ANTICANCER ACTIVITY OF STINGLESS BEE PROPOLIS ON HUMAN CANCER CELLS.

by

Hari Sagar S. K.

(2016-09-023)

THESIS

Submitted in partial fulfillment of the requirement for the degree of

B. Sc. - M. Sc. (INTEGRATED) BIOTECHNOLOGY

Faculty of Agriculture

Kerala Agricultural University, Thrissur

DEPARTMENT OF PLANT BIOTECHNOLOGY
COLLEGE OF AGRICULTURE
VELLAYANI, THIRUVANANTHAPURAM - 695 522
KERALA, INDIA 2022

DECLARATION

I hereby declare that the thesis entitled "Anticancer activity of stingless bee propolis on human cancer cells." is a bonafide record of research work done by me during the course of research and that the thesis has not previously formed the basis for the award of any degree, diploma, associateship, fellowship or other similar title, of any other University or Society.

Place: Vellayani

Date: 16/04/2022

Hari Sagar S. K.

(2016-09-023)

CERTIFICATE

Certified that this thesis entitled "Anticancer activity of stingless bee propolis on human cancer cells. "Is a record of research work done independently by **Mr. Hari Sagar S. K.** (2016-09-023) under my guidance and supervision and this has not previously formed the basis for the award of any degree, diploma, fellowship or associateship to her.

Place: Vellayani
Date: 16/04/2022

Dr. Shanas S.
(Chairman Advisory Committee)
Assistant Professor
(Agri. Entomology.)
IFSRS, Karamana
Thiruvananthapuram

CERTIFICATE

We, the undersigned members of the advisory committee of **Mr.HariSagar S. K.** (2016-09- 023) a candidate for the degree of B. Sc. -M. Sc. (Integrated) Biotechnology, agree that the thesis entitled **"Anticancer activity of stingless bee propolis on human cancer cells."** may be submitted by **Mr.HariSagar S. K.** in partial fulfillment of the requirement for the degree.

Dr. Shanas S.
(Chairman Advisory Committee)
Assistant Professor (Agri. Ento.)
IFSRS, Karamana
Thiruvananthapuram

Dr. K. B. Soni
(Member, Advisory Committee)
Professor and Head
Department of Plant Biotechnology
College of Agriculture, Vellayani
Thiruvananthapuram-695 522

Dr. Raghu Bhushan
Assistant Professor
Yenepoya Research Centre
Yenepoya Medical College
University Road Deralakatte
Mangalore – 575018 Karnataka

Dr. Swapna Alex
(Member, Advisory Committee)
Professor
Department of Plant Biotechnology
College of Agriculture, Vellayani,
Thiruvananthapuram-695 522

ACKNOWLEDGEMENT

At the foremost, I express my gratitude to ALMIGHTY GOD for being with me throughout each and every step that I have taken towards bringing the study into reality. I would also like to express my sincere gratitude to the people who helped me to complete the work.

I would like to express my deep and sincere gratitude to my supervisor Dr. Shanas S. Assistant Professor (Agri. Ento.), IFSRS, Karamana for giving me an opportunity to do research and providing invaluable guidance throughout this research. In addition to his support for completing the project, he gave me the confidence to describe things in the best and easiest way. His advice and suggestions were valuable for me in every aspect. It was such a different learning experience for me to be his student working with him.

I express my heartfelt gratitude and sincere thanks to Dr. Raghu Bhushan, Assistant Professor, Yenepoya Research Centre, Yenepoya Medical College, who was always there for me and for her valuable suggestions and affectionate encouragement throughout my research work.

I express my profound gratitude to Dr. Swapna Alex and Dr. K. B. Soni not only for their insightful comments and encouragement, but also for their valuable discussions, throughout support, intellectual advice, valuable guidance and critical suggestions they offered me related to the project.

I thank profusely all the lab members in Central University of Kerala, Kasargode and College of Agriculture, Vellayani especially Ms. Nija R. J., and Devika., PhD scholars at Central University of Kerala, for their help and cooperation throughout my work. Their research knowledge, continuous motivation have helped me to a very great extent to accomplish the task. I would like to express my sincere thanks and gratitude to my friends, Anaswara, Drishya, Tania, Malavika, Vishnu, Bharath, Gautham, Akshay and all classmates for their invaluable care, constant support and selfless help in the most difficult times.

Last but not the least, I express my profound gratitude to my parents Mr. HARI KUMAR.R and Mrs. SHEELA KUMARI.C for providing me unfailing support and continuous encouragement throughout my life. I acknowledge the favour of numerous

persons who, though not been individually mentioned here, who have all directly or indirectly contributed to this work.

Hari Sagar S. K.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF APPENDICES

LIST OF ABBREVIATIONS

%	Percentage
^{o}C	Degree Celsius
µg	Microgram
µl	Microlitre
ACTB	Beta actin
cm	centimeter
CAPE	Caffeic acid phenyl ester
DNA	Deoxyribonucleic acid
DMEM	Dulbecco's Modified Eagle's Medium
E8	Essential medium
et al.	*et alia*
EDTA	Ethylenediaminetetraacetic acid
g	gram
h	Hour
A549	adenocarcinomic human alveolar basal epithelial cells
IC50	Half maximal inhibitory concentration
kg	Kilogram
L	Litre
m	Meter
M	Molar
mg	milligram
min	Minute
ml	Millilitre

mM	millimolar
MTT	3- (4,5- dimethyl thiazol-2-yl)- 2,5- diphenyl tetrazolium bromide
OD	Optical density
PBS	Phosphate buffer Saline
qRTPCR	Quantitative real time polymerase chain reaction
RNA	Ribonucleic acid
rpm	rotations per minute

INTRODUCTION

1. INTRODUCTION

Stingless bee belongs to the Meliponinae, one of the three subfamilies of the family Apidae. There are over more than 600 species in Meliponini tribe that are found in different part of tropical regions all over the world; hence they are the world's largest and most diverse group of social insects (Popova *et al*., 2019). Propolis, commonly called as 'bee wax' is a complex mixture consist of plant derived compounds and bee – released compounds. It contains resin, pollen, essential oils, wax and other organic compounds. A lot of external factors such as geographic location, such as the season of the place, bee species, plant source, type and form (crude or purified) of propolis will affect the efficiency of propolis in practical usage (Farooqui *et al*., 2012). The chemistry of propolis depends on the plants that exists in the region inhabited by the bees and on the bee species. Hence the unique geography of Kerala being encroached upon by the Western Ghats provides a variety of propolis differing in chemical composition and medicinal values.

So far, over 300 chemical components have been identified in propolis. The biological activity of propolis is closely related to the presence of active compounds like flavonoids, phenolic acid, phenolic acid esters and terpenoids (Abdelrazeg *et al*., 2020). A plethora of studies shown that propolis extracts exhibit antibacterial, antifungal, antiviral, anti-inflammatory, antioxidant, anti-tumoral and tissue generation activities suggesting that this natural product could be a promising agent for the treatment and prevention of various diseases (Lavinas *et al*., 2019). Caffeic acid phenethyl ester (CAPE) is one of the major constituents of propolis with broad biological activities, including inhibition of cell growth and proliferation, inhibition of nuclear factor κ-B, induction of cell cycle arrest and apoptosis etc. (Huang *et al*., 2014).

There are several reports suggesting the anticancer activity of propolis. (Choudhari *et al*., 2013). Indian stingless bee propolis features a complex composition with 24 different chemical compounds. Propolis as extracted with the help of ethanol as solvent .The different concentration of ethanol has different uses. Ethanolic extract of propolis (EEP) is one of the richest sources of flavonoids and phenolic acids. Therefore the ethanolic extract increase the anti-cancer effect of propolis (Szliszka *et al.*, 2001). There are

various countries working on propolis and some of them are using propolis as a medicine also. India being a richest source of biodiversity also working for finding the effects of propolis in different diseases. In India there are different types of medicinal plants therefore the propolis obtained from these kinds of plants should have some medicinal properties.

There are different research groups across the world that are understanding the medicinal properties of propolis. India having a richest source of biodiversity, there are research questions undertaken to find the positive effects of propolis in curing different diseases.
Cancer is the second highest common disease in India and world leads to about 0.3 million deaths per year (Ali *et al*., 2011; Chandramohan *et al*., 2019). All types of cancers have been reported in Indian population including the cancers of skin, lungs, breast, rectum, stomach, prostate, liver, cervix, esophagus, bladder, blood, mouth etc. Propolis and its extracted compounds are found to show antitumor effects - cytotoxic to various tumor cell lines (Watanabe *et al.*, 2011). Various components of propolis have been found to inhibit the tumor cell growth. Red propolis polyphenols had displayed potent anti angiogenic properties by targeting the key steps in blood vessel development (Daleprane*et al*., 2013). Caffeic acid phenethyl ester (CAPE), a major component of propolis causes pronounced changes in breast cancer cell characteristics by inhibition of self-renewal, clonal expansion, decrease in CD44 content and potential reduction in malignancy (Wu *et al*., 2011). Strong reduction of tumor cell number (up to 80%) with propolis extracts at concentrations that are not toxic for patient, together with the fact that each tumor is unique, opens the question and possibility of unique personalized antitumor treatment with propolis extracts (Barbaric *et al.*, 2011). (Sawicka1 *et al*., 2012) studied the activity of propolis and its compounds like CAPE in the regulation of apoptotic process, and their influence on the proliferation of cancer cells. The study shows that propolis and its presented compounds induce apoptosis pathways in cancer cells. The anti-proliferative effects of compunds present in propolis like CAPE or Chrysin in cancer cells are the results of the suppression of complexes of cyclins, and leads to cell cycle arrest. The results of *in vitro* and *in vivo* studies suggest that propolis may inhibit tumor cell progression and be useful as potential chemotherapeutic and anticancer drugs (Choudhari *et al*., 2013).

The effect of stingless bee propolis is not very well understood. There are only few studies available about the effect of Indian stingless bee propolis on cancer cell. Hence the proposed work is expected to explore the effect of stingless bee propolis on cancer cells.

REVIEW OF LITERATURE

1. REVIEW OF LITERATURE

2.1 PROPOLIS

Stingless bees commonly called Meliponines are a large group of bees, which belongs to the tribe Meliponini that is widely occurring over the tropical and subtropical areas of the world (Velikova *et al*., 2000). They belong to the Apidae family, which is a family of social bees from the superfamily Apoidea (Al-Hatamleh *et al.,* 2020). The two major genera of stingless bees are genus Melipona and Trigona. They are closely related to the honeybees, bumblebees and orchid bees. Stingless bees are amongst the longest evolved bees that have been identified in 80 million years old parts of amber, estimated to have 400 to 500 different species but new species are identified every year (Kasote *et al.,* 2017).). Three new species of stingless bees *Tetragonula travancorica*, *Tetragonula calophyllae* and *Tetragonula perlucipinnae* were described from Kerala (Shanas and Faseeh, 2019).

Stingless bees use their head gland secretions, plant resins, wax, essential oils, pollen and exudates, including organic and inorganic earth components to produce propolis. It is one of the most fascinating bee products. The word propolis is derived from the Greek words pro meaning in defense, and polis meaning the city, that is defence of the city (or the hive) (Ghisalberti., 1979). Propolis is sticky at room temperature and is hard and brittle at low temperatures. They act as defensive substance and are used to build protective and supporting nest structures as well as honey pots (Massaro *et al.,* 2011).

Propolis is available in various forms such as toothpaste, tablets, capsules, face cream, ointments, mouthwash preparations, lotions, and solutions (Kartal*et al.,* 2003). Studies have already shown anticancer properties of propolis and its phenolic components by different mechanisms such as cell cycle arrest, induction of apoptosis (Sawichka*etal*., 2012), induction of mitochondrial stress (Benguedouar *et al* .,2008), inhibition of cancer cell proliferation and tumor growth (Chen *et al* .,2004). There are reports that suggesting the antioxidant properties of propolis samples using different chemical assays, such as DPPH radical scavenging assay (H. Izuta *et.al.,* 2009) and scavenging of superoxide anion

(Russo *et. al.* 2004). The activity of propolis depends on its chemical composition of propolis that mainly varies depending upon its origin, (Bankova *et al.*, 1989).

The color of propolis varies from yellow to dark brown based on the origin of the resin. Propolis is known for its antibacterial, antifungal, antiviral, anti-inflammatory, antioxidant, anti-tumoral and tissue generation activities (Bankova *et al.*, 2007; Popova *et al*., 2019). This type of propolis has long been used as traditional medicine in various countries like India, Mexico, Brazil, Argentina, and Vietnam, as a remedy for improving health and treating various diseases (Choudhari *et al*., 2012). In addition to this, commercial interest to propolis is growing rapidly, as it is used as a component of food additives, cosmetics and over-the-counter preparations. A lot of external factors such as geographic location, including the season and bee species, type and form (crude or purified) of propolis will affect the efficiency of propolis in practical usage (Farooqui *et al*., 2012). (Anjum, *et al.*2011) stated that chemical composition and pharmacological activities vary according to geographical and botanical origin of propolis, types of vegetable sources, time of collection and season of the year. India, being a vast country, has a number of different varieties of propolis varying in its chemical compositions and medicinal values (Wagh, 2013).

2.1.1 **Composition of Propolis**

The main components of propolis are oil and wax (30%-50%), resin (50%-70%), pollen (5%-10%) and there are other chemical compounds which include amino acids, minerals, sugars, vitamins B, C and E, flavonoids, phenol, as well as aromatic compounds which are discussed below (Wagh, 2013).

Resin

Resins are produced in the branches and leaves of trees. They are a sap like substance collected by the honeybees to protect their hive. It is used as a sealant, polisher and disinfectant. They are also used as a mummifier of dead insects in hives.

Wax

Wax is a highly absorbable material and is yellowish and soft. Fats contain acid, alcohol and esters. It is not resistant to heat and pressure but it is highly waterproof and stable.

Flower pollen

Flower pollen contain more than 96 nutrients. The exact composition of pollen depends upon the plant from which it is collected. Pollen is a rich source of vitamins, amino acids and minerals.

Phenols

Phenols are used in medicines as antiseptics. Phenols contain phenolic acids, tannins, flavonoids, coumarins and quinines .These compounds will provide anti-oxidant, ant mutagenic and anti-inflammatory properties of propolis.

Flavonoids

These are the important polyphenols in propolis which are helpful in finding the quality of propolis. Flavonoids are classified into different groups based on their chemical structure .They are flavanols, dihydrochalcones, isoflavans, flavonoid glycosides, neoflavanoids and isodihydroflavones. The composition and proportion of these types of substances varies and depends upon the place and time of collection.

Terpenes

Plants produce primary and secondary metabolites that have a wide range of functions. Primary metabolites include simple sugars, nucleic acids, amino acids and lipids. These primary metabolites are important for the cellular processes. Secondary metabolites are usually produced in response to stress and it includes alkaloids, terpenes and phenolic compounds. Among these terpene is an important compound and it helps in acting as a secondary messenger that helps in the expression of genes involved in defense mechanism of plants. Terpenes also have anti-fungal and anti-microbial effects.

Hydrocarbons

In recent years different types of hydrocarbons have been identified from propolis. They include alkane, alkene, di esters, fatty acids and steroids. Their composition may be different due to different geographical regions.

Minerals

Studies have shown that there are different minerals found in propolis. These are identified by atomic emission/ absorption spectroscopy. The different minerals present in propolis includes rare elements like magnesium, aluminum, carbon, manganese, nickel etc. and toxic elements like carbide, mercury, and lead.

Carbohydrates

There are not much details about the origin of carbohydrates like glucose and fructose in propolis. The main sources of glucose, fructose and sucrose are nectar and honey. The potential source of sugar in propolis includes resins that contain sugars, sugar alcohol etc.

Vitamins

Most researchers have shown that vitamins in propolis have different therapeutic properties. Different studies show that vitamins like B1, B2, E, C, and B 6 have been found in propolis. High-Performance Liquid Chromatography (HPLC) is used to detect the presence of Vitamins B1 (Thiamine) and B2 (Riboflavin) that is present in the propolis. Flower pollen is the main source of these vitamins.

2.1.2 **Bioactive components in propolis**

Isorhamnetin, Quercetin, Galangin, Apigenin, Pinobanksin, Kaempferol,,Islapinin, Ermanin, Pectolinarigenin, Sakuranetin, Isosakuranetin, Kaempferide, Rhamnazin, Quercetin- 3,3′-dimethyl ether, 3-acetyl pinoban pinobanksin, Betuletol, Rhamnetin, Alnusin, Alpinetin, Alnusitol, Pinostrobin, Pinocembrin, Chrysin, Tectochrysin, Acacetin, Rhamnocitrin, Rutin, Catechin, Luteolin, Naringenin (Walker and Crane, 1987).

Flavonoids, flavanones, flavones &flavonols:

Benzoic acid and derivatives:

Protocatechuic acid, Benzoic acid,,Trans-p-coumaryl benzoate, Salicylic acid, Gentisic acid, Gallic acid, Phenylmethyl ester of benzoic acid, Phenylmethyl ester of salicylic acid and Trans-coniferyl benzoate.(Walker and Crane, 1987)

Benzaldehyde derivatives:

Isovanillin p-hydroxybenzaldehyde, Vanillin, Caproic aldehydes and Protocatechualdehyde (Abdulkhani *et al*., 2017).

Cinnamyl alcohol, cinnamic acid & its derivatives:

Hydrocaeffic acid, Cinnamic acid methyl ester,Cinnamylidene acetic acid, Cinnamicacid,Cinnamic acid ethyl ester, Caffeicacid,Isoferulic acid, Cinnamyl alcohol andFerulic acid

Aliphatic hydrocorbons:

Pentacosane, Eicosane, Heneicosane, Eicosine, 1-octadecene and Tricosane.

Sugar:

D-ribofuranose, d-fructose, d-glucitol, d-gulose, Talose, Sucrose, d-glucose

Vitamins:

B1, B2 (complex), B6, C, E (Kuropatnicki*et al*., 2013)

Chalcones & dihydrochalcones

Alpinetinchalcone, Naringinenchalcone, Pinobanksinchalcones, Pinobanksin-3-acetate chalcone, Pinostrobinchalcone, Pinocembrin chalcones, Sakuranetinchalcone, 2′,6′,a-trihydroxy-4′-methoxy chalcone, 2′,6,dihydroxy-4′-methoxydihydro chalcone, 2′,4′,6-trihydroxydihydro chalcone (Marcucci, 1995)

Amino acids:

Alanine, β-alanine, α-amino butyric acid, δ-amino butyric acid, Arginine, Asparagine, Aspartic acid, Cystine, Cysteine, Glutamic acid, Glycine, Histidine, Hydroxyproline, Isoleucine, Leucine, Lysine, Methionine, Ornithine, Phenylalanine, Proline, Pyroglutamic acid, Sarcosine, Serine, Threonine, Tryptophane, Tyrosine, Valine

Esters:

Methyl palmitate, Cinnamyl-*trans*-4- coumarate, Ethyl palmitate, Stearic acid methyl ester, Phthalate ester, Benzyl benzoate, Benzyl-*trans*-4- coumarate, 3-Methyl-3-butenyl isoferulate, 3-Methyl-2-butenyl isoferulate, 3-Methyl-3-butenyl caffeate, 2-Methyl-2-butenyl caffeate, 3-Methyl-2-butenyl caffeate, Benzyl caffeate, Phenylethylcaffeate, Cinnamylcaffeate, Tetradecylcaffeate, Tetradecenylcaffeate, Tetradecenylcaffeate (isomer), Tetradecanylcaffeate, Hexadecylcaffeate (El Hady and Hegazi, 2002)

Other acids and derivatives

Ethyl ester of palmitic acid, Myristic acid, Sorbic acid, Butyl-2-methylpropyle ester of Phthalic acid, Phenylmethyl ester of 14- methylpentadecanoic acid, Stearic acid and Methyl ester of alnustic acid

Alcohol, ketones, phenols and heteroaromatic compounds:

Coumarine, Pterostilbene, Xanthorrhoeol, Scopoletol, Benzyl alcohol and Hexadecanol acetate.

Terpene, Sesquiterpene, alcohol & derivatives:

Geraniol, Neroledol, β-bisabolol, Guaiol, Farnisol, Dihydroeudesmol, α-acetoxybetulenol

Sesquiterpene& Triterpene hydrocorbons:

B-patchoulene, β-bisabolene, Caryophyllene, Patchoulane, Selenene, Aromadendrene, Squalene, β-bourbonene, Copaene, Calarene and Calamenene,

Sterols & steroid hydrocarbons:

Cholestrilene, Cholinasterol, Stigmasterol, β-dihydrofucosterol, Lanosterol, Cholesterol

Enzymes:

Glucose-6-phosphatase, Acid phosphatase, Adenosine triphosphatase, Succinic dehydrogenase

Waxy acids:

Lauric acid, Linoleic acid, Lignoceric acid, Montanic acid, archid acid, Behenic acid and Cerotic acid.

Aliphatic acids & aliphatic esters:

Isopentinyl acetate, acetic acid, Angelic acid, Butyric acid ,methylbutyric acid, Isobutyl acetate, Crotonicacid,Fumaric acid, Isopentyl acetate, andIsobutyric acid.

Aliphatic acids:

Lactic acid, Hydroxyacetic acid, Malic acid, 5-Hydroxy-n-valeric acid, 2,3-Dihydroxypropanoic acid, Pentonic acid- 2-deoxy-3,5-dihydroxy-γ-lactone, Pentonic acid- 2-deoxy-3,5- dihydroxy-γ-lactone (isomer), Succinic acid, 2,3,4,5-Tetrahydroxypentanoic acid- 1,4-lactone, 2,3,4,5-Tetrahydroxypentanoic acid- 1,4-lactone(isomer), Nonanoic acid, Palmitic acid, Oleic acid, Decanoic acid, Dodecanoic acid, Tetradecanoic acid, Heptadecanoic acid, Octadecenoic acid, Tetracosanoic acid, Eicosanoic acid, Hexacosanoic acid, 2- Hydroxyhexacosanoic acid (Hady and Hegazi, 2002)

Other compounds:

1,2,4-trihydroxy butane, 1,2,3-trihydroxy butanal, 1,2,3-trihydroxy butanal (isomer), Myristicin, 2,4-bis(dimethyl benzyl)-6-Phosphoric acid, 1,4-Dihydroxy benzene, 4 -Hydroxy-benzaldehyde,4-Hydroryacetophenone,t- butyl phenol, 1,8-dihydroxy-3-methyl anthraquinone, Myristicin (isomer).

2.2 **BEE PRODUCTS AND MEDICINAL PROPERTIES**

Honey has antibacterial, antifungal, antiviral, ant oxidative, probiotic, anti-inflammatory, ant carcinogenic properties. Pollen has antibacterial, antifungal, ant oxidative, immunomodulation, radio protective, antianaemic, ant atherosclerosis, antidiarrheal and it also helps to prevent osteoporosis (Ekeuku *et al*,2021) The Royal jelly has antibacterial, antifungal, antiviral, ant oxidative, bio stimulating, immunomodulation, radio protective, ant carcinogenic, ant fatigue, ant stress, ant hypoxia, antitumor, anti-inflammatory, both antihypertensive,anti-hypotensive, vasodilatative, atherosclerosis, cardio-protective, tranquilizing, neuroprotective, anti-osteoporosis activities(Fratini et al,2016) The Propolis has antibacterial, antifungal, antiviral, ant oxidative, ant parasitic, immunomodulation, anti-inflammatory, analgesic, hepatoprotective, ant carcinogenic, local anesthetic, pain soothing, spasmolytic, hepatodetoxiquant and hepatoprotecting, anticoagulant, improving blood circulation(Zulhendri *et al*,2021) The Bee venom has antibacterial, anti-inflammatory, immunoactivity, immunosuppressive, analgesic, radio protective, ant carcinogenic, accelerates heartbeat, increases blood circulation, lowers blood pressure, improves hemoglobin synthesis, anticoagulant, lowers cholesterol levels, membrane effects on blood cells, influences immuno-active blood cells and hormone levels, antiarrhythmic, heart stimulating therapeutic effects, improvement in hypertonia and atherosclerosis. Propolis is very widely used in dentistry (Oliviera and Murgo, 2006). Propolis inhibits in the mouth different pathogenic microbes such as bacteria, fungi and viruses (Hayacibara et al., 2005) (Santos et al., 2003; Sauvager, 1992; Silici et al., 2007). Studies have shown that propolis-containing local therapy can eradicate HPV infections. Propolis can also be used in pediatric diseases, radiculitis, polyradiculoneuritis, gastric ulcers and baldness.

2.3 EXTRACTION OF PROPOLIS

Various methods have been used for the extraction of active components from propolis such as maceration, soxhlet, ultrasonic (sonication), and microwave extraction. Among these different extraction methods, many experiments were carried out to identify which method yielded better propolis quality. One of the studies indicated that purification process through centrifugation, filtering and vacuum induces improvement of propolis quality by increasing its antioxidant activity, increasing its total phenolic and flavonoid

content (Graikini *et al*, 2019). The findings also suggested that unlike methanol, is a non-toxic solvent (food grade), ethanol extraction was more effective. Hence, most ethanol extraction rather than methanol, is used in the experimental process and commercial products. Moreover, most efficient method of improving propolis quality, is purification using centrifugation because the wax is easily removed as a uniform mass after agglomeration during the process. Therefore,it is concluded that purification of propolis through centrifugation is an essential procedure that should be applied both for experimental and commercial purposes as it can increase antioxidant activity, flavonoid content and total polyphenolic content consequently the value of propolis as a medicine (Graikini et al., 2019). Another study also indicated that ethanolic extraction produced a higher yield values than other solvents such as propylene glycol, distilled water, VCO and olive oil. The propolis produced from solvent extraction with ethanol was solid sticky property. The 70% ethanol was found to extract most of the active components of propolis as it dissolves well in ethanol but not beeswax does not get dissolved in ethanol and gets settled down as sediment (Pujirahayu et al., 2019). Basyirah in 2018 demonstrated that the percentages of yield depended on method of extraction, solvent used to extract, time, temperature (Basyirah et al., 2018).

2.4 PROPOLIS FOR CANCER TREATMENT

Cancer is one of the major public health problems worldwide. Cancer being more than a single-cause disease, it is a group of related diseases that are related to hereditary, environmental, inflammation and other factors (Wang *et al*., 2017). It is caused by a series of successive gene mutations which change the cell functions. It is also appreciated as a disease of change - a condition which is characterized by plasticity and heterogeneity that evolves at phenotypic, genetic, and pathological levels, and progresses through different stages clinically (Yoo *et al*., 2003). Chemical compounds play an obvious role of forming gene mutations and cancer cells. In addition, smoking is also one of the major factors that lead to lung cancer as it includes several carcinogenic chemical compounds (Poon *et al*., 2014). Interestingly, environmental chemical substances which have carcinogenic properties also influence directly or indirectly the cytoplasm and nucleus of cells, and lead to genetic disorders and gene mutations. Viruses, bacteria and radiation rays are other carcinogenesis factors that also lead to cancer.

Among various diseases, cancer has become a big threat to human beings globally (Singh *et al*., 2018). It is the second leading cause of death globally, and is responsible for an estimated 9.6 million deaths in 2018 (Siegel *et al*., 2019; Mattiuzzi *et al*., 2019). According to WHO, globally about 1 in 6 deaths occur due to cancer. It is also the second most common disease in India, responsible for maximum mortality with about 0.3 million deaths per year (Ali *et al*., 2011). All types of cancers have been reported in Indian population including the cancers of skin, lungs, breast, rectum, stomach, prostate, liver, cervix, oesophagus, bladder, blood, mouth etc. (Chandramohan *et al*., 2019). Hence, cancer has become one of the serious problems affecting the health of all human societies. Unfortunately, it is a type of disease at the tissue level and this is a major challenge for its specific diagnosis, followed by efficacy of treatment (Hassanpour. 2017). Moreover, metastasis of cancer is the major cause of morbidity and mortality rate (Guan. 2015). Hence, understanding the unequal global cancer profile enables us to explore the etiology and potential high-risk factors of cancer and to work out strategies for better cancer prevention, early detection and treatment (Cai *et al*., 2019).

Researchers has reported that bee products have the ability to inhibit cancer by inhibiting tumour growth, metastasis and induce apoptosis (Tamura *et. al*., 1985). These studies suggest that bee products (or their active components) could be used in the treatment of cancer (Yusuf *et. al.*, 2007). They are studies which shows that Ethanolic extracts of propolis (EEP) are rich in phenols and flavonoids. Natural phenolic compounds are able to scavenge free radicles; therefore, they may exert chemo protective activity in cancer (Olivera *et al*., 2015).

For over many years, natural products have been served well in combating cancer (Nobili *et al*., 2009; Demain *et al*., 2010). There are numerous medications available to treat different types of cancer, however, there is no drug that is totally effective and safe. Among the available treatments such as chemotherapy, radiotherapy, surgery etc, main restrictions of chemotherapy is its toxicity on the healthy and normal cells of the organism, with severe side effects such as such as oral mucositis, gastrointestinal toxicity, hepatotoxicity, nephrotoxicity, hematopoietic system injury, cardiotoxicity, and neurotoxicity, fatiche, anemia, hair loss, easy bruising and bleeding (Prakash *et al*., 2013; Zhang *et al*., 2018). Moreover, when chemo- and radio-therapy are used systemically or

over a broad directed tissue area to kill cancerous cells, they also typically harm healthy cells in the process causing undesirable side effects that limit the treatment (duration and/or dose) and effectiveness, or in the worst cases can kill the patient faster than the cancer would have done. Metastasis and tumor growth are the major reasons of increase in death rate of cancer patients and despite of the diversity in the availability of chemotherapeutic drugs, the adverse effects, regressions and failures in therapeutic responses still persist (Majolo, *et al.* 2019). The current cancer treatment protocol shows many side effects and is now becoming ineffective due to the development of resistance (Desai *et al*., 2008; Lakshmi *et al*., 2015). So, it is of fundamental importance to find a solution to control both the onset and progress of cancer. Accordingly, research for alternative anticancer drugs has become a popular topic, especially for natural products. Natural products are viewed as generally being much safer than the existing treatments.

Propolis and its extracted compounds are found to be cytotoxic to various tumor cell lines. Various components of propolis have been found to inhibit the tumor cell growth. Propolis have active compounds such as CAPE and chrysin, which are mainly responsible for the antitumor therapeutic activities of propolis. Its inhibitory effects in cancer cells have been confirmed (Sawicka *et al*., 2012). Moreover some phenolic compounds and diterpenoids isolated from propolis also have shown potential antitumorgenic properties (Banskota *et al*., 2001). Many studies have indicated that different types of propolis extracts significantly showed potential to inhibit cell growth and reduce the differentiation or proliferation of tumor cells. Apoptosis is also initiated by propolis. The mechanism of propolis-induced apoptosis is found to be independent of the type of cancer cells studied, but dependent on the concentration and amount of propolis extract used (Szliszka *et al.*, 2011). Several studies have demonstrated that propolis induces apoptosis by the caspase cascade and TRIAL (Tumor necrosis factor-related apoptosis inducing ligand) signals (Lirdprapamongkol *et al*., 2013). The antioxidant activity of propolis is also associated with its anticancer activity. Hence these studies suggest the potential application of the use of these natural compounds as part of an alternative medical treatment of human tumors (Premratanachai *et al*., 2014).

Various studies have been carried out to investigate and understand the mechanism of action of propolis extracts on cancer cells. Choudhari *et al.* (2013) investigated the anti-cancer activity of different concentrations of ethanolic extract of propolis (EEP) by

testing the cytotoxic and apoptotic effect in four different cancer cell lines such as HT-29 (human colon adenocarcinoma), MCF-7 (human breast cancer), B16F1 (murine melanoma) and Caco-2 (human epithelial colorectal adenocarcinoma). Their results showed ≥50% mortality in all cell lines tested (i.e., IC50 value) and the extracts exhibited a time dependent and concentration dependent cytotoxic effect (Choudhari *et al.,* 2013). It was reported that propolis effectively inhibits the proliferation of U937 cells in a dose-dependent manner by inducing apoptosis and blocking cell cycle progression in the G2/M phase. In the study it was revealed that propolis causes a significant reduction in the expression of cyclin A and cyclin B of U937 cells and also decreases the expression of Cdc2 causing cell cycle arrest. In addition to this, propolis extracts also increased the expression of p21 and p27. Moreover, the down-regulation of anti-apoptotic protein Bcl-2 and selective activation of caspase-3 was found to mediate apoptosis in U937 cells indicating that propolis can be effectively used as a cancer preventive agent (Motomura *et al*., 2008).

Ethanolic extracts of Chinese and Brazilian propolis from *Trigonalaeviceps* demonstrated anti-proliferative activities on human colon carcinoma cell lines such as CaCo2, HCT116, HT29 and SW480 (Ishihara *et al*., 2009) and it was found that the inhibitory effects of Chinese propolis extracts exhibited stronger growth inhibitory effects than that of Brazilian propolis. This indicated that biological activities and number of active components of propolis depend on the phyto-geographical factors such as plant sources, specific location of plant distribution, and seasonal time periods. Among the different compounds identified in propolis samples, caffeic acid phenethyl ester (CAPE) shows significant role in the inhibition of the growth and induce G1-phase arrest and apoptosis by decreasing mRNA and protein expression of cyclin D1 and c-myc.

Red propolis polyphenols had displayed potent anti-angiogenic properties by targeting the key steps in blood vessel development (Daleprane *et al*., 2013).CAPE, a major component of propolis inhibited growth of human breast cancer cells - MDA-231 and MCF-7, due to its cytotoxicity in tumor but not normal cells remained unaffected and also CAPE induced cell cycle arrest, apoptosis and suppressed VEGF formation (Wu *et al*., 2011). CAPE is also found to exhibit anti-angiogenic activities in the chick embryo chorioallantoic membrane (CAM) (Song *et al*., 2002). Strong reduction of tumor cell

number (up to 80%) with propolis extracts at concentrations that are not toxic for the patient, together with the fact that each tumor is unique, opens the question and possibility of unique personalized antitumor treatment with propolis extracts (Barbaric *et al.*, 2011). Supplementation of ethanolic extract of propolis induced promising anti-tumor response in mice model of differentiated type gastric adenocarcinoma and also the models also showed a upregulation of CDKN1A, which encodes for the p21 protein, accompanied by significant down-regulation in the levels of CCND1, CDK1, and CDK2 genes encoding for the Cyclin D1, Cdk1, and Cdk2 proteins respectively. (Desamero *et al.*, 2019). Propolis nanofood is found to inhibit pancreatic cell growth in murine xenograft models by a potent anti-angiogenic response (Kim *et al*., 2008). The methanolic extract of the Netherlands propolis exhibited antiproliferative activity towards liver-metastatic murine colon 26-L5 carcinoma and also indicated that the caffeates in the propolis as a whole contribute to the antiproliferative effects (Banskota *et al*., 2002). One of the studies conducted using Indian bee propolis revealed that the ethanolic extract of propolis exhibited the most effective and promising result against human breast cancer (MCF - 7), colon cancer (HCT 116), and prostate cancer (PC 3) cell lines. The inhibitory effects varied upon the propolis origin suggesting that these effects are attributed to the chemical composition of propolis, which is highly dependent on the geographical location of the flora (Shubharani *et al.*, 2014). Propolis-induced apoptosis may be via the activation of caspase-8 and caspase-9, and the activation of caspase-6, leading to DNA fragmentation, and finally apoptosis and also extracts exhibited a dose-dependent inhibition of cellular growth and activation of apoptosis in the MCF-7 cell line (Seda *et al*., 2010). A study using Turkish ethanolic extract of propolis (EEP) demonstrated that the extract exhibited powerful antiproliferative effects against all studied human cancer cell lines investigated such as Liver, Colon, Breast, Cervix and Prostate Cancer Cell Lines (Turan *et al.*, 2015). Artepillin C (ARC), an active component of Brazilian green propolis, is found to activate p53 tumor suppressor protein by abrogating its complex with mortalin, a p53 inactivating protein, thereby possesses anticancer activity (Bhargava *et al*., 2018).

Radiation has tremendous therapeutic benefits for humans; it is the most common modality for treating human cancers (Paul *et al*., 2011). However, it is associated with the risk of serious adverse effects. Radiotherapy destroys cells both normal and malignant

cells in the area exposed to the radiation by damaging their genetic material, such that patients may experience harmful symptoms during the course of therapy for a few weeks after therapy or months or years later. Radioprotectors are certain compound such as SH compounds (cysteine and WR-2721 (amifostine)) which are designed to reduce the damage in normal tissue due to the radiation. These compounds are generally antioxidants because of their ability to direct free radicals scavenging or stabilizing the reactive oxygen species (ROS) by interacting with the reactive compound of the radical and these compounds are generally given along with radiation (at the time or before) for their effectiveness (Maurya *et al*., 2006; Benkovic *et al*., 2008). Radiotherapy is the most common method for treating human cancers. Several clinical approaches have been made to reduce the early and late complications of the radiotherapies and one of the most common among them is the use of an effective and non-toxic radio-protector. Radio-protectors are those compounds that are developed to reduce the damage in normal tissue caused by radiation. These compounds are usually antioxidants and must be present before or at the time of radiation for their effectiveness (Maurya *et al*., 2006). But sometimes the use of these substance leads to the harmful effects, hence in order to overcome the harmful effects of synthetic compounds, many naturally occurring substances have been studied as candidates for effective radioprotection (Kim *et al*., 2006). Supplementation of propolis with radiotherapy treatment offers a quite measurable protection against DNA damage caused by ionizing radiation in BC patient's leukocytes and inhibits RRM2 overexpression. Moreover, propolis has beneficial effects on the serum antioxidant capacity and improves the digestive utilization of iron and the regeneration efficiency of hemoglobin.

Ethanolic extracts of Chinese and Brazilian propolis from *Trigona laeviceps* demonstrated anti-proliferative activities on human colon carcinoma cell lines such as CaCo2, HCT116, HT29 and SW480 (Ishihara *et al*., 2009) and it was found that the inhibitory effects of Chinese propolis extracts exhibited stronger growth inhibitory effects than that of Brazilian propolis. This indicated that biological activities and amount of active components of propolis depend on the phyto-geographical factors such as plant sources, specific location of plant distribution, and seasonal time periods. Among the different compounds identified in propolis samples, caffeic acid phenethyl ester (CAPE)

shows significant role in the inhibition of the growth and induce G1-phase arrest and apoptosis by decreasing mRNA and protein expression of cyclin D1 and c-myc.

Several studies suggests that propolis possess promising radioprotective effect and is possible to offer measurable protection against DNA damage. Flavonoids present in propolis extract can also increase the function of the endogenous antioxidant enzyme systems such as superoxide dismutase, glutathione peroxidase, catalase, and glutathione reductase. In addition, propolis also boosted immune activity. It was found that treatment with propolis extracts increased the number of cytotoxic T-cells and suppressor T-cells, which likely involved an increase in INF-γ levels. This increase in IFN-γ levels, activates cell-mediated immune responses, such as proliferation and activation of type I helper T (Th1) cells (Takagi *et al.*, 2005). Moreover, when a group of patients received propolis treatment plus radiotherapy showed a significant improvement of Hb concentration, WBCs count, and platelets count and increase in serum iron (Benkovic *et al*., 2009). It also enhanced the proliferation of hematopoietic cells in the bone marrow and spleen. Hence supplementation of propolis along with radiotherapy treatment offers a considerable protection against DNA damage caused by ionizing radiation. Moreover, propolis has beneficial effects on the serum antioxidant capacity and improves the digestive utilization of iron and the regeneration efficiency of hemoglobin. (Ebeid *et al*., 2016)

2.5 GENE EXPRESSION PROFILE OF A549 CELLS

β-Actin, a cytoskeletal protein is one of the most widely used housekeeping genes on the basis their consistency of expression (Glare *et al*., 2002). ACTB (Actin beta) are highly conserved proteins that are involved in cell motility, structure, integrity, and intercellular signaling. Housekeeping genes are genes expressed constitutively in every cell. Housekeeping genes are used in molecular biology assays based on the assumption that their levels of expression remain the same from cell to cell, sample to sample, treatment to treatment.

NODAL (Nodal Growth Differentiation Factor) is a Protein Coding gene. Nodal is a TGF-beta related embryonic morphogen that is expressed in multiple human cancers. Nodal was shown to be expressed in human breast carcinoma cell lines, but poorly

expressed in normal mammary epithelial and myoepithelial cells. In cancer cells NODAL promotes EMT formation. It is normally expressed during embryogenesis, and promotes mesendoderm specification and left-right asymmetry. However, its re-expression induces increased aggressiveness and tumorigenicity in cancer cells in melanoma, glioma, and prostate cancer.Nodal predominantly binds to activin-like kinase type II (ActRIIB) and type I (ALK4/7) receptors, which leads to phosphorylation of ALK4/7. Activation of the receptors promotes intracellular phosphorylation of Smad2/3, which then interacts with SMAD4, followed by translocation to the nucleus, thereby regulating target genes. Nodal expression is correlated with tumor progression, poor prognosis and angiogenesis .Moreover, recent studies have shown that Nodal may regulate breast cancer progression and metastasis. Nodal signaling promotes tumorigenicity.

CDX2 is a Drosophila caudal-related homeobox transcription factor that is important for the establishment and maintenance of intestinal epithelial cells.CDX2 expression is confined to the intestinal epithelium and has been shown to regulate intestinal gene transcription, differentiation, and proliferation. In human colon cancer cell lines that express low levels of CDX2, in vitro overexpression of CDX2 reduced proliferation, increased compaction and differentiation, and significantly altered the transcription of genes regulating these processes. In lung cancer cells, restored CDX2 expression suppressed cell proliferation by arresting the cell G1/S transition.CDX2 inhibits the proliferation of colon cancer cells by suppressing Wnt signaling activity. In pancreatic cancer cells, CDX2 inhibits cell proliferation by directly repressing cyclin D1 transcriptional activity.CDX2 might regulate PTEN, an important tumor suppressor, thereby inhibiting the gastric cancer aggressive biological phenotype via PI3K/Akt pathway.

VIM gene can be used as a biomarker for the early detection of several cancers.VIM gene is transcriptionally inactive in normal colon epithelial cells. VIM gene encodes vimentin, a member of the intermediate filament family that is especially found in connective tissue. Vimentin is known to be involved in various biological processes including maintaining cell shape and stabilizing cytoskeletal interactions. Viemntin also plays vital roles in cell adhesion, migration, and signaling. When overexpressed in solid cancers, vimentin drives epithelial to mesenchymal transition (EMT) and ultimately, metastasis. Increased vimentin expression has been reported in various epithelial cancers including prostate

cancer, gastrointestinal tumors, CNS tumors, breast cancer, malignant melanoma, lung cancer and other types of cancers. Increased vimentin expression has been reported in various epithelial cancers including prostate cancer, gastrointestinal tumors, CNS tumors, breast cancer, malignant melanoma, lung cancer and other types of cancers. Although its expression is more emphasized in the EMT process, it is equally possible that tumorigenic events including tumor cell migration and invasion are a consequence of vimentin over-expression in these cells.

DUSP6 is a negative regulator of the ERK signaling pathway and plays an important role in chemotherapy-resistance.DUSP6 is a member of a subfamily of protein tyrosine phosphatases known as dual-specificity phosphatases (DUSPs), which dephosphorylates extracellular signal-regulated protein kinase 1/2 (ERK1/2) to negatively regulate ERK signaling. Through its regulation of ERK signaling it modulates cell proliferation, differentiation and apoptosis. Many studies have confirmed a role for DUSP6 in the negative regulation of ERK signaling pathway and the reduction in cellular proliferation rates.DUSP6 plays an important role in chemotherapy- resistance by causing cellular quiescence through its regulation of the ERK signaling pathway.DUSP6 regulates ERK1/2 activity during the cell cycle, which leads to G0/G1 arrest and chemotherapy-resistance.

MATERIALS AND METHODS

3. MATERIALS AND METHODS

The study entitled **"**Anticancer activity of stingless bee propolis on human cancer cells" was conducted at the Department of Plant Biotechnology, College of Agricultutre, Vellayani, Thiruvananthapuram and at Department of Genomic Science, Central University of Kerala, Kasaragod, during October 2020 – June 2021. Details relating to the experimental materials and methodology adopted for the study are detailed in this chapter.

3.1 PROPOLIS INFORMATION

The propolis sample from following species of stingless bees were collected and designated as P1, P2 and P3.

- P1- *Lisotrigona sp.*
- P2- *Tetragonula calophyllae*
- P3- *Tetragonula travancorica*

3.3 EXTRACTION OF PROPOLIS

The protocol developed by Devequi-Nunes *et al.,* (2018) was followed. The ethanolic extracts of propolis were made by adding 10 mL of ethanol (95%) to 3 g of crushed and homogenized propolis. The extraction was performed at 70°C for 30 minutes under constant agitation in a Shaker incubator, at 710-rpm. After that step, the extract was then centrifuged at 8800 rpm at 5°C for 10 min and the supernatant was transferred to glass test tubes (15x160 mm). 10 mL ethanol (90%) was added to the residue in the centrifuge tube, and the centrifugation was repeated. All the extracts were maintained at a temperature of 5°C to avoid degradation.

3.4 REAGENTS

DMEM, DPBS, Trypsin-EDTA (0.25%), phenol, 10% FBS, L-Glutamine (200mM), Penicillin-Streptomycin.

3.5 CELL LINE INFORMATION

A549 Cell (Adenocarcinomic human alveolar basal epithelial cells)

3.6 CELL CULTURE AND MAINTENANCE

3.6.1 Plating-A549 Cells.

A549 cells were plated in to 96 well plated once the cells reached 75% confluence. Splited the cells after DPBS wash and added Trypsin-EDTA for the detachment and kept in 5% CO2 incubator for 30 seconds. Check under the microscope to confirm all cells detached from the surface. Then, add complete DMEM media to stop trypsinization immediately. Mix it thoroughly and transfer to a 15 ml centrifugation tube and spin at 15000rpm for 3 minutes at room temperature. Remove the supernatant and re-suspend the pellet in 1 ml media. Count the cells and seed 5000 cells per well of 96 well plate and incubate at 37ºC for 24 hours in a humidified atmosphere of 5% CO2 incubator for 2 days to reach 80% confluence.

- **Cell Counting:**

For cell counting 10 µl of the cell suspension were transferred into a 0.5 ml microfuge tube.10 µl of 0.4% trypan blue solution was added to the cell suspension (creating a dilution factor of 2) in the centrifuge tube .Then mixed thoroughly .With a cover-slip in place, transferred 10 µl of the trypan blue-cell suspension to a chamber on the hemocytometer (by carefully touching the edge of the cover-slip with the pipette tip and allowing the chamber to fill by capillary action).Then Counted the viable cells (non-viable cells stain blue, viable cells will remain opaque) in the 1mm center square and the four corner squares. Calculated the total number of cells per ml

Cells per ml = the average count per square X the dilution factor X 10^4

$= (563/4) \times 2 \times 10^4$

$= 281.5 \times 10^4$

Seeding Density = 5000 cells per well of 96 well plate

Cells taken per well = $5000 / 281.5 \times 10^4 = 2$ µl

Therefore, 2µl of A549 cells to each well of 96 well plate, which means 5000 cells of A549 per well.

- **Determination of the cytotoxicity of propolis by MTT assay**

After 2 days cells become 80 percentage confluent**,** then started the treatments with 3 different propolis like P1, P2 and P3 of different concentrations like 150, 300, 450, 600 and 900µg/ml. Also kept appropriate control which was treated the cells with 95% ethanol and also kept cell alone for blank correction (after MTT assay). Incubated the plate at 37°C up to 24 hours in a humidified at 5% CO2 atmosphere.

- **MTT Assay**

After 24 hour treatments, then started MTT assay using CellTiter 96® Non-Radioactive Cell Proliferation Assay (MTT) kit (Promega- G4000). Added 15µl of the Dye Solution to each well. Incubated the plate at 37°C for up to 2 hours 15 minutes in a humidified, 5% CO2 atmosphere. After incubation, added 100µl of the Solubilization Solution/Stop Mix to each well. One hour after addition of the Solubilization Solution/Stop Mix, the contents of the wells may be mixed to get a uniformly colored solution. However, care should be taken to avoid bubble formation. Bubbles on the surface may interfere with the accurate recording of absorbance values. Recorded the absorbance at 570nm wavelength using a 96-well plate reader (EnSpire Multimode Plate Reader). The use of a reference wavelength will reduce background contributed by cell debris, fingerprints and other nonspecific absorbance. Reference wavelength of 630nm was used. Recorded all the absorbance values and calculated the cell viability and IC-50 of each propolis used for treatments.

3.6.2 Cell plating for treatments (A549/P10)

Plating is done when the cells reach 75 % confluence (T75 flask), remove the media and wash the cells with DPBS to remove unwanted debris. Aspirate it and add 2.5 ml Trypsin-EDTA and incubate for 30 seconds at 37ºC. After incubation, check under the microscope to verify the cells got detached from the adherent surface. Once it detached, add 3 ml of DMEM complete media (complete growth medium) and pipette up and down. Then collect the cells in a 15 ml centrifugation tube by gently pipetting and spin at 12,000 rpm for 3 minutes. Remove the supernatant and re-

suspend the pellet in 1 ml DMEM complete media. Plate 65000 cells per each well of whole 6 well plate and one 3 cm dish after cell counting using hemocytometer.

- **Cell Treatments – Propolis (A549/P10)**

Cells were maintained on DMEM complete media till the confluence becomes 50-60%. The media is aspirated and we have given the treatments of propolis (P1, P2 and P3) of concentrations that we have obtained from MTT assay (IC50 value of P1, P2 and P3). Controls were taken as the untreated ones and ethanol treated. After 24 hours, harvested the cells and taken the RNA lysates for further processing after taking all the images by inverted microscope (Lawrence and Mayo phase contrast inverted microscopy). RNA lysates were stored in -80℃ for further studies. And for checking, we have kept the cells for 48 hours after treatments, but all the cells got detached and dead.

3.6.3 RNA isolation of propolis treated cells.

QIAzol Lysis Reagent, is a monophasic solution of phenol and guanidine thiocyanate, which facilitated the lysis of cells, inhibition of RNases, and the removal of the cellular DNA and proteins from the lysate by organic extraction. The addition of chloroform into the lysate leads to the separation of homogenate into upper - aqueous phase (RNA), interphase contains DNA and proteins to the lower by centrifugation. The upper, aqueous phase is extracted, and ethanol is added to provide appropriate binding conditions for all RNA molecules from 18 nucleotides (nt) and upwards.

1. Using at least 10^6 cells, aspirate the media and washed once with 1 ml of DPBS.
2. Aspirated the DPBS and added 1 ml QIAzol.
3. Scraped the plate briefly, then mixed well by pipetting up and down using a 1 ml pipette and collected the cell lysate into a 1.5 ml centrifuge tube.
4. Placed the tube containing the homogenate on the benchtop at room temperature (15–25°C) for 5 min.
5. Added 0.2ml chloroform per 1 ml QIAzol Lysis Reagent pipetted in step 1. Securely caped the tube containing the homogenate, and shaked it vigorously for 15 s.

6. Placed the tube containing the homogenate on the benchtop at room temperature for 2–3 min.
7. Centrifuged at 12,000 x g for 30 min at 4°C.
8. Transferred the upper, aqueous phase to a new tube. Be careful to avoid the interphase. Add 0.5 ml isopropanol per 1 ml QIAzol Lysis Reagent pipetted in step 1. Mixed thoroughly by vortexing.
9. Placed the tube on the benchtop at room temperature for 10 min.
10. Centrifuged at 12,000 x g for 10 min at 4°C.
11. Carefully aspirated and discarded the supernatant.
12. Added at least 1 ml of 75% ethanol per 1 ml QIAzol Lysis Reagent pipetted in step 1. Centrifuged at 7500 x g for 5 min at 4°C.
13. Removed the supernatant completely, and briefly dry the RNA pellet at 55°C (in drybath).
14. Redissolved the RNA in an appropriate volume of RNase-free water.

3.6.4 cDNA Synthesis

Reverse transcription protocol (iScript™cDNA Synthesis Kit, Cat# 1708891)

The RNA isolated from control and treated samples were subjected to cDNA conversion.

Table 1. Reaction setup for cDNA synthesis

Component	**Volume per reaction, µl**
5x iScript reaction mix	4.0
iScript reverse transcriptase	1.0
Nuclease-free water	Calculate according to the amount of RNA template
RNA template (100 fg – 1 µg total RNA)*	Calculate according to the concentration of RNA
Total volume	**20 µl**

Table 2. Reaction protocol for cDNA synthesis

Incubate the complete reaction mix in a thermal cycler,

Priming	5 min at 25°C
Reverse transcription	20 min at 46°C
RT inactivation	1 min at 95°C
Optional step	Hold at 4°C

3.6.5 Gene expression using qRT-PCR

Quantitative Real-time PCR is currently considered as the most powerful and standard method for detection of plant pathogens and gene expression studies. This technique allows the monitoring of the reaction during the amplification process by the use of a fluorescent signal that increases proportionally to the number of amplicons generated and to the number of targets present in the sample. The cDNA of control and treated were taken for qPCR. Expression of five genes (Nanog, Actin β, Sox 17, N-CAM, T) were studied. The real-time quantitative PCR (qPCR) was performed with the Applied Biosystems PCR system in a total volume of 10 µl.

Table 3. Reaction setup for qRT-PCR

COMPONENT	**VOLUME 10 µL/ WELL**
Power Up™ SYBR™ Green Master Mix (2X) (Cat no: A25776)	5 µL
Forward + reverse primers, reconstituted and diluted according to manufacturer's instruction.	Variable
c-DNA	Calculate such that each reaction has 20ng c-DNA
Nuclease-Free Water	Calculate such that the whole reaction becomes 10 µL
Total volume	**10 µL**

Table 4. Reaction protocol for qRTPCR

Incubate the complete reaction mix in a thermal cycler (Roche Light Cycler 480),

STEP	TEMPERATURE	TIME
INITIALIZATION	95^0 C	10mins
DENATURATION	95^0 C	5 Sec
ANNEALING	58^0 C	30 Sec
EXTENSION (not mandatory)	72^0 C	30 Sec
Total number of cycles: 40, and then held at 40^0 C after completing the reactions.		

After the completion of the real time PCR reactions, the threshold cycle (Ct) was recorded the gene expression level was calculated using comparative Ct method or delta-delta Ct method. All samples were run in triplicates and results are presented as mean values ± SD. A melt curve analysis was performed at every run to assess the product specificity.

The relative gene expression level of control and treated cells is represented as $2^{-\Delta\Delta CT}$ method.

ΔCt = Ct (target gene) – Ct (reference gene)

$\Delta\Delta Ct = \Delta Ct$ (sample) – ΔCt (control)

RESULTS

4. RESULTS

The results of the study entitled “Anticancer activity of stingless bee propolis on human cancer cells” are presented in this chapter.

4.1 LOCATION OF PROPOLIS

Propolis samples of different species were collected from the following locations in Kerala.

- P1-*Lisotrigona sp.* from Kollam District
- P2-*Tetragonula calophyllae* from ThiruvananthapuramDistrict
- P3-*Tetragonula travancorica*from ThiruvananthapuramDistrict

4.2 EXTRACTION OF PROPOLIS

The ethanolic extracts of propolis were prepared by adding 10 mL of ethanol (95%) to 3 g of crushed and homogenized propolis. The extract was centrifuged at 8800 rpm at 5°C for 10 min and the supernatant was collected.

4.3CELL VIABILITY ASSAYS (IC50 of samples on the A549 Cells)

4.3.1. Sample P1

IC50 value =485.822 µg/ml

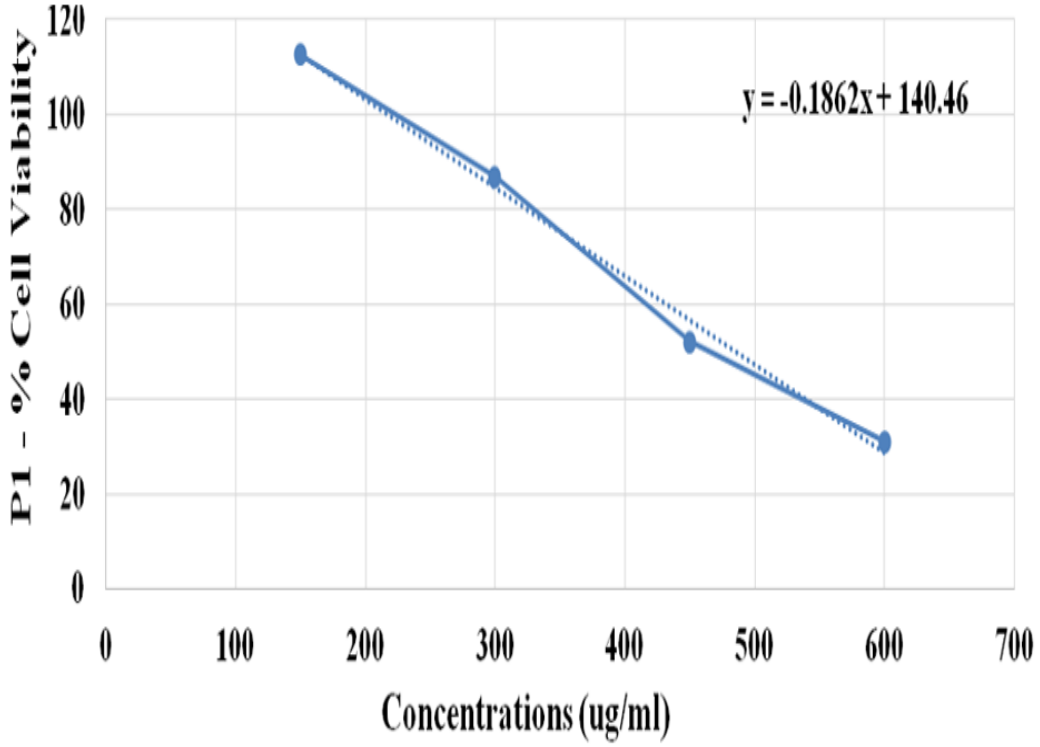

Fig. 1: Viability of A549 cells treated with P1

4.3.2 Sample P2

IC50 value = 236.6095 µg/ml

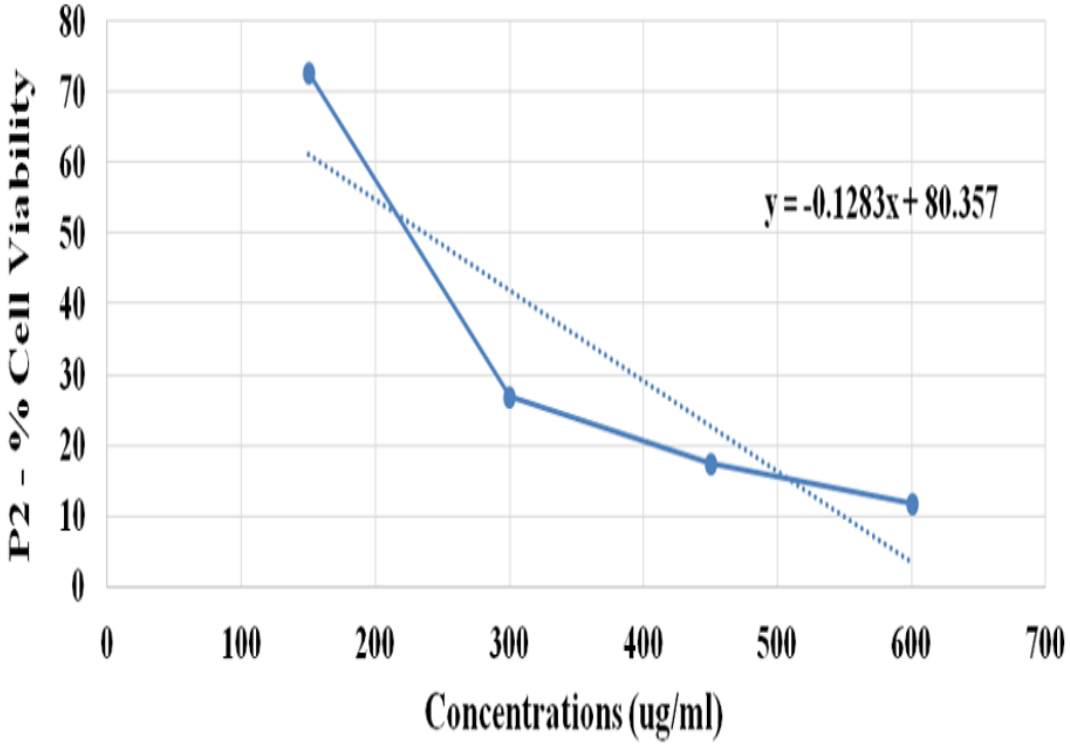

Fig. 2: Viability of A549 cells treated with P2

4.3.3 Sample P3

IC50 value = 294.223µg/ml

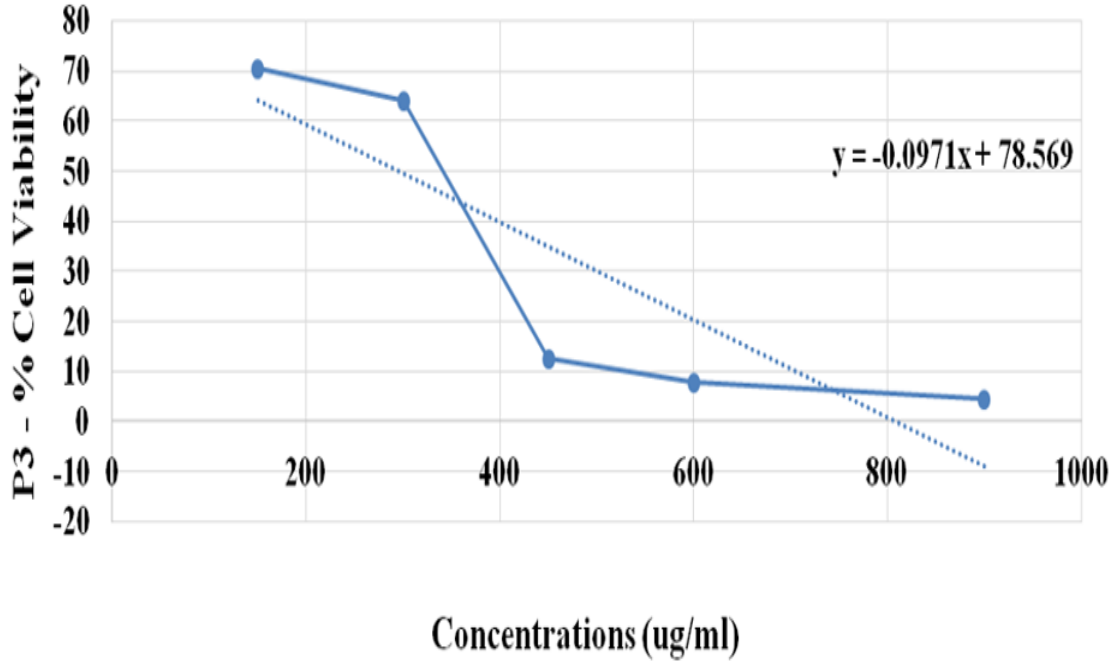

Fig. 3: Viability of A549 cells treated with P3

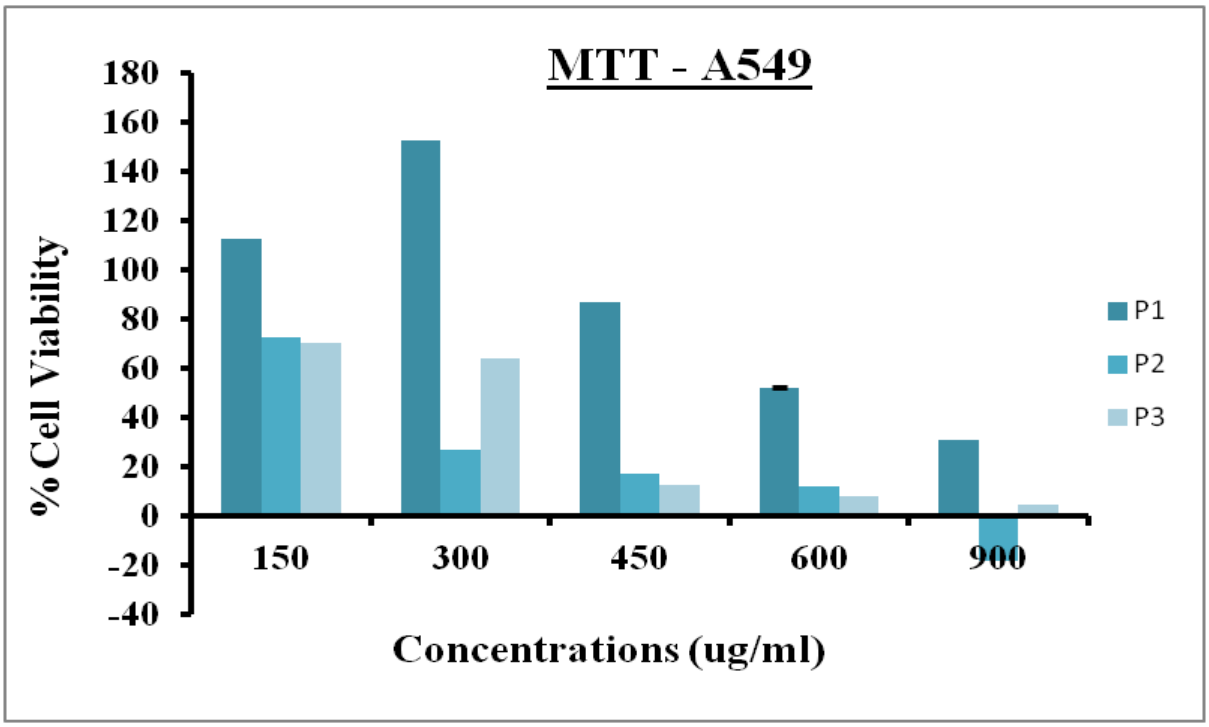

Figure 4: Comparison of percentage cell viability of the cell treated with three propolis

4.3.4. Morphological changes observed in MTT assay

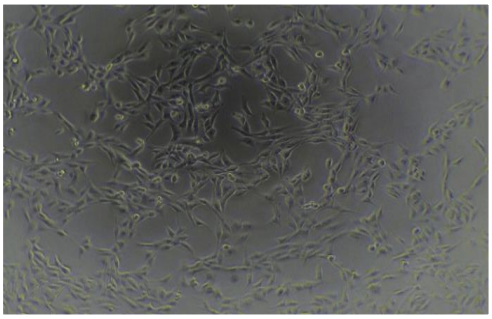

Fig. 5: Morphology of A549 cells treated before treatment with propolis.

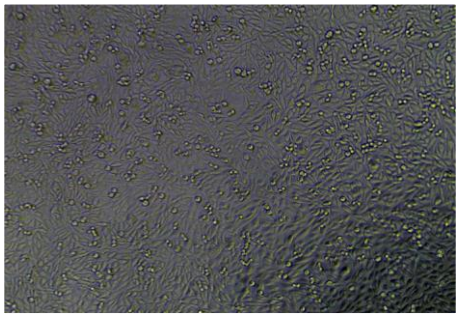

Fig. 6: Morphology of A549 cells treated after treatment (24 hours) with propollis.

Morphology of A549 cells treated after treatment (24 hours) in different concentration.

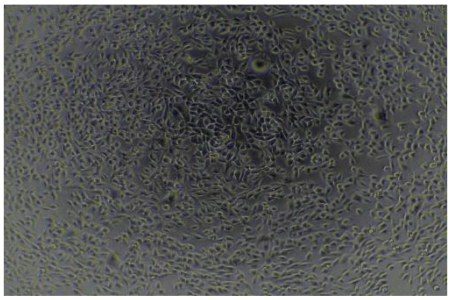

Fig7: Cells treated with 150µg/ml of propolis.

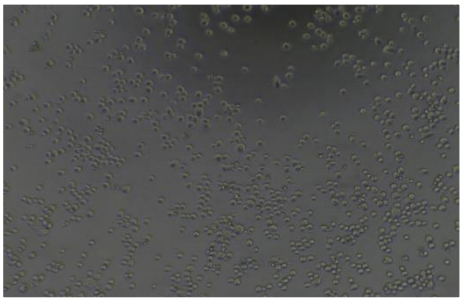

Fig8: Cells treated with 300µg/ml of propolis.

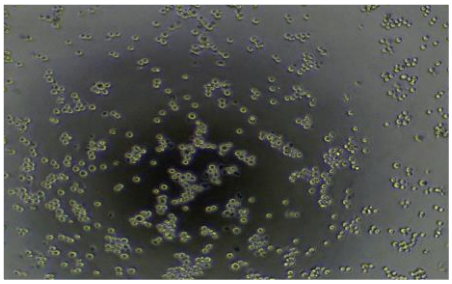

Fig9: Cells treated with 450µg/ml of propolis.

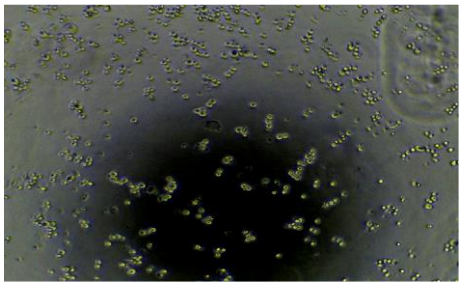

Fig10: Cells treated with 600µg/ml of propolis.

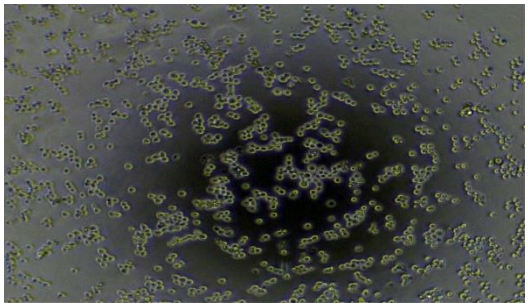

Fig11: Cells treated with 900µg/ml of propolis.

4.4 MORPHOLOGICAL CHANGES

Cells treated with all the three propolis samples showed morphological changes .Cells appeared to have shrunk and were floating and it showed the inhibition of cell growth. The cells appeared as a pebble like structure and it shows cell death. Cells treated with three propolis samples showed cell death.

Day 0 - Control

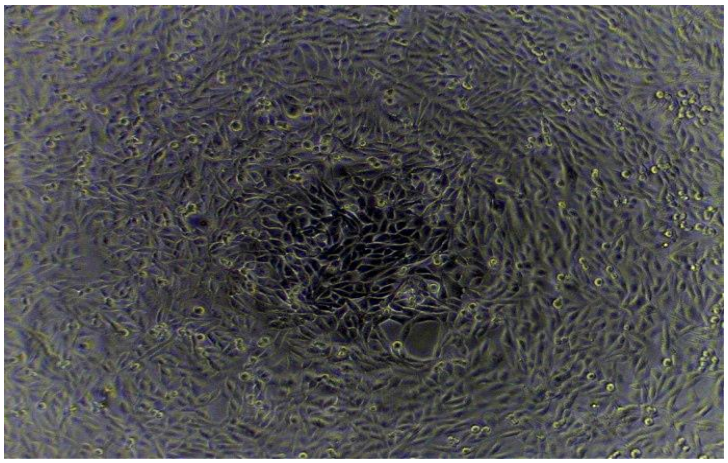

Figure 12: Morphology of A549 cells - day 0 (control)

Day 1 – P1 Ethanol control

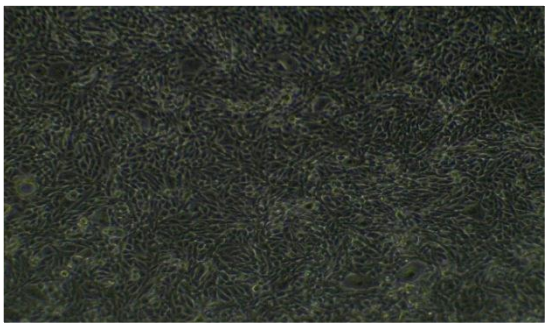

Fig13: Morphology of A549 cells - ethanol control

Day 1- P1 treatment

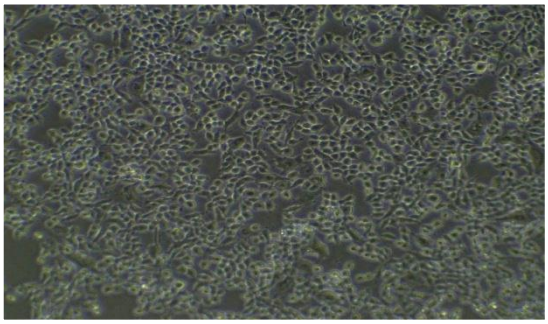

Fig14: Morphology of A549 cells treated with P1

Day 1 – P2 Ethanol control

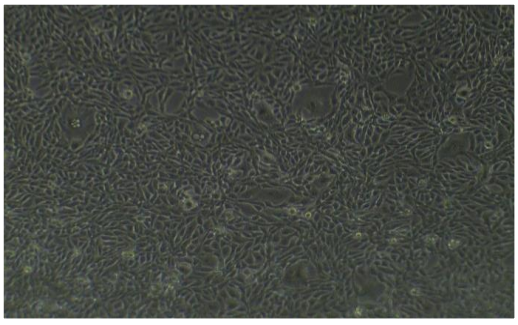

Fig15: Morphology of A549 cells - ethanol control

Day 1- P2 treatment

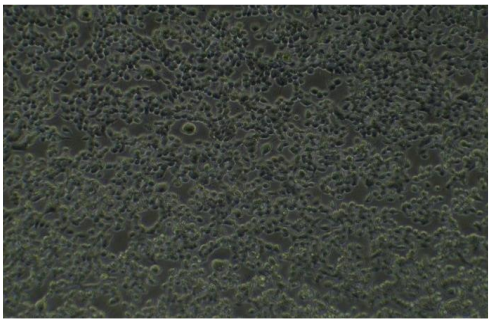

Fig16: Morphology of A549 cells treated with P2

Day 1 – P3 Ethanol control

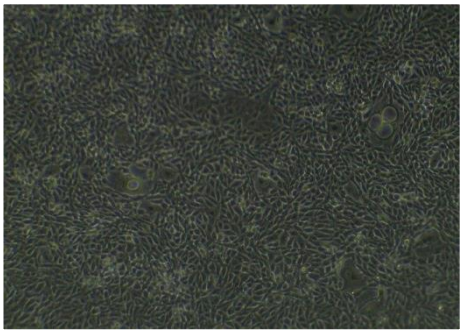

Fig17: Morphology of A549 cells - ethanol control

Day 1- P3 treatment

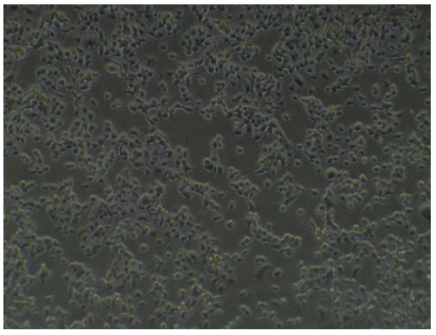

Fig18: Morphology of A549 cells treated with P3

4.5 Gene expression study

RNA was isolated from both control and propolis treated cells. The quantity of the isolated RNA was determined using Nanodrop spectrophotometer. Good quality of RNA was obtained (i.e., $A_{260/280}$ value was between 1.8 and 2).

Table No. 5. Quality and quantity of the isolated RNA

SL NO.	SAMPLE	A_{260}/A_{280} VALUE	CONCENTRATION (ng/µl)
1	Normal control	1.90	155.4
2	P1 Control	1.89	227.2
3	P1 Treated	1.72	648.7
4	P2 Control	1.99	435.7
5	P2 Treated	1.82	157.6
6	P3 Control	1.93	243.5
7	P3 Treated	1.91	1691.7

The RNA isolated from control and propolis treated cells were subjected to cDNA conversion using - Reverse transcription protocol (iScript^{TM} cDNA Synthesis Kit). The cDNA of control and treated cells were subjected qPCR for studying the expression of different genes.

4.5.1 Expression of CDX2 gene

Table No. 6: Fold change ratio of target gene *CDX2*

SAMPLES	LOG 2 RATIO (AGAINST ACTIN B)
P1	27.51
P1 Eth	1
P2	10.41
P2 Eth	1
P3	0.03
P3 Eth	1

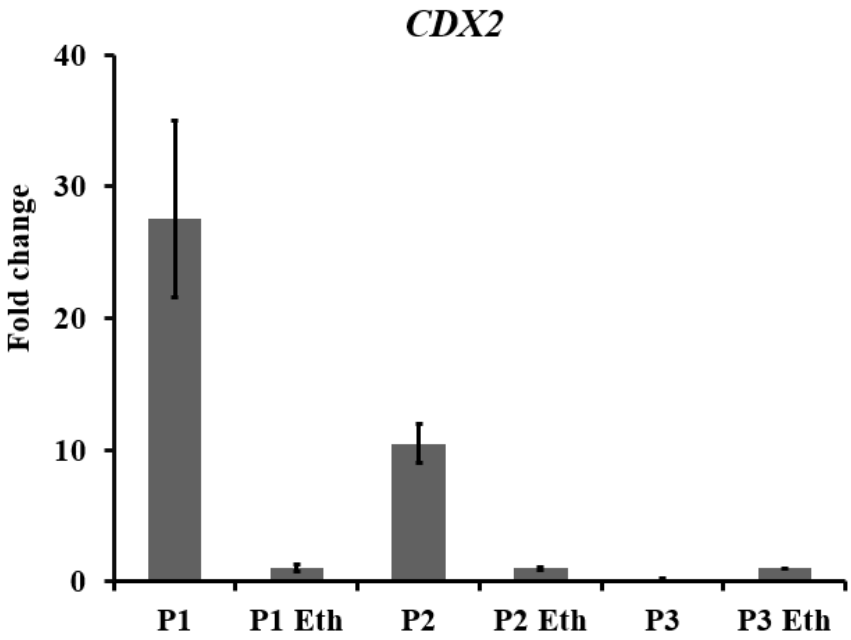

Fig. 19: Graphical representation of gene expression of *CDX2* in the propolis treated samples

4.5.2 Expression of *VIM gene*

Table No. 7: Fold change ratio of target gene *VIM*

SAMPLES	LOG 2 RATIO (AGAINST ACTIN B)
P1	-3.58
P1 Eth	0
P2	-1.35
P2 Eth	0
P3	-0.21
P3 Eth	0

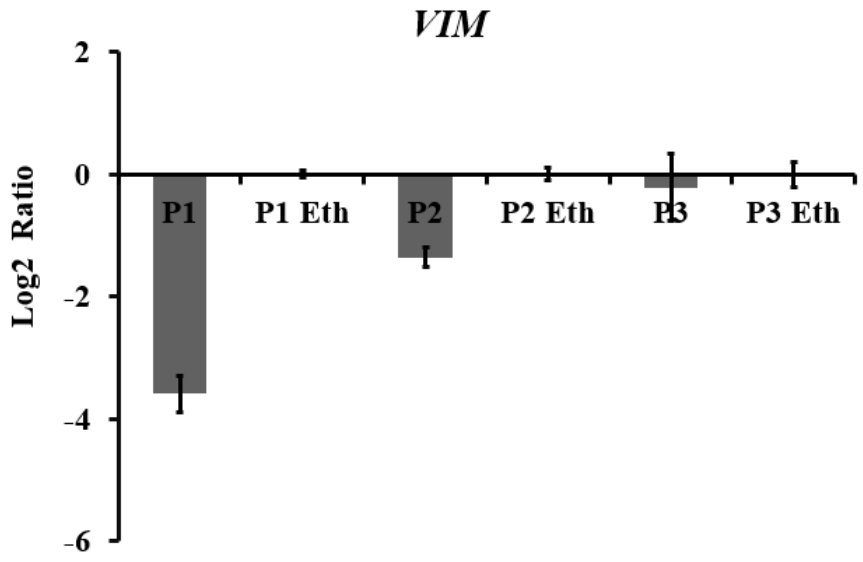

Fig. 20: Graphical representation of gene expression of *VIM* in the propolis treated samples

4.5.3 Expression of *NODAL gene*

Table No. 8: Fold change ratio of target gene *NODAL*

SAMPLES	LOG 2 RATIO (AGAINST ACTIN B)
P1	-1.07
P1 Eth	0
P2	-5.28
P2 Eth	0
P3	-2.11
P3 Eth	0

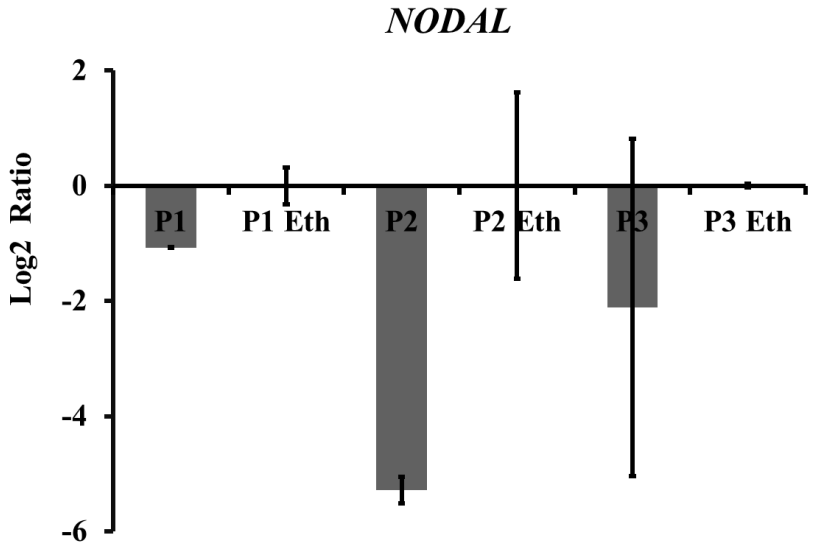

Fig. 21: Graphical representation of gene expression of *NODAL* in the propolis treated samples

4.5.4 Expression of *DUSP4 gene*

Table No. 9: Fold change ratio of target gene *DUSP4*

SAMPLES	LOG 2 RATIO (AGAINST ACTIN B)
P1	-0.23
P1 Eth	0
P2	0.21
P2 Eth	0
P3	-1.21
P3 Eth	0

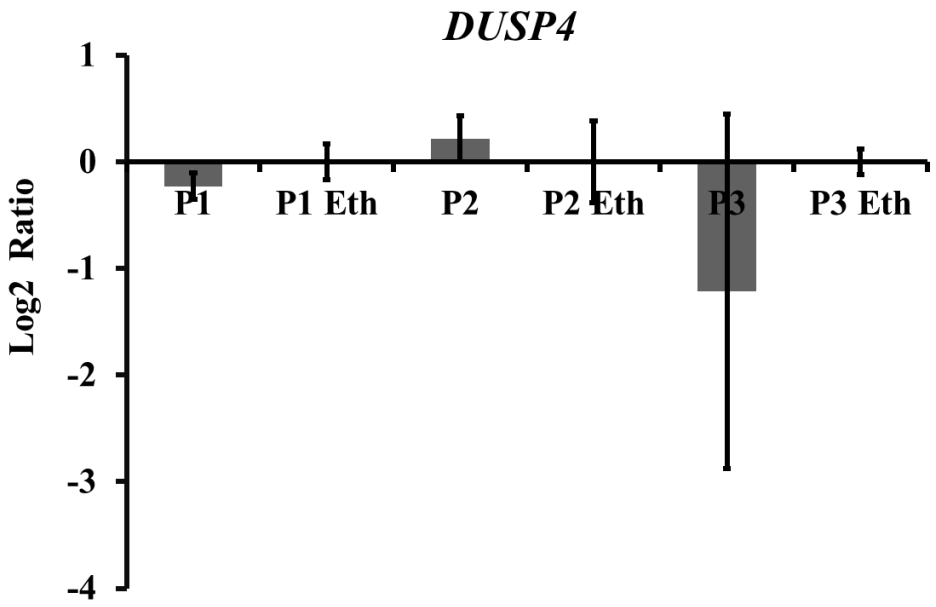

Fig. 22: Graphical representation of gene expression of *DUSP4* in the propolis treated samples

DISCUSSSION

5. DISCUSSION

Propolis is a resinous material with unique biological properties. The inhibitory effects of propolis in cancer cells have been studied (Sawicka *et al*., 2012).Sawicka *et al.* (2012) studied the mechanism of activity of propolis and its active compounds such as CAPE and chrysin in the apoptotic process, and their influence on the proliferation of cancer cells. The study showed that propolis and its compounds induce apoptosis pathways in cancer cells. The ant proliferative effects of propolis, CAPE or chrysin in cancer cells are the results of the suppression of complexes of cyclins that leads to cell cycle arrest. The results of *in vitr*o and *in vivo* studies suggest that propolis, has antibacterial, antioxidant, anti-inflammatory and anti tumoral properties. The chemical composition and pharmacological activities of propolis vary according to the geographical location and biological origin of propolis. Many studies have suggested that propolis plays an important role in acting as an anti-cancer agent. There are no studies related to the comparison of different kinds of propolis on cancer cells. There are also studies which shows that stingless bee propolis has much more effect than other kinds of propolis.

5.1 The effect of propolis on A549 cell

Our morphological observations of cells upon exposure to different concentrations of EEP, using optical microscopy.Cells when treated with crude propolis showed visible morphological changes compared with ethanol control indicating that, propolis has some effect on these cells as they prevent cell proliferation etc. Cells treated with three propolis samples showed morphological changes like cells appeared rounding up, a loss of cell extensions, to be shrunken, rounding up ,detachment and were floating, apoptotic blebbing, and reduction in size as well as cell density as compared to untreated cells. Surface topology was severely altered with respect to surface roughness and cell height.

5.2 Cell viability

The influence of propolis on the proliferation and for calculating the IC-50 value of propolis on A549 cell. MTT assay was performed. The effective concentration of the

three propolis samples on A549 cancer cells was analysed by MTT assay. The Seeding density of cell was determined through cell counting by Trypan blue exclusion test. Three hundred cells per well of 96 well platewas used for treatment. Five different concentration of propolis - 150µg/ml, 300µg/ml, 450µg/ml, 600µg/ml and 900µg/ml respectively were added to A549 cells for a period of 24 hrs. Cells exposed to complete growth media without any propolis were used as normal control and cell treated with 95% ethanol were taken as control. IC 50 value was determined for the three propolis samples. Cells treated with the propolis extracted from *Lisotrigona* sp. (P1), *T. calophyllae* (P2) and *T. travancorica* (P3) showed IC50 values of 485.822 ug/ml, 236.609ug/ml and 294.223 ug/ml respectively. The cells treated with the three different propolis inhibited the proliferation of cancer cell.When the cell were treated with propolis, a marked difference in the morphology of cells was observed. Hence it was confirmed that propolis has an influence on A549 cells. The Anti-cancerous effect of propolis is imparted by different mechanisms like inhibiting the cell proliferation, enhancing apoptosis and also by altering the expression of genes involved in tumor formation.

5.3 Gene expression

VIM gene can be used as a biomarker for the early detection of several cancers (Costa, V.L *et al*, 2010) VIM gene is transcriptionally inactive in normal colon epithelial cells. *VIM* gene encodes vimentin, a member of the intermediate filament family that is especially found in connective tissue. *Vimentin* is known to be involved in various biological processes including maintaining cell shape and stabilizing cytoskeletal interactions. *Vimentin* also plays vital roles in cell adhesion, migration, and signaling. When overexpressed in solid cancers, *vimentin* drives epithelial to mesenchymal transition (EMT) and ultimately, metastasis. Increased *vimentin* expression has been reported in various epithelial cancers including prostate cancer, gastrointestinal tumors, CNS tumors, breast cancer, malignant melanoma, lung cancer and other types of cancers. Increased vimentin expression has been reported in various epithelial cancers including prostate cancer, gastrointestinal tumors, CNS tumors, breast cancer, malignant melanoma, lung cancer and other types of cancers. Although its expression is more emphasized in the EMT process, it is equally possible that tumorigenic events including tumor cell migration and invasion are a consequence of *vimentin* over-expression in these cells. In this study *Vimentin* (*VIM*) is a mesenchymal marker, which is seen to be highly down-

regulated with propolis P1, followed by P2. Cells treated with P1 propolis were found to highly down regulate the expression of *VIM*. In *VIM* p1 (*Lisotrigona* sp.) is showing high down regulation which is depicting the inhibition of cell migration.

CDX2 is a Drosophila caudal-related home box transcription factor that is important for the establishment and maintenance of intestinal epithelial cells.*CDX2* expression is confined to the intestinal epithelium and has been shown to regulate intestinal gene transcription, differentiation, and proliferation. Overexpression of *CDX2* reduces proliferation, increased compaction and differentiation, and significantly alters the transcription of genes (Escaffit *et al*., 2006) .In lung cancer cells, restored *CDX2* expression suppressed cell proliferation by arresting the cell G1/S transition.*CDX2* inhibits the proliferation of colon cancer cells by suppressing *Wnt* signaling activity. In pancreatic cancer cells, *CDX2* inhibits cell proliferation by directly repressing cyclin D1 transcriptional activity.*CDX2* might regulate PTEN, an important tumor suppressor, thereby inhibiting the gastric cancer aggressive biological phenotype via PI3K/Akt pathway. In the present study *CDX2* expression was high in P1 and P2-treated samples, indicating inhibition of proliferation and tumor formation.

NODAL (Nodal Growth Differentiation Factor) is a Protein Coding gene. Nodal is a TGF-beta related embryonic morphogen that is expressed in multiple human cancers. Nodal was shown to be expressed in human breast carcinoma cell lines, but poorly expressed in normal mammary epithelial and myoepithelial cells. In cancer cells nodal promotes EMT formation. It is normally expressed during embryogenesis, and promotes mesendoderm specification and left-right asymmetry. However, its re-expression induces increased aggressiveness and tumorigenicity in cancer cells in melanoma, glioma, and prostate cancer. Nodal predominantly binds to activin-like kinase type II (ActRIIB) and type I (ALK4/7) receptors, which leads to phosphorylation of ALK4/7. Activation of the receptors promotes intracellular phosphorylation of Smad2/3, which then interacts with SMAD4, followed by translocation to the nucleus, thereby regulating target genes (Gong, W *et al.*, 2016). Nodal expression is correlated with tumor progression, poor prognosis and angiogenesis. Moreover, recent studies have shown that nodal may regulate breast cancer progression and metastasis. Nodal signaling promotes tumorigenicity. In this study indicated that EMT is affected with the propolis samples tested. However, the p3 propolis (*Tetragonula travancorica*) did not show any significant effect on these cells. After p2

treatment expression of VIM has been highly downregulated showing inhibition of EMT formation.

DUSP6 is a negative regulator of the ERK signaling pathway and plays an important role in chemotherapy-resistance.DUSP6 is a member of a subfamily of protein tyrosine phosphatases known as dual-specificity phosphatases (DUSPs), which dephosphorylates extracellular signal-regulated protein kinase 1/2 (ERK1/2) to negatively regulate ERK signaling (Marchetti *et al*,. 2005) Through its regulation of ERK signaling it modulates cell proliferation, differentiation and apoptosis. Many studies have confirmed a role for DUSP6 in the negative regulation of ERK signaling pathway and the reduction in cellular proliferation rates.DUSP6 plays an important role in chemotherapy- resistance by causing cellular quiescence through its regulation of the ERK signaling pathway.DUSP6 regulates ERK1/2 activity during the cell cycle, which leads to G0/G1 arrest and chemotherapy-resistance.DUSP6, involved in FGF signaling also shows down-regulation after treated with propolis.

There are only small numbers of studies about the effect of Indian stingless bee propolis. Caffeic acid phenethyl ester (CAPE) is one of the major constituents of propolis with broad biological activities, including inhibition of cell growth and proliferation, inhibition of nuclear factor κ-B, induction of cell cycle arrest and apoptosis etc (Huang *et al*., 2014) and studies also reported that ethanolic extract of propolis has more effect on cancer cell than normal propolis. The finding obtained in this study was harmonious with previously published studies, which reported that propolis has anti-cancerous properties.

Natural compounds have been used in conventional medicine for the treatment of a wide range of diseases, further investigating their apoptotic and cytotoxic effects on cancer cell may provide a deeper understanding for curing various diseases.

In the following study, propolis, a natural compound, supported the inhibition of proliferation of cancer cell. The findings highlight that the propolis samples from all the three stingless bee species have an inhibitory effect on the cancer cell lines.

Propolis extracted from the nest of *Lisotrigona* sp. showed inhibitory effect compared to *Tetragonula* spp . Propolis from *Lisotrigona* sp highly down regulate the expression of cancer cell by inhibiting cell migration and cell proliferation. The EMT formation is also

suppressed. Hence better understanding of the chemical composition of propolis, investigating its mechanism and regulatory effects will pave the way as invaluable candidate in future regenerative medicines.

SUMMARY

6. SUMMARY

The investigation on “Anticancer activity of stingless bee propolis on human cancer cells.” was undertaken in the Department of Plant Biotechnology, College of Agriculture, Vellayani, Thiruvananthapuram and Department of Genomic Science, Central University of Kerala during the period from 2020-2021. The experiment was conducted to study the effect of propolis collected from two different genera of Stingless bee – *Lisotrigona* and *Tetragonula* on the A549 cancer cell.

Propolis is a resinous material with unique biological properties due to the presence of components like Caffeic acids, cinnmaic acids, ferulic acids and its derivatives. In this study *in vitro* experiments were carried out and optimum dose of propolis for the treatment of cell were determined. As per the findings during the study, propolis sample extracted from the nest of *Lisotrigona sp.*is showing more tendency to differentiate into mesoderm and endoderm lineage compared to propolis extracted from *Tetragonula spp.* The finding obtained in this study was in agreement with previously published studies, which reported that propolis show anti-cancer activity. The result of the study proved that, the propolis obtained from *Lisotrigona sp*. bees probably has more therapeutic value in terms of its effect on human cancer cells viz., more inhibitory effect on cancer cell line, compared to the propolis obtained from *Lisotrigona sp*. Propolis from Lisotrigona *sp* highly downregulate the expression of cancer cell by inhibiting cell migration and cell proliferation .There is also significant morphological changes happened to the cancer cell which also confirms that propolis has inhibitory effect on cancer cell. Propolis as a natural product can be used for cancer treatment in future.

REFERENCE

7. REFERENCE

Abdel Razek, M. M., Moussa, A.Y., El-Shanawany, M. A., and Singab, A. N. B. 2020. A New Phenolic Alkaloid from *Halocnemum strobilaceum* endophytes: antimicrobial, antioxidant and biofilm Inhibitory activities. *Chem. Biodivers.* 17(10): 37-50.

Abdulkhani, A., Hosseinzadeh, J., Ashori, A., and Esmaeeli, H. 2017. Evaluation of the antibacterial activity of cellulose nanofibers/polylactic acid composites coated with ethanolic extract of propolis. *Polym. Compos.* 38(1): 13-19.

Al-Hatamleh, M. A., Ear, E. N. S., Boer, J. C., Ferji, K., Six, J. L., Chen, X., Elkord, E., Plebanski, M., and Mohamud, R. 2020. Synergistic Effects of Nanomedicine Targeting TNFR2 and DNA Demethylation Inhibitor—An Opportunity for Cancer Treatment. *Cell.* 9(1): 33.

Ali, I., Salim, K. A., Rather, M. A., Wani, W., and Haque, A. 2011. Advances in nano drugs for cancer chemotherapy. *Curr. Cancer. Drug. Targets*. 11(2): 135-146.

Anjum, S. I., Ullah, A., Khan, K. A., Attaullah, M., Khan, H., Ali, H., Bashir, M. A., Tahir, M., Ansari, M. J., Ghramh, H. A., and Adgaba, N. 2019. Composition and functional properties of propolis (bee glue): A review. *Saudi J. Biol. Sci.* 26(7): 1695-1703.

Bankova, V. S., Popov, S. S., and Marekov, N. L. 1989. Isopentenyl cinnamates from poplar buds and propolis. *Phytochem.* 28(3): 871-873.

Bankova, V., and Popova, M. 2007. PHCOG REV.: Review Article Propolis of Stingless Bees: a Promising Source of Biologically Active Compounds. *Pharmacogn. Rev.* 1(1): 874-876.

Banskota, A. H., Nagaoka, T., Sumioka, L. Y., Tezuka, Y., Awale, S., Midorikawa, K., Matsushige, K., and Kadota, S. 2002. Antiproliferative activity of the Netherlands propolis and its active principles in cancer cell lines. *J. Ethnopharmacol.* 80(1): 67-73.

Banskota, A. H., Tezuka, Y., Adnyana, I. K., Ishii, E., Midorikawa, K., Matsushige, K., and Kadota, S. 2001. Hepatoprotective and anti-Helicobacter pylori activities of constituents from Brazilian propolis. *Phytomedicine.* 8(1): 16-23.

Barbarić, M., Mišković, K., Bojić, M., Lončar, M. B., Smolčić-Bubalo, A., Debeljak, Ž., and Medić-Šarić, M. 2011. Chemical composition of the ethanolic propolis extracts and its effect on HeLa cells. *J. Ethnopharmacol.* 135(3): 772-778.

Basyirah, Azemin, A., Rodi, M. M. M., and Mohd, K. S. 2018. Chemical composition and antioxidant activity of stingless bee propolis from different extraction methods. *Int. J. Eng. Technol.* 7(4): 90-95.

Benguedouar, Lamia., Boussenane, Hanane., Wided, Kebsa., Alyane, Mohamed., Hassiba, Rouibah., Mesbah, Lahouel. 2008. Efficiency of propolis extract against mitochondrial stress induced by antineoplasic agents (doxorubicin and vinblastin) in rats. *Indian J. Exp. Biol.* 46(8): 112-119.

Benkovic, V., Knezevic, A. H., Dikic, D., Lisicic, D., Orsolic, N., Basic, I., Kosalec, I., Kopjar, N. 2008. Radioprotective effects of propolis and quercetin in gamma-irradiated mice evaluated by the alkaline comet assay. *Phytomedicine.* 15(10): 851–858.

Benkovic, V., Knezevic, A. H., Orsolic, N., Basic, I., Ramic, S., Viculin, T., Knezevic, F., and Kopjar, N. 2009. Evaluation of radioprotective effects of propolis and its flavonoid constituents: in vitro study on human white blood cells. *Phytother. Res.* 23(8): 1159-1168.

Bhargava, P., Grover, A., Nigam, N., Kaul, A., Ishida, Y., Kakuta, H., Kaul, S. C., Terao, K., and Wadhwa, R. 2018. Anticancer activity of the supercritical extract of Brazilian green propolis and its active component, artepillin C: Bioinformatics and experimental analyses of its mechanisms of action. *Int. J. Oncol.* 52(3): 925-932.

Cai, S. J., Liu, Y., Han, S., and Yang, C. 2019. Brusatol, an NRF2 inhibitor for future cancer therapeutic. *Cell Biosci.* 9(1): 1-3.

Chandramohan, V., Bao, X., Yu, X., Parker, S., McDowall, C., Yu, Y. R., Healy, P., Desjardins, A., Gunn, M. D., Gromeier, M., and Nair, S. K. 2019. Improved efficacy against malignant brain tumors with EGFRwt/EGFRvIII targeting immunotoxin and checkpoint inhibitor combinations. *J. Immunother. Cancer.* 7(1): 1-14.

Chen, C. N., Weng, M. S., Wu, C. L., and Lin, J. K. 2004. Comparison of radical scavenging activity, cytotoxic effects and apoptosis induction in human melanoma cells by Taiwanese propolis from different sources. *Evid. Based Complementary Altern. Med.* 1(2): 175-185.

Choudhari, M. K., Haghniaz, R., Rajwade, J. M., and Paknikar, K. M. 2013. Anticancer activity of Indian stingless bee propolis: an *in vitro* study. *Evid. Based Complementary Altern. Med.* 5(2): 65-69.

Choudhari, M. K., Punekar, S. A., Ranade, R. V., and Paknikar, K. M. 2012. Antimicrobial activity of stingless bee (*Trigona* sp.) propolis used in the folk medicine of Western Maharashtra, India. *J. Ethnopharmacol.* 141(1): 363-367.

Costa, V. L., Henrique, R., Danielsen, S. A., Duarte-Pereira, S., Eknaes, M., Skotheim, R. I., Rodrigues, Â., Magalhães, J. S., Oliveira, J., Lothe, R. A., and Teixeira, M. R. 2010. Three epigenetic biomarkers, GDF15, TMEFF2, and VIM, accurately predict bladder cancer from DNA-based analyses of urine samples. *Clin. Cancer Res.* 16(23): 5842-5851.

Daleprane, J. B., and Abdalla, D.S. 2013. Emerging roles of propolis: antioxidant, cardioprotective, and antiangiogenic actions. *Evid. Based Complementary Altern. Med.*13(9): 85-88.

Demain, A. L., and Vaishnav, P. 2010. Natural products for cancer chemotherapy. *Microb. Biotechnol.* 4(6): 687-699.

Desai, A. G., Qazi, G. N., Ganju, R. K., El-Tamer, M., Singh, J., Saxena, A. K., Bedi, Y. S., Taneja, S. C., and Bhat, H. K. 2008. Medicinal plants and cancer chemoprevention. *Curr. Drug Metab.* 9(7): 581-591.

Desamero, M. J., Kakuta, S., Tang, Y., Chambers, J. K., Uchida, K., Estacio, M. A., Cervancia, C., Kominami, Y., Ushio, H., Nakayama, J., and Nakayama, H. 2019. Tumor-suppressing potential of stingless bee propolis in *in vitro* and *in vivo* models of differentiated-type gastric adenocarcinoma. *Sci. Rep.* 9(1): 1-13.

Devequi-Nunes, D., Machado, B. A. S., Barreto, G. D. A., Rebouças Silva, J., da Silva, D. F., da Rocha, J. L. C., Brandão, H. N., Borges, V. M., and Umsza-Guez, M. A. 2018. Chemical characterization and biological activity of six different extracts of propolis through conventional methods and supercritical extraction. *PLoS One.* 13(12): 76-78.

Ebeid, S. A., Abd El Moneim, N. A., El-Benhawy, S. A., Hussain, N. G., and Hussain, M. I. 2016. Assessment of the radioprotective effect of propolis in breast cancer patients undergoing radiotherapy. New perspective for an old honey bee product. *J. Radiat. Res. Appl. Sci.* 9(4): 431-440.

Ekeuku, S. O., and Chin, K. Y. 2021. Application of Propolis in Protecting Skeletal and Periodontal Health—A Systematic Review. *Molecules. 26*(11): 3156-3159.

Escaffit, F., Paré, F., Gauthier, R., Rivard, N., Boudreau, F., and Beaulieu, J. F. 2006. Cdx2 modulates proliferation in normal human intestinal epithelial crypt cells. *Biochem. Biophys. Res. Commun.* 342(1): 66-72.

Farooqui, M., Hassali, M. A., Shatar, A. K. A., Shafie, A. A., Saleem, F., and Aljadhey, H. 2012. Complementary and alternative medicines (CAM) disclosure to the health care providers: a qualitative insight from Malaysian cancer patients. *Complement. Ther. Clin. Pract.* 18(4): 252-256.

Fratini, F., Cilia, G., Mancini, S., and Felicioli, A. 2016. Royal Jelly: An ancient remedy with remarkable antibacterial properties. *Microbiol. Res.* 192: 130-141.

Ghisalberti, E. L. 1979. Propolis: a review. Bee world, 60(2): 59-84.

Glare, E. M., Divjak, M., Bailey, M. J., and Walters, E. H. 2002. β-Actin and GAPDH housekeeping gene expression in asthmatic airways is variable and not suitable for normalising mRNA levels. Thorax, 57(9): 765-770.

Gong, W., Sun, B., Zhao, X., Zhang, D., Sun, J., Liu, T., Gu, Q., Dong, X., Liu, F., Wang, Y., and Lin, X. 2016. Nodal signaling promotes vasculogenic mimicry formation in breast cancer via the Smad2/3 pathway. *Oncotarget*, 7(43): 70152.

Graikini, D., Papachristoforou, A., and Mourtzinos, I. 2019. Comparison of qualitative characteristics of propolis extracts using different purification methods. *J. Apic. Res.* 58(5): 792-799.

Graikini, D., Papachristoforou, A., and Mourtzinos, I. 2019. Comparison of qualitative characteristics of propolis extracts using different purification methods. *J. Apic. Res.* 58(5): 792-799.

Guan, X. 2015. Cancer metastases: challenges and opportunities. *Acta Pharm. Sin. B.* 5(5): 402-418.

Hady, F. K., and Hegazi, A. G. 2002. Egyptian propolis: 2. Chemical composition, antiviral and antimicrobial activities of East Nile Delta propolis. Zeitschrift für Naturforschung C, 57(4): 386-394.

Hassanpour, S. H., and Dehghani, M. 2017. Review of cancer from perspective of molecular. *J. Cancer Res. Pract.* 4(4): 127-129.

Hayacibara, M. F., Koo, H., Rosalen, P. L., Duarte, S., Franco, E. M., Bowen, W. H., Ikegaki, M., and Cury, J. A. 2005. *In vitro* and *in vivo* effects of isolated fractions of Brazilian propolis on caries development. *J. Ethnopharmacol.* 101(3): 110-115.

Huang, X., and Jan, L. Y. 2014. Targeting potassium channels in cancer. *J. Cell Biol.* 206(2): 151–162.

Ishihara, M., Naoi, K., Hashita, M., Itoh, Y., and Suzui, M. 2009. Growth inhibitory activity of ethanol extracts of Chinese and Brazilian propolis in four human colon carcinoma cell lines. *Oncol. Rep.* 22(2): 349-354.

Izuta, H., Shimazawa, M., Tsuruma, K., Araki, Y., Mishima, S., and Hara, H. 2009. Bee products prevent VEGF-induced angiogenesis in human umbilical vein endothelial cells. *BMC Complement. Altern. Med.* 9(1): 1-10.

Kartal, M., Yıldız, S., Kaya, S., Kurucu, S., and Topçu, G. 2003. Antimicrobial activity of propolis samples from two different regions of Anatolia. *J. Ethnopharmacol.* 86(1): 69-73.

Kasote, D. M., Pawar, M. V., Bhatia, R. S., Nandre, V. S., Gundu, S. S., Jagtap, S. D., and Kulkarni, M. V. 2017. HPLC, NMR based chemical profiling and biological characterisation of Indian propolis. *Fitoterapia.* 122(1): 52-60.

Kim, D. M., Lee, G. D., Aum, S. H., and Kim, H. J. 2008. Preparation of propolis nanofood and application to human cancer. *Biol. Pharm. Bull.* 31(9): 1704-1710.

Kim, W. Y., and Sharpless, N. E. 2006. The regulation of INK4/ARF in cancer and aging. *Cell.* 127(2): 265-275.

Kuropatnicki, A. K., Szliszka, E., and Krol, W. 2013. Historical aspects of propolis research in modern times. *Evid. Based Complement. Alternat. Med.* 20(1): 3-6.

Lakshmi, S., Hughes, T. A., and Priya, S. 2015. Exosomes and exosomal RNAs in breast cancer: A status update. *Eur. J. Cancer.* 144: 252-268.

Lavinas, F. C., Macedo, E. H. B., Sá, G. B., Amaral, A. C. F., Silva, J. R., Azevedo, M., Vieira, B. A., Domingos, T. F. S., Vermelho, A. B., Carneiro, C. S., and Rodrigues, I. A. 2019. Brazilian stingless bee propolis and geopropolis: promising sources of biologically active compounds. *Rev. Bras. Farmacogn.* 29: 389-399.

Lirdprapamongkol, K., Sakurai, H., Abdelhamed, S., Yokoyama, S., Maruyama, T., Athikomkulchai, S., Viriyaroj, A., Awale, S., Yagita, H., Ruchirawat, S., and Svasti, J. 2013. A flavonoid chrysin suppresses hypoxic survival and metastatic growth of mouse breast cancer cells. *Oncol. Rep.* 30(5): 2357-2364.

Majolo, F., Delwing, L. K. D. O. B., Marmitt, D. J., Bustamante-Filho, I. C., and Goettert, M. I. 2019. Medicinal plants and bioactive natural compounds for cancer treatment: Important advances for drug discovery. *Phytochem. Lett.* 31: 196-207.

Marchetti, S., Gimond, C., Chambard, J. C., Touboul, T., Roux, D., Pouysségur, J., and Pagès, G. 2005. Extracellular signal-regulated kinases phosphorylate mitogen-

activated protein kinase phosphatase 3/DUSP6 at serines 159 and 197, two sites critical for its proteasomal degradation. *Mol. Cell. Biol.* 25(2): 854-864.

Marcucci, M. C. 1995. Propolis: chemical composition, biological properties and therapeutic activity. *Apidologie.* 26(2): 83–99.

Massaro, F. C., Brooks, P. R., Wallace, H. M., and Russell, F. D. 2011. Cerumen of Australian stingless bees (*Tetragonula carbonaria*): gas chromatography-mass spectrometry fingerprints and potential anti-inflammatory properties. *Naturwissenschaften.* 98(4): 329-337.

Mattiuzzi, C., and Lippi, G. 2019. Current cancer epidemiology. *J. Epidemiol. Glob. Health.* 9(4): 217.

Maurya, P., Meleady, P., Dowling, P., and Clynes, M. 2006. Proteomic approaches for serum biomarker discovery in cancer. *Anticancer Res.* 27(3): 1247-1255.

Motomura, K., Nagumo, S., Komoike, Y., Koyama, H., and Inaji, H. 2008. Accuracy of imprint cytology for intraoperative diagnosis of sentinel node metastases in breast cancer. *Ann. Surg.* 247(5): 839-842.

Nobili, S., Lippi, D., Witort, E., Donnini, M., Bausi, L., Mini, E., and Capaccioli, S. 2009. Natural compounds for cancer treatment and prevention. *Pharmacol. Res.* 59(6): 365-378.

Oliveira, A. C. S., Murgo, D. O. A. 2006. Stage of the application of propolis in dentistry anticancer research, 27(3): 1247-1255.

Oliveira, C., Pinheiro, H., Figueiredo, J., Seruca, R., and Carneiro, F. 2015. Familial gastric cancer: genetic susceptibility, pathology, and implications for management. *Lancet Oncol.* 16(2): 60-70.

Paul, S., Emmanuel, T., Matchawe, C., Alembert, T. T., Sophie, L., Maurice, T. F., Joel, Y. G. A., and De, T. A. 2011. Pentacyclic triterpenes and crude extracts with antimicrobial activity from Cameroonian brown propolis samples. *J. Appl. Pharm. Sci.* 4(7): 1-5.

Poon, I. K., Chiu, Y. H., Armstrong, A. J., Kinchen, J. M., Juncadella, I. J., Bayliss, D. A., and Ravichandran, K. S. 2014. Unexpected link between an antibiotic, pannexin channels and apoptosis. Nature, 507(7492): 329-334.

Popova, P., Notabi, M. K., Code, C., Arnspang, E. C., and Andersen, M. Ø. 2019. Co-delivery of siRNA and etoposide to cancer cells using an MDEA esterquat based drug delivery system. *Eur. J. Pharm. Sci.* 127: 142-150.

Prakash, O. M., Kumar, A., and Kumar, P. 2013. Anticancer potential of plants and natural products. *Am. J. Pharmacol. Sci.* 1(6): 104-115.

Premratanachai, P., and Chanchao, C. 2014. Review of the anticancer activities of bee products. *Asian Pac. J. Trop. Biomed*. 4(5): 337-344.

Pujirahayu, N., Suzuki, T., and Katayama, T. 2019. Cycloartane-type triterpenes and botanical origin of propolis of stingless Indonesian bee Tetragonula sapiens. Plants, 8(3): 57-60.

Russo, A., Cardile, V., Sanchez, F., Troncoso, N., Vanella, A., and Garbarino, J. A. 2004. Chilean propolis: antioxidant activity and antiproliferative action in human tumor cell lines. *Life Sci.* 76(5): 545-558.

S. Shanas., and P. Faseeh. 2019. A new subgenus and three new species of stingless bees (*Hymenoptera: Apidae: Apinae: Meliponini*) from *India.Entomon*. 44(1): 33- 48.

Santos, F. A., Bastos, E. M. A. F., Maia, A. B. R. A., Uzeda, M., Carvalho, M. A. R., Farias, L. M., and Moreira, E. S. A. 2003. Brazilian propolis: physicochemical properties, plant origin and antibacterial activity on periodontopathogens. *Phytother. Res.*17(3): 285-289.

Sauvager, F. 1992. *In vitro* antiviral activity of propolis. *Apidologie.* 23(3): 231-240.

Sawichka, H., Car, M. H., Borawska., and Niklinski. 2012. "The anticancer activity of propolis," *Folia Histochem. Cytobiol.* 50(1): 25–37

Seda., Sorkun, K., Gurhan, S. I. D., Ozdal-Kurt, F., Turkoz, E., Gencay, O., and Salih, B. 2010. Propolis from Turkey induces apoptosis through activating caspases in human breast carcinoma cell lines. *Acta Histochem.* 112(6): 546-556.

Shubharani, R., Sivaram, V., and Kishore, B. R. 2014. *In-vitro* cytotoxicity of Indian bee propolis on cancer cell lines. *Int. J. Pharm. Bio. Sci.* 5(4): 698-706.

Siegel, R. L., Miller, K. D., and Jemal, A. 2019. Cancer statistics, 2019. CA: a cancer journal for clinicians, 69(1): 7-34.

Silici, S., Ünlü, M., and Vardar-Ünlü, G. 2007. Antibacterial activity and phytochemical evidence for the plant origin of Turkish propolis from different regions. *World J. Microbiol. Biotechnol.* 23(12): 1797-1803.

Singh, S. S., Vats, S., Chia, A. Y. Q., Tan, T. Z., Deng, S., Ong, M. S., Arfuso, F., Yap, C. T., Goh, B. C., Sethi, G., and Huang, R. Y. J. 2018. Dual role of autophagy in hallmarks of cancer. *Oncogene.* 37(9): 1142-1158.

Song, Y. S., Park, E. H., Jung, K. J., and Jin, C. 2002. Inhibition of angiogenesis by propolis. *Arch. Pharm. Res*. 25(4): 500-504.

Szliszka, E., Czuba, Z.P., Domino, M., Mazur, B., Zydowicz, G. and Krol, W., 2009. Ethanolic extract of propolis (EEP) enhances the apoptosis-inducing potential of TRAIL in cancer cells. Molecules, 14(2), pp.738-754.

Szliszka, E., Zydowicz, G., Janoszka, B., Dobosz, C., Kowalczyk-Ziomek, G., and Krol, W. 2011. Ethanolic extract of Brazilian green propolis sensitizes prostate cancer cells to TRAIL-induced apoptosis. *Int. J. Oncol.* 38(4): 941-953.

Takagi, Y., Choi, I. S., Yamashita, T., Nakamura, T., Suzuki, I., Hasegawa, T., Oshima, M., and Gu, Y. H. 2005. Immune activation and radioprotection by propolis. *Am. J. Chin. Med.* 33(2): 231-240.

Tamura, K., Unoki, T., Sagawa, K., Aratake, Y., Kitamura, T., Tachibana, N., Ohtaki, S., Yamaguchi, K., and Seita, M. 1985. Clinical features of OKT4+/OKT8+ adult T-cell leukemia. *Leuk. Res.* 9(11): 1353-1359.

Turan, I., Demir, S., Misir, S., Kilinc, K., Mentese, A., Aliyazicioglu, Y., and Deger, O. 2015. Cytotoxic effect of Turkish propolis on liver, colon, breast, cervix and prostate cancer cell lines. *Trop. J. Pharm. Res.* 14(5): 777-782.

Velikova, M., Bankova, V., Marcucci, M. C., Tsvetkova, I., and Kujumgiev, A. 2000. Chemical composition and biological activity of propolis from Brazilian meliponinae. *Z. Naturforsch.* 55(9): 785-789.

Wagh, V. D. 2013. Propolis: a wonder bee's product and its pharmacological potentials. *Adv. Pharmacol. Sci.* 20(1): 3-6.

Walker, P., and Crane, E. 1987. Constituents of propolis. *Apidologie*. 18(4): 327-334.

Wang, K., Jin, X., You, M., Tian, W., Leu, R. K. L., Topping, D. L., Conlon, M. A., Wu, L., and Hu, F. 2017. Dietary propolis ameliorates dextran sulfate sodium-induced colitis and modulates the gut microbiota in rats fed a western diet. *Nutrients.* 9(8): 875.

Watanabe, M. A. E., Amarante, M. K., Conti, B. J., and Sforcin, J. M. 2011. Cytotoxic constituents of propolis inducing anticancer effects: a review. *J. Pharm. Pharmacol.* 63(11): 1378-1386.

Wu, J., Omene, C., Karkoszka, J., Bosland, M., Eckard, J., Klein, C. B., and Frenkel, K. 2011. Caffeic acid phenethyl ester (CAPE), derived from a honeybee product propolis, exhibits a diversity of anti-tumor effects in pre-clinical models of human breast cancer. *Cancer Lett.* 308(1): 43-53.

Yoo, M. H., & Hatfield, D. L. 2003. The cancer stem cell theory: is it correct?. *Mol. Cells.* 26(5): 514–516.

Yu, J., Huang, C., Sun, Y., Su, X., Cao, H., Hu, J., Wang, K., Suo, J., Tao, K., He, X., and Wei, H. 2019. Effect of laparoscopic vs open distal gastrectomy on 3-year disease-free survival in patients with locally advanced gastric cancer: the CLASS-01 randomized clinical trial. *Jama.* 321(20): 1983-1992.

Yusuf, M. A., Badar, F., Meerza, F., Khokhar, R. A., Ali, F. A., Sarwar, S., and Faruqui, Z. S. 2007. Survival from hepatocellular carcinoma at a cancer hospital in Pakistan. *Asian Pac. J. Cancer Prev.* 8(2): 272.

Zhang, H., Fu, Y., Niu, F., Li, Z., Ba, C., Jin, B., Chen, G., and Li, X. 2018. Enhanced antioxidant activity and in vitro release of propolis by acid-induced aggregation using heat-denatured zein and carboxymethyl chitosan. *Food Hydrocoll.* 81: 104-112.

Zulhendri, F., Chandrasekaran, K., Kowacz, M., Ravalia, M., Kripal, K., Fearnley, J., and Perera, C.O. 2021. Antiviral, antibacterial, antifungal, and antiparasitic properties of propolis: A Review. *Foods*. 10(6): 1360-1364.

APPENDICES

8. APPENDIX

APPENDIX 1

Reagents required for maintenance of A549 cells

- 0.5 M EDTA. pH 8.0
- DMEM Complete medium preparation,

DMEM	– 44 ml
10% FBS	– 5 ml
L-Glutamine (200mM)	– 0.5 ml
Penicillin-Streptomycin	– 0.5 ml

APPENDIX II

Preparation of 95% Ethanol

100% ethanol	95ml
Distilled water	5ml

APPENDIX III

Preparation of Propolis for treatment

Propolis	3g
95% Ethaanol	10 ml

APPENDIX IV

Reagents required for MTT assay

- **MTT stock** - MTT salt, 5 mg in 1 ml PBS.

ABSTRACT

ANTICANCER ACTIVITY OF STINGLESS BEE PROPOLIS ON HUMAN CANCER CELLS

Hari Sagar S. K.

(2016-09-023)

ABSTRACT OF THESIS

Submitted in partial fulfilment of the requirement for the degree of

B. Sc. - M. Sc. (INTEGRATED) BIOTECHNOLOGY

Faculty of Agriculture

Kerala Agricultural University, Thrissur

DEPARTMENT OF PLANT BIOTECHNOLOGY

COLLEGE OF AGRICULTURE

VELLAYANI, THIRUVANANTHAPURAM-695522

KERALA, INDIA

2022

9.ABSTRACT

The present research work entitled “Anticancer activity of stingless bee propolis on human cancer cell line” was carried out in the Department of Plant Biotechnology, College of Agriculture, Vellayani, Thiruvananthapuram and Department of Genomic Science, Central University of Kerala during 2020-2021, with the objective to study the effect of propolis collected from two different genera of Stingless bees, viz., *Lisotrigona* sp. and *Tetragonula* spp. on human cancer cell line- A549.

Crude propolis samples were collected from the nest of three species of stingless bees *Lisotrigona* sp. (Kollam), *Tetragonula calophyllae* (Thiruvananthapuram) and *Tetragonula travancorica* (Thiruvananthapuram). Propolis samples were macerated at room temperature and extracted with 95% ethanol. MTT assay was performed and IC-50 values were calculated in-order to determine the influence of propolis on the proliferation of human cancer cell line. Five different concentrations of propolis, viz., 150µg/ml, 300µg/ml, 450µg/ml, 600µg/ml and 900µg/ml respectively, were added to A549 cells for a period of 24 hrs.

Cells treated with the propolis extracted from *Lisotrigona* sp, *T. calophyllae* and *T. travancorica* obtained IC50 values of 485.822 ug/ml, 236.6095 ug/ml and 294.223 ug/ml respectively. It was observed that, propolis treatment had an inhibitory effect on A549 cells after 24 hrs treatment in relative to the control. When the cells were treated with the right concentration of propolis, marked difference in their morphology was observed. The cells appeared to shrink and were floating.Quality and quantity of the samples were analyzed through nanodrop, which gave an absorbance value of 1.8 to 2.0 for all the samples. The concentration of the samples observed was between 30-200 $ng\mu l^{-1}$. The RNA samples were then subjected to cDNA conversion (iScriptTM cDNA Synthesis Kit). The cDNA of control and treated samples were then subjected qRT PCR. The real-time quantitative PCR (qPCR) was performed with the Applied Biosystems PCR system in a total volume of 10 µl and expression of different genes was studied.

Gene expression analysis revealed that propolis taken from *Lisotrigona* sp. treated VIM was downregulated and it indicates that the cell migration and EMT formation are suppressed. propolis taken from *T. calophyllae* downregulated the expression of NODAL and it shows the inhibition of EMT formation. propolis taken from *Lisotrigona* sp. showed upregulation of CDX2 expression indicating that propolis from *Lisotrigona* sp. inhibits cell proliferation.

To conclude, the result of the study proved that, propolis obtained from the stingless bee *Lisotrigona* sp. showed more inhibitory effect on cancer cell line- A549 compared to *Tetragonula* spp.