020). An endangered medicinal plant, *Ilex khasiana* exhibits potent antiparasitic activity against intestinal tapeworm. Pharmacognosy Journal, 12(4).

Li, L., Zhang, Y., Zhang, P., Pi, H., Ruan, H., & Wu, J. (2011). Appraisal of anti-inflammatory and free radical scavenging activities of ethanol extract of *Ilex*

ficoidea Hemsl. and *Ilex centrochinensis* SY Hu. Environmental Toxicology and Pharmacology, 32(2), 122-127.

Lin, S. Y., Wang, Y. Y., Chen, W. Y., Chuang, Y. H., Pan, P. H., & Chen, C. J. (2014). Beneficial effect of quercetin on cholestatic liver injury. The Journal of Nutritional Biochemistry, 25(11), 1183-1195.

Liu, X., & Locasale, J. W. (2017). Metabolomics: a primer. Trends in biochemical sciences, 42(4), 274-284.

Mihigo, S. O., Ndombele, L., Masesane, I. B., & Sichilongo, K. (2015). Preliminary GC-MS Profiling and Anti-bacterial activity Investigation of *Acanthospermum hispidum* DC (Asteraceae). Int J Chem Aquat Sci, 1, 20-9.

Moon, E. J., Lee, Y. M., Lee, O. H., Lee, M. J., Lee, S. K., Chung, M. H., ... & Kim, K. W. (1999). A novel angiogenic factor derived from Aloe vera gel: β-sitosterol, a plant sterol. Angiogenesis, 3(2), 117-123.

Muthian, G., & Bright, J. J. (2004). Quercetin, a flavonoid phytoestrogen, ameliorates experimental allergic encephalomyelitis by blocking IL-12 signaling through JAK-STAT pathway in T lymphocyte. Journal of clinical immunology, 24(5), 542-552.

Nirmal, S. A., Pal, S. C., Mandal, S. C., & Patil, A. N. (2012). Analgesic and anti-inflammatory activity of β-sitosterol isolated from *Nyctanthes arbortristis* leaves. Inflammopharmacology, 20(4), 219-224.

Pagliacci, M. C., Smacchia, M., Migliorati, G., Grignani, F., Riccardi, C., & Nicoletti, I. (1994). Growth-inhibitory effects of the natural phyto-oestrogen genistein in MCF-7 human breast cancer cells. European Journal of Cancer, 30(11), 1675-1682.

Pandey, A., & Tripathi, S. (2014). Concept of standardization, extraction and pre phytochemical screening strategies for herbal drug. Journal of Pharmacognosy and Phytochemistry, 2(5).

Park, C., Moon, D. O., Rhu, C. H., Choi, B. T., Lee, W. H., Kim, G. Y., & Choi, Y. H. (2007). β-Sitosterol induces anti-proliferation and apoptosis in human leukemic U937 cells through activation of caspase-3 and induction of Bax/Bcl-2 ratio. Biological and Pharmaceutical Bulletin, 30(7), 1317-1323.

Rutz, A., Dounoue-Kubo, M., Ollivier, S., Bisson, J., Bagheri, M., Saesong, T., & Allard, P. M. (2019). Taxonomically informed scoring enhances confidence in natural products annotation. Frontiers in plant science, 1329.

Sermakkani, M., & Thangapandian, V. (2012). GC-MS analysis of *Cassia italica* leaf methanol extract. Asian J Pharm Clin Res, 5(2), 90-94.

Sermakkani, M., & Thangapandian, V. (2012). GC-MS analysis of *Cassia italica* leaf methanol extract. Asian J Pharm Clin Res, 5(2), 90-94.

Shailajan, S., Menon, S., Sayed, N., & Tiwari, B. (2012). Simultaneous quantitation of three bioactive triterpenoids Lupeol, Stigmasterol and β-sitosterol from Carissa carandas using RP-HPLC method and its validation as per ICH guidelines. Int J Green Pharm, 6(3), 241-7.

Shebeko, S. K., Zupanets, I. A., Popov, O. S., Tarasenko, O. O., & Shalamay, A. S. (2018). Effects of quercetin and its combinations on health. In Polyphenols: Mechanisms of Action in Human Health and Disease (pp. 373-394). Academic Press.

Sherma, J., & Fried, B. (Eds.). (2003). Handbook of thin-layer chromatography. CRC press.

Shilajan, S., & Swar, G. (2013). Simultaneous estimation of three triterpenoids-ursolic acid, β-sitosterol and lupeol from flowers, leaves and formulations of

Rhododendron arboreum Smith. using validated HPTLC method. International Journal of Green Pharmacy (Medknow Publications & Media Pvt. Ltd.), 7(3).

Tasyriq, M., Najmuldeen, I. A., In, L. L., Mohamad, K., Awang, K., & Hasima, N. (2012). 7α-Hydroxy-β-sitosterol from *Chisocheton tomentosus* induces apoptosis via dysregulation of cellular Bax/Bcl-2 ratio and cell cycle arrest by downregulating ERK1/2 activation. Evidence-based complementary and alternative medicine: eCAM, 2012.

Ulbricht, C. E. (2016). An evidence-based systematic review of beta-sitosterol, sitosterol (22, 23-dihydrostigmasterol, 24-ethylcholesterol) by the Natural Standard Research Collaboration. Journal of Dietary Supplements, 13(1), 35-92.

Valerio, M., & Awad, A. B. (2011). β-Sitosterol down-regulates some pro-inflammatory signal transduction pathways by increasing the activity of tyrosine phosphatase SHP-1 in J774A. 1 murine macrophage. International immunopharmacology, 11(8), 1012-1017.

Vundru, S. S., Kale, R. K., & Singh, R. P. (2013). β-sitosterol induces G1 arrest and causes depolarization of mitochondrial membrane potential in breast carcinoma MDA-MB-231 cells. BMC complementary and alternative medicine, 13(1), 1-9.

Wenjuan, Q., Xiue, W., Junjie, Z., Fukuyama, Y., Yamada, T., & Nakagawa, K. (1986). Triterpenoid glycosides from leaves of *Ilex cornuta*. Phytochemistry, 25(4), 913-916.

Xiao, Z. P., Wang, X. D., Wang, P. F., Zhou, Y., Zhang, J. W., Zhang, L., & Zhu, H. L. (2014). Design, synthesis, and evaluation of novel fluoroquinolone–flavonoid hybrids as potent antibiotics against drug-resistant microorganisms. European journal of medicinal chemistry, 80, 92-100.

Yeshurun, D., & Gotto Jr, A. M. (1976). Drug treatment of hyperlipidemia. The American journal of medicine, 60(3), 379-396.

Yousuf, M., Khan, P., Shamsi, A., Shahbaaz, M., Hasan, G. M., Haque, Q. M. R., ... & Hassan, M. I. (2020). Inhibiting CDK6 activity by quercetin is an attractive strategy for cancer therapy. ACS omega, 5(42), 27480-27491.

Zhang, X., Ke, M., & Ou, F. (1981). Extraction and isolation of total flavonoid glycosides of Mao Don Quing (*Ilex pubescens*) bark. Zhongcaoyao, 12, 399-399.

Zhao, Y., Chang, S. K., Qu, G., Li, T., & Cui, H. (2009). β-Sitosterol inhibits cell growth and induces apoptosis in SGC-7901 human stomach cancer cells. Journal of agricultural and food chemistry, 57(12), 5211-5218.

ABBREVIATIONS

µg	: Microgram
ng	: Nanogram
ALT	: Alanine transaminase
AST	: Aspartate transaminase
BHT	: Butylated hydroxytoluene
BSA	: Bovine serum albumin
BSI	: Botanical Survey of India
cm	: Centimeter
DIC	: Diclofenac sodium
DLA	: Dalton's lymphoma ascites
DOX	: Doxorubicin
DPPH	: 2, 2-diphenyl-1-picryl hydrazine
DTNB	: 5-5'-dithiobis [2-nitrobenzoic acid]
EDTA	: Ethylenediamine tetra-acetic acid
GC-MS	: Gas Chromatography – Mass Spectrometry
GSH	: Glutathione
GST	: Glutathione-S-transferase
HPTLC	: High Performance Thin layer chromatography
IKC	: *Ilex khasiana* Chloroform extract
IKM	: *Ilex khasiana* Methanol extract
IKM Bu	: IKM Butanol fraction
IKM Hex	: IKM Hexane fraction
IKM Dee	: IKM Diethyl Ether fraction
IKM C	: IKM Chloroform fraction
IKP	: *Ilex khasiana* Petroleum Ether extract
MDA	: Malondialdehyde
MIC	: Minimum Inhibitory Concentration
mm	: Millimeter

MTT	: (3-(4, 5-dimethylthiazole-2-yl)-2, 5-diphenyl tetrazolium bromide)
NADH	: b-nicotinamide adenine dinucleotide
NBT	: Nitroblue tetrazolium
PMS	: Phenazine methosulphate
R_f	: Retention factor
ROS	: Reactive oxygen species
SOD	: Superoxide Dismutase
TBA	: Thiobarbituric acid
TCA	: Tricarboxylic acid

PLANT AUTHENTICATION CERTIFICATE

Typed copy

भारत सरकार
GOVERNMENT OF INDIA
पर्यावरण एवं वन मंत्रालय
MINISTRY OF ENVIRONMENT AND FORESTS

टेलीफोन/Telephone: 2224139, 2223971,2223618 ई मेल E-Mail-bshvishl@yahoo.co.in Fax No. 0364-2224139

BOTANICAL SURVEY OF INDIA
OFFICE OF THE JOINT DIRECTOR
EASTERN CIRCLE
SHILLONG -793003

भारतीय वनस्पति सर्वेक्षण
संयुक्त निदेशक कार्यालय
पूर्वी प्रक्षेत्र : शिलांग-793003

No.BSI/EC/~~Estt.~~/2008/ 677 Tech **Dated: 16.10.2008**

To

Mr.M.Sawmliana
Chanmari West
Aizawl, Mizoram
PIN-796007

Sub- Identification of Plant specimens

Sir

With reference to your letter No. nil dated 12-10-2008 and dated 29-2-2008 regarding identification of 10 specimens, please find enclosed herewith a list of 8 identified species. The remaining two specimens (Nos.2 and 6) are yet to be identified which will be communicated to you as soon as it is done.

Further, with reference to your letter dated 21-06-2007 I am to inform you to correct the identification of Sl.No.15 as *Dendrobium peguanum* Lindl. instead of *D.diodon* Rchb.f. subsp.*kodayarensis* Gopalan & Henry. The three specimens serial No.14, 16 and 22 could not be identified as yet. It will be tried to get identified at CNH whenever somebody goes there and will be communicated to you.

धन्यवाद/ Thanking you,

भवदीय /Yours faithfully

16/10/08

(डा. टी. एम. हिनयूटा) / (Dr. T.M. HYNNIEWTA)
संयुक्त निदेशक / Joint Director

List of Plants identified sent by M. Saumliana from Mizoram

No.1
Local Name : Ngai
Botanical Name : *Agave sisalana* Perrine ex Engel.
Family : Agavaceae
Locality : Buhban
Altitude : Ca.1,200 m.
Date : 27-7-2006(Flowering), 2-10-2006(Fruiting)
Habit : A small tree upto about 4 m high.
Note : Growing wild. Occasionally planted in homestead compound for ornamental.

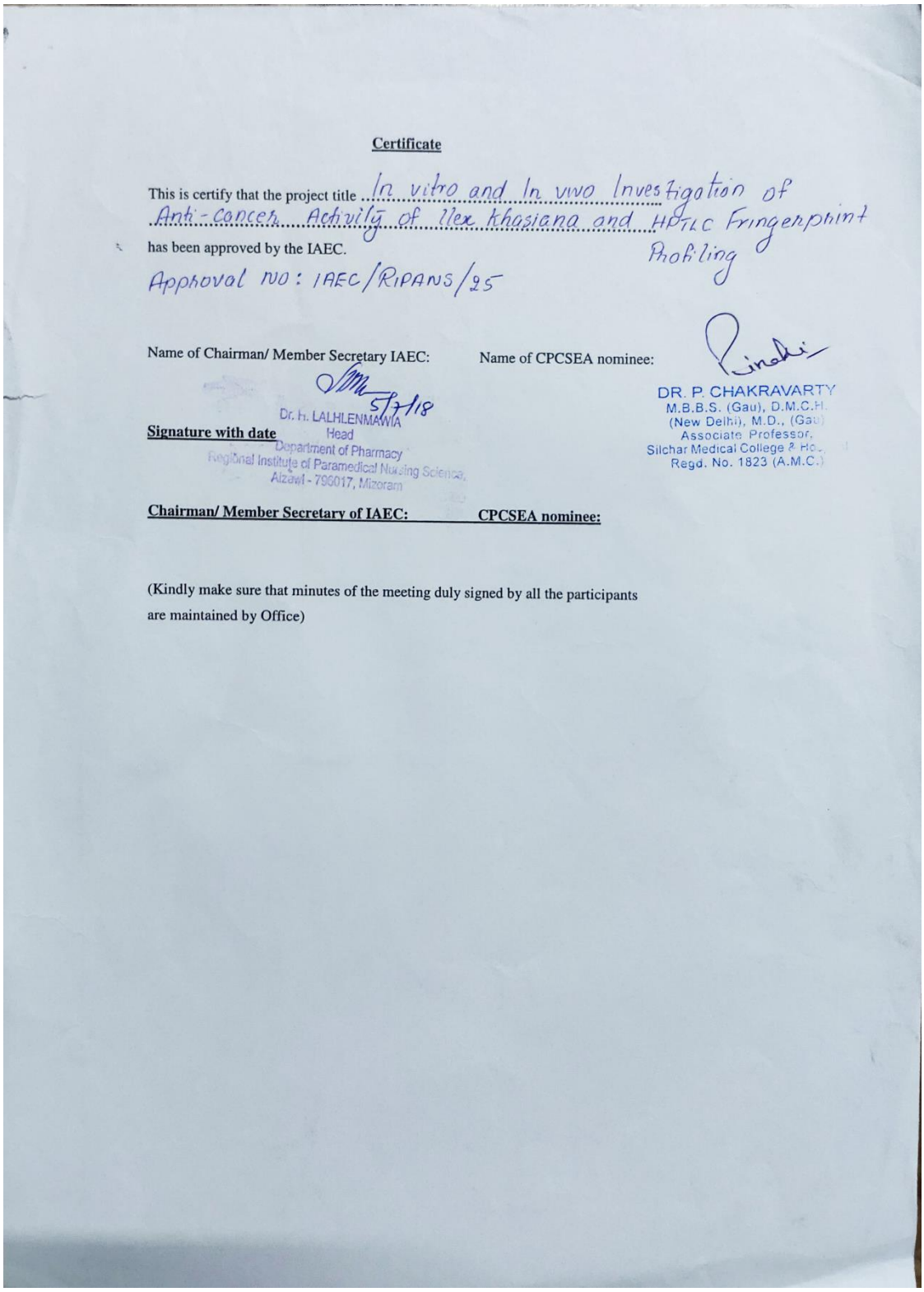
No.3
Local Name : Zo-kangthai
Botanical Name : *Laportea terminalis* .Wight.
Family : Urticaceae
Location : Tawi WLS
Altitude : Ca.1,400 m.
Date : 19-12-2007
Habit : A herb with stinging hairs.

No.4
Local Name :
Botanical Name : *Clematis buchananiana* DC.
Family : Ranunculaceae
Locality : Between Kaunpui and Maite road(or Tawi WLS)
Altitude : Ca.1,400 m.
Date : 19-12-2007
Habit : A climber with yellow flowers.

No.5
Local Name :
Botanical Name : *Ilex khasiana* Purkay.
Family : Aquifoliaceae
Locality : Aizawl(Luangmual)
Altitude : 1,000 m.
Date : a.15-3-2006(Fruiting), b.25-12-2007(Flowering)
Habit : A middle-sized evergreen tree ; leaves bitter & astringent ; fruit red when ripe.
Uses : Fruits are eaten by wild animals like Palm Civets, Squirrels 7 and birds.

No.7
Local Name : Thiallawn
Botanical Name : *Rhaphidophora glauca* Schott.
Family : Araceae
Locality : Tawi WLS
Altitude : 1,500 m.
Date : 24-1-2008

ANIMAL ETHICS APPROVAL CERTIFICATE



Certificate

This is certify that the project title ...In vitro and In vivo Investigation of Anti-cancer Activity of Nex khasiana and HPTLC Fringerprint Profiling... has been approved by the IAEC.

Approval NO: IAEC/RIPANS/25

Name of Chairman/ Member Secretary IAEC:

5/7/18

Dr. H. LALHLENMAWIA
Head
Department of Pharmacy
Regional Institute of Paramedical Nursing Science,
Aizawl - 796017, Mizoram

Signature with date

Name of CPCSEA nominee:

DR. P. CHAKRAVARTY
M.B.B.S. (Gau), D.M.C.H.
(New Delhi), M.D., (Gau)
Associate Professor,
Silchar Medical College & Ho...
Regd. No. 1823 (A.M.C.)

Chairman/ Member Secretary of IAEC: **CPCSEA nominee:**

(Kindly make sure that minutes of the meeting duly signed by all the participants are maintained by Office)

Certificate

This is certify that the project title In vitro and in vivo Investigation of Anti-Cancer Activity of Ilex khasiana and HPTLC Fingerprint Profiling.

has been approved by the IAEC.

Approval NO: IAEC/RIPANS/26

Name of Chairman/ Member Secretary IAEC:

Dr. h. LALHLENMAWIA
Head
Department of Pharmacy
Regional Institute of Paramedical Nursing Science
Aizawl - 796017, Mizoram

Name of CPCSEA nominee:

DR. P. CHAKRAVARTY
M.B.B.S. (Gau), D.M.C.H.
(New Delhi), M.D., (Gau)
Associate Professor,
Silchar Medical College & Hos
Regd. No. 1823 (A.M.C.)

Signature with date

Chairman/ Member Secretary of IAEC: **CPCSEA nominee:**

(Kindly make sure that minutes of the meeting duly signed by all the participants are maintained by Office)

Provisional Certificate

This is to certify that the project entitle "***In vitro* and *in vivo* investigation of anti-cancer activity and HPTLC fingerprint of *Ilex khasiana*" (Permit No. : MZUBS/2021/01)** has been approved by MZUBS provisionally. Later this proposal will be submitted and presented before the MZUBS meeting schedules during the end of 2021 for permission.

Topic: *In vitro* and *in vivo* investigation of anti-cancer activity and HPTLC fingerprint of *Ilex khasiana*

Supervisor: Prof. H.T. Lalremsanga

Institution: Department of Zoology, Mizoram University

Major objectives of the proposed work:

1. Screening of *Ilex khasiana* for its anti-microbial activity against selected microbes.

(S.K Mehta) 28/10/2021
Chairman, MZUBS

(Dr. S.K. MEHTA)
Professor
Dept. of Botany
Mizoram University
Aizawl - 796004

BIO-DATA OF CANDIDATE

Name: Charles Lalnunfela

Father's name: C. Lalengvara (L)

Mother's name: Darthantluangi

Date of Birth: 19th July, 1989

Marital status: Single

Nationality: Indian

Religion: Christianity

Contanct: +91 9612 110 610

Email ID: c.lalnunfela@gmail.com

Education qualifications:

Name of exam	Year	Board	Subject	Percentage	Division
HSLC	2006	MBSE	General	75.80	Distinction
HSSLC	2009	MBSE	Science	57.40	Second
B.Sc	2012	Madras University	Adv. Zoology & Biotechnology	72.10	First
M.Sc	2014	Madras University	Biomedical Genetics	65.23	First

Address:

House no. D-3, Complex Road, Zonuam, Aizawl, Mizoram.

CHARLES LALNUNFELA

LIST OF PUBLICATIONS

1. **Lalnunfela, C., Lalremsanga, H. T., Lalhriatpuii, T. C., Lalnunzira, D., & Lalchhandama, K.** (2019). Unveiling the Unexplored and Critically Endangered *Ilex khasiana* for its Antioxidant Properties. Journal of Natural Remedies, 19(4), 214-220.

2. **Lalnunfela, C., Lalremsanga, H. T., Lalhriatpuii, T. C., Lalthanpuii, P. B., & Lalchhandama, K.** (2020). *Ilex khasiana* - the silent holly species of aquifoliaceae on its pharmacognostical importance as a free radical scavenger and antibacterial agent. International Journal of Research in Pharmaceutical Sciences, 11(2), 2438-2444

3. **Lalnunfela, C., Lalthanpuii, P. B., Lalhriatpuii, T. C., & Lalchhandama, K.** (2020). An endangered medicinal plant, *Ilex khasiana* exhibits potent antiparasitic activity against intestinal tapeworm. Pharmacognosy Journal, 12(4).

4. **Lalruatfela, B., Lalthanpuii, P. B., Lalnunfela, C., & Lalchhandama, K.** (2020). Nematocidal effects of tobacco infusion (tuibur) against intestinal helminth parasites of chicken. Journal of Environmental Biology, 41(4), 840-844.

5. **Lalthanpuii, P. B., Laldinpuii, Z. T., Lalhmangaihzuala, S., Vanlaldinpuia, K., Lalruatfela, B., Lalnunfela, C., ... & Lalchhandama, K.** (2020). Chemical profiling of alkylamides from the" herbal Botox", Acmella oleracea, cultivated in Mizoram and their pharmacological potentials. Journal of Environmental Biology, 41(4), 845-850.

CONFERENCES/SEMINARS/WORKSHOP ATTENDED

International:

1. International Conference on Biodiversity, Environment and Human Health Innovations and Emerging Trends (BEHIET) – 2018.
2. International Conference on Recent Advances in Animal Sciences (ICRAAS) – 2019.

National:

1. Workshop on "Biostatistics and Bioinformatics" – BIF, Dept. of Biotech. MZU, 2016.
2. Seminar on "Recent advances and scope in herbal technology: Challenges and prospect" - DPS, AU, 2016.
3. Science communication workshop (SCICOMM 101) – DBT India, MZU, 2017
4. Seminar on "Animal handling, maintenance and care" -Biotech-HUB, MZU, 2018.
5. Hands on Training on "HPTLC Technique with Anchrom, Mumbai" - RIPANS, 2021.

State:

1. Seminar on Science for Nation Building -MAS & DST-MZR, 2018.
2. Mizoram Science Congress - MAS, MISTIC, STAM & BIOCONE, 2016.
3. Seminar on Science for the people and the people for science - MAS & MISTIC, 2019.
4. Mizoram Science Congress - MAS, MISTIC, STAM & BIOCONE, 2020.

PAPER PRESENTED

International:

1. ***"Ilex khasiana* - the silent holly species of aquifoliaceae on its pharmacognostical importance as a free radical scavenger and antibacterial agent."** on the International Conferences of Recent Advances in Animal Sciences (ICRAAS) organized by Department of Zoology, Pachhunga University College, 6-8th November, 2019.

National:

1. **"Unfurling the treasury of *Ilex khasiana* and quantification of Quercetin using HPTLC Method"** on Mizoram Science Congress - MAS, MISTIC, STAM & BIOCONE, 3rd – 4th December, 2020.

PARTICULARS OF THE CANDIDATE

NAME OF THE CANDIDATE	**: CHARLES LALNUNFELA**
DEGREE	**: DOCTOR OF PHILOSOPHY**
DEPARTMENT	**: ZOOLOGY**

TITLE OF THE THESIS	***: IN VITRO* AND *IN VIVO* INVESTIGATION OF ANTI-CANCER ACTIVITY OF *ILEX KHASIANA* AND HPTLC FINGERPRINT PROFILING.**
DATE OF ADMISSION	**: 22. 08. 2016**

APPROVAL OF RESEARCH PROPOSAL

DRC	**: 04. 04. 2017**
B.O.S	**: 19. 05. 2017**
SCHOOL BOARD	**: 26. 05. 2017**
MZU REGISTRATION	**: 1607299**
Ph.D REGISTRATION NO. & DATE	**: MZU/Ph.D/1011 of 26.05.2017**

(Prof. H.T. LALREMSANGA)
Head
Department of Zoology
Mizoram University