TO INCREASE THE EFFICACY OF VITAMIN D2 ON CELL LINES BY USING *CISSUS QUADRANGULARIS*

Dissertation submitted in partial fulfilment of the requirement for the degree of

MASTER OF SCIENCE

IN

BIOTECHNOLOGY

By

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DECLARATION

I hereby declare that the work reported in the M.Sc. dissertation entitle "**INCREASING THE EFFICACY OF VITAMIN D2 ON CELL LINES BY USING *CISSUS QUADRANGULARIS*"** submitted at Jaypee University of Information Technology, Waknaghat, Solan, Himachal Pradesh, India, isa authentic record of my work carried out under the supervision of Dr. Udaybanu M , Dept. of Biotechnology and Bioinformatics, Jaypee University of Information Technology, Waknaghat, Solan, Himachal Pradesh-173234, India. I have not submitted this work elsewhere for any other degree or diploma.

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SUPERVISOR'S CERTIFICATE

This is to certify that the work reported in the M.Sc. dissertation entitle "**INCREASING THE EFFICACY OF VITAMIN D2 ON CELL LINES BY USING *CISSUS QUADRANGULARIS***" submitted by Bindu Sai Vadaga (207832) at Jaypee University of Information Technology, Waknaghat, Solan, Himachal Pradesh, India, is bonafide record of his original work has not been submitted elsewhere for any other degree or diploma.

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ACKNOWLEDGEMENT

On the very outset of this report, I would like to extend my sincere and heartfelt obligation towards all the personages who have helped me in this endeavour. Without their active guidance, help, cooperation and encouragement, I would not have made headway in the project. I am ineffably indebted to Professor Dr. Uday banu M for conscientious guidance and encouragement to accomplish this project. I am grateful to PhD guide Miss Diksha Manhas for guiding me and sharing his knowledge with me throughout the project from starting to the completion. I am extremely thankful and pay my gratitude to my lab partner Mr. Shubham Pal and Miss Harshita shringi for their support on completion of this project in its presently. I extend my gratitude to JUIT and Dr. Sudhir syal HOD of Biotechnology and Bioinformatics JUIT, for giving me this golden opportunity to do this wonderful project who has provided me with all the facilities to complete this wonderful project. I also acknowledge with a deep sense of reverence, my gratitude towards my parents who have always supported me morally as well as economically. At last, and but not the least my gratitude goes to all my friends and fellow peers who had directly or indirectly helped me in different aspects of this project

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Abbrevations:

DNA- Deoxyribonucleic acid

H_2SO_4- Sulphuric Acid

NaOH- Sodium Hydroxide

RNA- Ribonucleic Acid

IU- International Units

UV – Ultraviolet

Vit D2- Vitamin D2

Vit D3- Vitamin D3

VDD-Vitamin D deficiency

VDR- Vitamin D Receptor

ABSTRACT

Vitamin D deficiency remains a global public health issue. The major function of vitamin D in vertebrates is maintenance of calcium homeostasis, but vitamin D insufficiency has also been linked to an increased risk of hypertension, autoimmune diseases, diabetes, and cancer. Natural sources of vitamin D (dietary and UVB exposure) are limited, and thus mechanisms are needed to allow individuals to achieve the new dietary recommendations. The two forms of vitamin D are ergocalciferol (vitamin D 2) synthesized by exposure of ergosterol in plants to UVB radiation and cholecalciferol (vitamin D 3) synthesized in the skin of human by UVB radiation. However, the efficacy of vitamin D2 is less as compared to Vitamin D3. As Vitamin D3 is derived from animal-based sources, vegans and vegetarians may not prefer animal sourced supplements. For this reason, D2 derived from plant-based sources is a good choice for them. *Cissus quadrangularis* plant have been used to cure osteoporosis and, in some studies, they have been showed that when used in combination with Vitamin D3, it increases the efficacy of it. So, in this study by using extract of this plant attempt has been made to increase the efficacy of Vitamin D2.

CHAPTER 1

INTRODUCTION

1.1 Basic Introduction

Vitamin D is distinctive as compared to all the other vitamins because of its diverse source available [1]. It is a steroid fat-soluble prohormone which increases the interstitial absorption of phosphate, $Ca^{+2,}$ and magnesium level in our body. They can be of two major forms on the basis of food source.

1.2 Types of Vitamin D

The first form of Vitamin is Vitamin D2 which is also called as Ergocalciferol. The second form of Vitamin is Vitamin D3 which is also called as Cholecalciferol Vitamin D2 is an artificial derivative steroid which is present in plants mostly in fungi. It is made by some plant when exposed to UVB light. It is synthesized when plant sterol ergosterol is irradiated when exposed to UVB light [2]. Vitamin D3 also called as Cholecalciferol is a natural derivative

sunshine hormone which is synthesized in our body in the exposed to sunlight. Major source of it is egg yolk, oily fish etc. It is synthesized in lower epidermis by UV irradiation by 7-dehydrocholesterol [2]. Both of the Vitamin function as prohormone, the only difference between them structural change in side chain. The only difference between them is that double bond is present between Carbon 22 and Carbon 23 and at Carbon 24 methyl group is present on Vitamin D3. Both of the vitamin is well absorbed in small intestine.

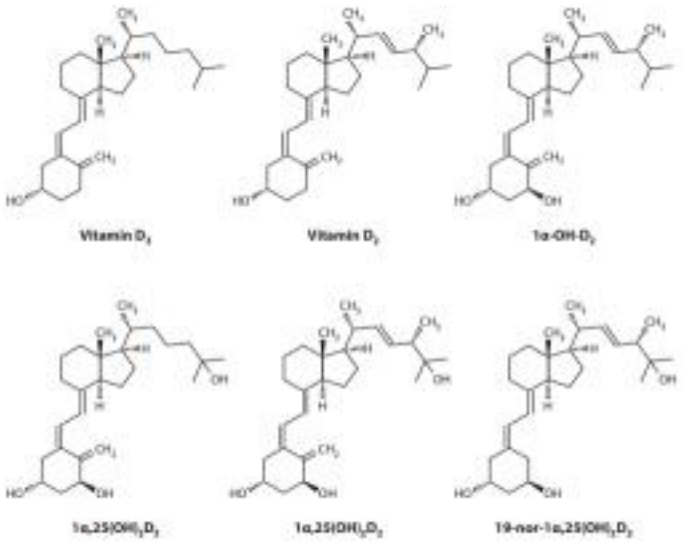


Figure 1.1: Structure of Ergocalciferol and Cholecalciferol from [3]

1.2 Deficiency of Vitamin D

It is most common problem globally which is mostly observed in older and sick individuals [4]. Majority of the population (1Billion) suffer from this deficiency worldwide. As most of the people get Vitamin D by exposure to sunlight and with small amount in the form of food. Another reason for low vitamin is reduced outdoor activities, and environmental factor like air pollution which affect the synthesis of it in our body. It not only affects muscoskeleton but also causes acute and chronic diseases. Vitamin D deficiency cause many diseases which include rickets, hyperthyroidism, osteoporosis, cancer, diabetes etc [3]. Dietary source of Vitamin D is important during winter season as there is insignificant synthesis in our body [5].

1.3 Role of Vitamin D

The major role of it is in gut where calcium absorption takes place and serum phosphate and calcium are maintained in level in the body which allows mineralization of bone and hypercalcaemic tetany is prevented. It is also needed in osteoblast and osteoclast for bone growth and bone remodelling. Due to low levels of Vitamin D, bones conformation is changed, now these changes include in which it becomes delicate or brittle, thin, and bends out of shape. It can also lead to rickets which is caused in children and in case of adults osteomalacia occurs. Vitamin D, in combination with calcium will prevent the osteoporosis in old people [6].

Some functions of this prohormone which take place in the body include reduction in inflammation and it also control some of activities of the cell such as proliferation, neuro muscular immunological, and metabolism of sugar or glucose [7].

Table 1.1: Different age group and recommended intake of Vitamin D

AGE	**RECOMMEDED IN TAKE (International Units per day)**
Toddlers and Teenagers (0-18 years)	400-600
Adult (19-70 years)	600
Older (70 years above)	800

Pregnancy and lactation	600

1.5 Basic Difference between Vit D2 and Vit D3

However, both of the prohormones (D2 and D3) functions and metabolism is almost same but there are problems being faced when it comes to Vitamin D2. As Vitamin D needs to bind

VDR known as vitamin D receptor in order to carry out its function in increasing the 25 - Hydroxyvitamin D level in our body. 25-Hydroxyvitamin D is the marker of Vitamin D in our body and generally when this prohormone is being checked 25(OH) D is being measured out in our body. Despite the fact that both D2 and D3 have been said to have similar hydroxylation process that will form the similar metabolite which is known as calcitriol. It has also been suggested in some of study that both of the Vitamin (D2 and D3) can raise the serum 25-

Hydroxyvitamin D level differently which, the established marker for status of vitamin D. It has been shown that Vitamin D2 is less efficient in raising the serum 25-Hydroxyvitamin D than D3 [2].

When given as 4 thousand international units per day is given for two weeks or when fifty thousand international unit is given as a single dose, vitamin D2 was not able to raise the blood 25-Hydroxvitamin D level and was less efficient. It was also seen that when single dose of fifty thousand international units was given to normal healthy person the level of Vitamin D decreased rapidly in the serum which proves that it not only is less effective but gets degraded fast. While some of the drawbacks of Vitamin D2 include that it has lower affinity for Vitamin binding receptor then Vitamin D3 and its metabolites. Vitamin D2 has shorter duration of action and is more sensitive to humidity and fluctuations in temperature.

1.6 *Cissus quadrangularis* plant to increasing the efficacy of Vitamin D2

Cissus quadrangularis is an edible climbing shrub with a thick quadrangular fleshy stem present in the hotter portions of some countries like Malaya, India, Java, Sri Lanka, and Africa. The plant is also called as "Asthisamdhani" in Sanskrit and also known as settler of bone and in Hindi language it is also called as "Hadjod" as it will join. In ayurveda, some the uses of this plant have some medicinal properties which is used in treating some of the diseases like piles, dysentery, syphilis, osteoporosis, irregular menstruation, obesity and some heart diseases.

Therefore, it is used in bone health and weight loss and studies has shown that extract of cissus quadrangularis when mixed in some proportion with Vitamin D3 increased it activity and efficacy in the body. In this study we are trying the same for Vitamin D2 and trying to increase the efficacy of Vitamin D2 by using this extract.

1.6 Objective

1) To study the differences in potency or efficacy of Vitamin D2 and Vitamin D3.
2) To increase the efficacy of Vitamin D2 on cell by using *cissus quadrangularis*.

CHAPTER 2

REVIEW OF LITERATURE

2.1 Vitamin D role

In 1930s, the ability of ultraviolet irradiation of yeast extract to produce an anti-rachitic substance known as vitamin D was discovered. This structure was the called as ergocalciferol [8]. In response to sun exposure, the human body can produce vitamin D. Some people, however, may need to supplement their consumption with certain meals or supplements [9].

Vitamin D was first characterised as a substance that could heal rickets and was given the letter 'D' because it was the fourth vitamin identified. It has two isoforms which is basically based of source of food – D2 and D3 [10]. Both of the prohormones can assist by meeting vitamin D needs. Both of the Vitamin is absorbed first by the lymph system and then enters into the circulation of venous. When it is obtained from the diet and skin, it is present in inactive form and must be converted into active form to 25- Hydroxyvitamin D in the presence of enzyme 25-OHase in the liver [11]. To create biologically active form, 25-Hydroxyvitamin d is hydroxylated in kidney by the enzyme 25 Hydroxy-D-1-OHase which is also called as CYP27B1 and gets converted into the form 1,25Hydroxy-2D [11]. Ca^{+2} absorption which take place in intestine is stimulated by 1,25-Hydroxy-2D. [12] Only ten to fifty % of Ca^{+2} which is dietary and sixty % of P (phosphorous) absorption take place without vitamin D. Absorption of Ca and P can be raised to thirty to forty and eighty percent respectively when this vitamin is present is level [7].

2.2 HISTORY

2.2.1 DISCOVERY OF VITAMIN D:

Vitamin D is thought to have originated from world primordial living organism about Nine hundred Palaeozoic era. UV (ultraviolet) light battered primitive life forms which damaged Ultra Violet sensitive Deoxyribonucleic acid, ribonucleic acid and amino acid or proteins [12]. Ultra violet light around 240 and 320 nm is taken by pro-vitamin D making it a potential sunscreen. Provitamin D2 or ergosterol-produced from phytoplankton and zooplankton were much more inclined to have their nucleic acid safeguarded from Ultra violet rays, which allows them their genes to be passed into next generation [12]. Provitamin D2 was produced by phytoplankton which includes *Emiliania huxleyi* (E. huxleyi) 500 million years ago. Pro vitamin ergocalciferol was transformed to pre-vitamin ergocalciferol, which had been isomerized in the presence of Ultra- violet rays B thermally into vitamin D2. Animals further on the food chain

are thought to get their vitamin D via phytoplankton and zooplankton. It is needed for proper serum Ca^{+2} levels and in land vertebrates bone metabolism functioning. The skin of poikilothermic animals was discovered to contain multiple provitamin Ds, with 7-dehydrocholesterol being the most common (7-DHC, provitamin D3). Provitamin D3 levels in the skin of northern grass frogs as well as lizards have been found to be 1 to 2 magnitudes higher than in humans. As a result, some poikilotherms' skin can create a lot of Vitamin D3. This study shows that in poikilothermic terrestrial animals, cutaneous vitamin D production might just have played a significant evolutionary function [12]. Huldschinsky, a German physician, used a quartz lamp with mercury vapor which radiated Ultraviolet B rays to treat rickets patients in the early twentieth century. The children's condition improved radiologically after 6 weeks of treatment, as seen by increased mineralization on their X-rays. Hess and Unger discovered in 1921 that children's exposure to sunlight 3-4 times per week reduced their clinical and radiological rickets symptoms [13]. Mellanby was the first to establish that giving fish oil capsules to puppies may decrease the probability of causing rickets [14]. Black and Steenbock discovered that when Ultraviolet irradiation of meals were ingested by animals, they exhibited an antirachitic effect [15]. Cowell later established in 1925 in which milk which has bovine when treated for twenty minutes under a Hg lamp when ingested by children and teenager can used for curing of disease [16]. Vitamin D was added to a range of foods & beverages within two decades. Vitamin D has antirachitic effect in both people and animals, according to these findings. Endogenously generated vitamin D was once thought to be identical to vitamin D produced by irradiation yeast [13].

2.2 Sources

2.2.1 Sun exposure:

The majority of individuals on this planet obtain little amount of Vitamin D from sunlight. UVB radiation of wavelength 290- 320 nm penetrates into cutaneous layer of skin and pre vitamin D is formed by conversion of 7-dehydrochloestrol which in turn gets into converted into form that is active in the body [17].

The following are some of the factors that influence vitamin D synthesis when exposed to sunlight.:

a) Melanin content

b) Environmental factor like air pollution, smog, clouds etc

c) Sunscreen, which does not allow the sunlight to pass through skin depending upon the SPF content, for eg. SPF of 8 will block the sunlight on skin.

d) Season, for example during winter the synthesis of vitamin in our body is less

e) Length of the day and time of the day (is often said that vitamin D synthesis is maximum till 10 AM).

2.2.2 Food:

Some of the food naturally contain Vitamin D in it which include fish like tuna, rohu, salmon tuna and liver oil from fishes are considered to be finest source of it. Whereas yolk of egg, milk products like cheese and beef also contain small amount of V D in it. However, mushroom is good form of D2. Nowadays mushrooms are treated with UV so that the Vitamin D content is increased which has been approved by FDA. In most of the countries fortified food are used as good form of it. Some products like curd and milk are fortified by Vitamin D [17], [18].

2.2.3 Dietary supplements:

D2 or D3 vitamin both can be found in dietary supplements. D2 is typicaly produced when mushroom or fungi is exposed to Ultraviolet rays and in case of D3 is made naturally by our body in the presence of sunlight by conversion from sheep lanolin [19] or animal free version of Vitamin D3 was also made from lichen.

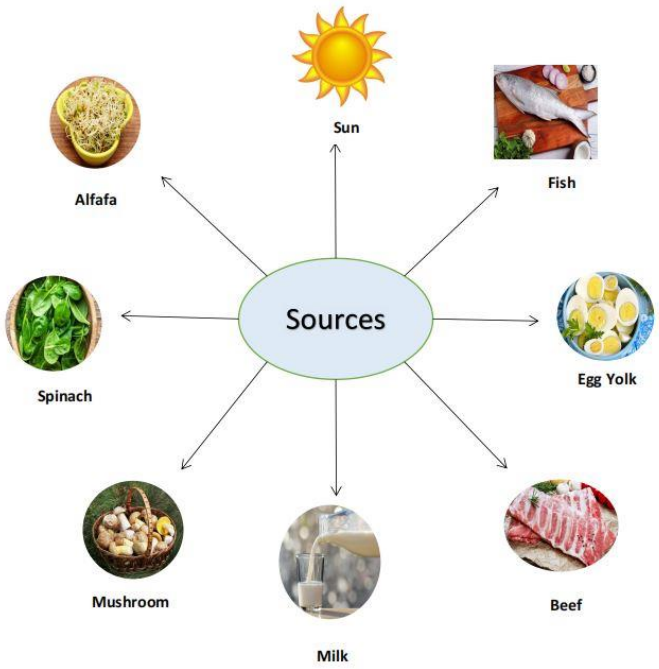


Fig 2.1: Food that contain Vitamin D

2.3 Vitamin d analogs

It contains 5 naturally occurring analogy, and 4 artificially synthesized analogs, which are synthesised.

2.3.1 Natural analogs:

Vitamin D1- it is a 1:1 chemical complex made up of ergocalciferol or Vitamin D2 and lumisterol. Vitamin D2 is made by invertebrates, fungi like yeast, some plant like alfafa by Ultraviolet. Vitamin D3 which is the natural form formed in our body when Ultraviolet B rays causes the reaction of 7-dehydrocholesterol. Vitamin D4 - 22-dihydroergocalciferol is the scientific name for vitamin D4. Vitamin D5 -Sitocalciferol (vitamin D5) is an analogue of 7-dehydrositosterol [20].

2.3.2 Synthetic analog:

Maxocalcitol -is the first analogue discovered with a wider duration of treatment 1,25 Hydroxy2D3 [21]. Calcipotriol- it was discovered during vitamin D treatment trials for osteoporosis [20]. DHT – also called as Dihydrotachysterol is a lab made version of D vitamin which is thought that it is stronger to naturally occurring form of vitamin s It does not require hydroxylation inside the kidneys to become

active [20]. Paracalcitol-class of vitamin to receive approval from FDA to secondary hyperparathyroidism, it is different from chemical calcitriol because of the absence of C-19 exocyclic one and it has D2 side chain [22]. Tacalcitol - D3 derivative. Keratinocytes inside the skin are known to be hampered by it [20].

2.4 Deficiency of Vitamin D

It is a global health problem [23]. Vitamin D deficiency occurs when 25- Hydroxyvitamin D level is < 0.8 International Units in the body [19], [24]. It is common in many countries like Australia, Africa, India and America [19]. Vitamin D's most well-known role in bone metabolism and skeletal health is through calcium and phosphorus hemostasis [25]. This deficiency has been related to increase in chronic disease like diabetes type 2, cancer, heart related disorder, some disorder including at metabolic level [26]. Prohormone D affects inflammation, the system which include rennin and angio-tensin, and blood pressure, all of which contribute to cardiovascular disease.

Bone metabolism, calcium, and phosphorous level are all affected by VDD. It has reduced the nutritional Ca^{+2} and P level absorption which causes the Parathyroid level to rise [11], [19], [24]. Osteoclastic activity is increased by parathyroid hormone which causes the deterioration of bone and loss of some mineral density from bone which led to cause some disease like osteoporosis and osteopenia [19], [27]. Mineral deficiency in the bones is caused improper Ca^{+2} and P levels in the body. Therefore, wide number of bone malformations take place in toddler and teenagers who are at their growing age and develop a condition called as Rickets [28]. It led weaking in muscles, affected youngsters have difficulties standing and walking, whilst the older people experience dizziness, and increased regular fall, increasing the risk of fracture [29], [30].

2.4 Groups which are at high risk of VDD

2.4.1 High melanin content:

Melanin is a pigment responsible for dark pigmentation of the skin present in epidermis later. People with darker complexion has greater melanin content than people with fair complexions. However, this pigment prevents to produce Vitamin D. It has been observed that people with dark skin tone or high melanin have low serum 25 Hydroxyvitamin D level in their body [31].

2.4.2 Older adults:

Among all the groups, this group is at high risk of developing deficiency as with age skin’s ability to

synthesize decreases. It is generally preferred that elderly people are given High dose of V D.

2.4.3 Infants(breastfeed):

Human milk alone does not usually offer enough vitamin D for new-borns to meet their needs because it contains less than 25 to 78 IU/L (0.6 to 2.0 mcg/L) [17]. Vitamin D levels in human milk are linked to status of prohormone level present in mother; studies have shown t moms who take daily vitamin supplements containing at least 2,000 IU have higher amount vitamin D3 in their breastmilk [32].

2.4.4 Limited sun exposure:

In this group, people with limited sun exposure are unable to synthesize vitamin D in their body, as it is made in our body when sunlight passes the epidermis of our body. Also, applying sunscreen also inhibits the mechanism of action [18].

2.4.5 Fat malabsorption patients:

Because vitamin D is high in saturated fat, its absorption is based on the gut's capacity to absorb dietary fat [33]. Fat malabsorption causes numerous medical problems, which include obesity, some liver disorder, Inflammatory bowel syndrome like Crohn’s and UC, and celiac [17], [34]. In addition, they are more prone of deficiency, patients with certain illnesses may avoid or consume only modest amounts of specific food like milk and curd fortified with this vitamin. Vitamin D supplementation may be necessary for people who have trouble absorbing dietary fat [34].

Fig 2.2: Disease caused by VDD

2.5 Diseases caused by VDD

2.5.1 Osteoporosis:

Is a disease which occurs in elder generation. Some of changes include weakening of bones followed by less bone density because of less level of Ca^{+2} in the body.

2.5.2 Cancer:

Vit D inhibits carcinogenesis because it decreases proliferation of cells which in turn increases the differentiation of cells. Angiogenesis is also affected by it. Vit D has some properties which are immunomodularity, anti-inflammatory, proapoptotic and antiangiogenic [18]. Connection between Vit d and cancer has been observed in some of studies which tells that people with low levels of Vit D in their body are more prone to cancer (prostate cancer) and people with high

level of Vit D are at major risk of developing colon cancer [35].

2.5.3. Type 2 diabetes:

As Vit D plays a major role of metabolism of glucose. VDR in pancreatic beta cells enhance insulin production, whereas VDR show resistance to insulin in liver and muscular cells [36]. Low 25(OH) level is indicator of diabetes type II and low level of 25(OH) in a person are more prone of developing this disease.

2.5.4 cardiovascular disease or heart disease:

Patients that have low Vit D level are at major risk of developing heart disease. It aids in the regulation of the blood, maintains some pathway(fibrotic)and inflammatory, as well as vascular cell growth [37]. Dysfunctioning of vascular system, hyperlipidemia stiffness of artiness and hypertrophy of ventricular (left), are all linked to vitamin D insufficiency.

Vit D have been related to issues of heart and increases the probability of cardiovascular disease for these reasons [38].

2.5.6 Rickets:

It is a condition in which bone softening and weaking occurs in children due to vitamin deficiency. As Vitamin D is important for bone and skeletal health. In intestine, it encourages enterocyte development and Ca^{+2} and P uptake. It led to bone mineralization. Vit D maintain the Ca^{+2} and P level in the body(blood) by resorption of bone in hypophosphatemia and hypocalcaemia. Hypocalcaemia and hypophosphatemia are caused by vitamin D deficiency or resistance [39].

2.5.7 Gastrointestinal Disease:

Due to abnormal enterohepatic circulation, absorption decrease of Vit d and 25, Hydroxyvitamin is observed when diseases occur in small intestine or liver which has more cholesterol, diseases in pancreas and obstruction in biliary hepatic system. Vitamin D malabsorption is caused by when emulsification of fats and absorption mediated by chylomicron is disrupted by steatorrhea. Many people may suffer from low bone mass, rickets in children, in adults osteomalacia, or just have low bone density. Celiac, IBS, allergies of some food, cystic fibrosis and cholestasis, are all common examples [40].

2.5.8 Liver Disease:

Calcidiol [25Hydroxyvit D] is formed when Vit d is hydroxylated in liver. As a result, it has been that sick person has low level of 25 (HydroxyVit D) which has issue in liver and parenchyma. These people will only show biochemical or histological signs of osteomalacia if there is a nutritional deficiency or a blockage of the enterohepatic circulation [40].

2.5.9 Obesity:

Low circulating vitamin D levels are frequent in obese people, and they are at major risk of developing heart or cardiovascular diseases and some malignancy. BMI, waist circumference, and fat present are all related inversely to level of 25- HydroxVit D [20].

2.5.10 Parkinson Disease:

This disease is the leading disability cause among the elderly. Regrettably, the disease's susceptibility factors remain mostly unknown. Recently, it has been postulated that low vit D intake may play major a role in development of this disease. Low vit D levels have been linked to the development of this disease which is a study based on the Health Survey by Mini finland [41].

2.5.11 Depression:

Vit D is engaged with variety of activities of brain and VDR can be found is region of brain which include glia and parts of the that are hypothesised to implicated in pathogenesis of depression [42]. After oneyear, overweight patients who received twenty to forty thousand international units' weekly dosage of Vit D experienced a substantial improvement in scale scores of depressions compared to those who received a placebo The findings reveal a connection between vit D and the incidence to depression [20].

2.6 Vit D Production

Vitamin D3 production is not enzymatic process, first 7-dehydrocholestrol with the help of 2 step process get converted into Vitamin D when UV light of 280- 320 (UVB) from the sun where B ring is broken and get converted into pre-vitamin D. Now this isomerization occurs of pre-vit to vit D to vitamin D3 which is thermos sensitive but non catalytic process. Vit D2 is synthesized in the presence of Ultraviolet rays where irradiation of ergosterol take place present in some fungi and plants. Ergosterol first get converted into pre-vitamin D2 when UVB light

range is provided. Pre-vitamin D2 is further isomerized into vitamin D2[43].

2.7 Vit D Metabolism

Once Vit D is obtained which is made in the epidermis layer of skin or obtained from dietary source (D3 and D2 respectively). It then enters the circulation by Vitamin D- binding proteins. It is then transferred into liver where hydroxylation of Vitamin D at 25 position occurs and form 25-Hydroxylase D [19].

25HydroxyVit D then circulates into kidney where with the help of 1-α- hydroxylase enzyme gets hydroxylated at 1 position to form 1,25- dihydroxy vitamin is the active or hormonal form of Vit d. 1,25 dihydroxyvit D enters target cell coupled to vitamin D–binding protein, binds to the VDR inside the cytoplasm, after which it enters inside the nucleus and heterodimers which has receptor of retinoic acid increases the transcription rate of Vit D whose genes are needed for Ca^{+2} and P, bone metabolism of bone, and some other functions gene inhibition which is important for growth of cancer.[44].

The 24- hydroxylase catabolizes circulating 1, dihydroxyvit D to generate 1,24,25, dihydroxyvit D an inactive vitamin D molecule. By boosting the expression of the 24-hydroxylase, 1,25dihydroxyvit D promotes catabolism Anticonvulsants & rifampin, for example, have recently been discovered to promote 1, dihydroxyvit D catabolism which stimulates the receptor of pregnane, leading to increased production of enzyme 24-hydroxylase [45].

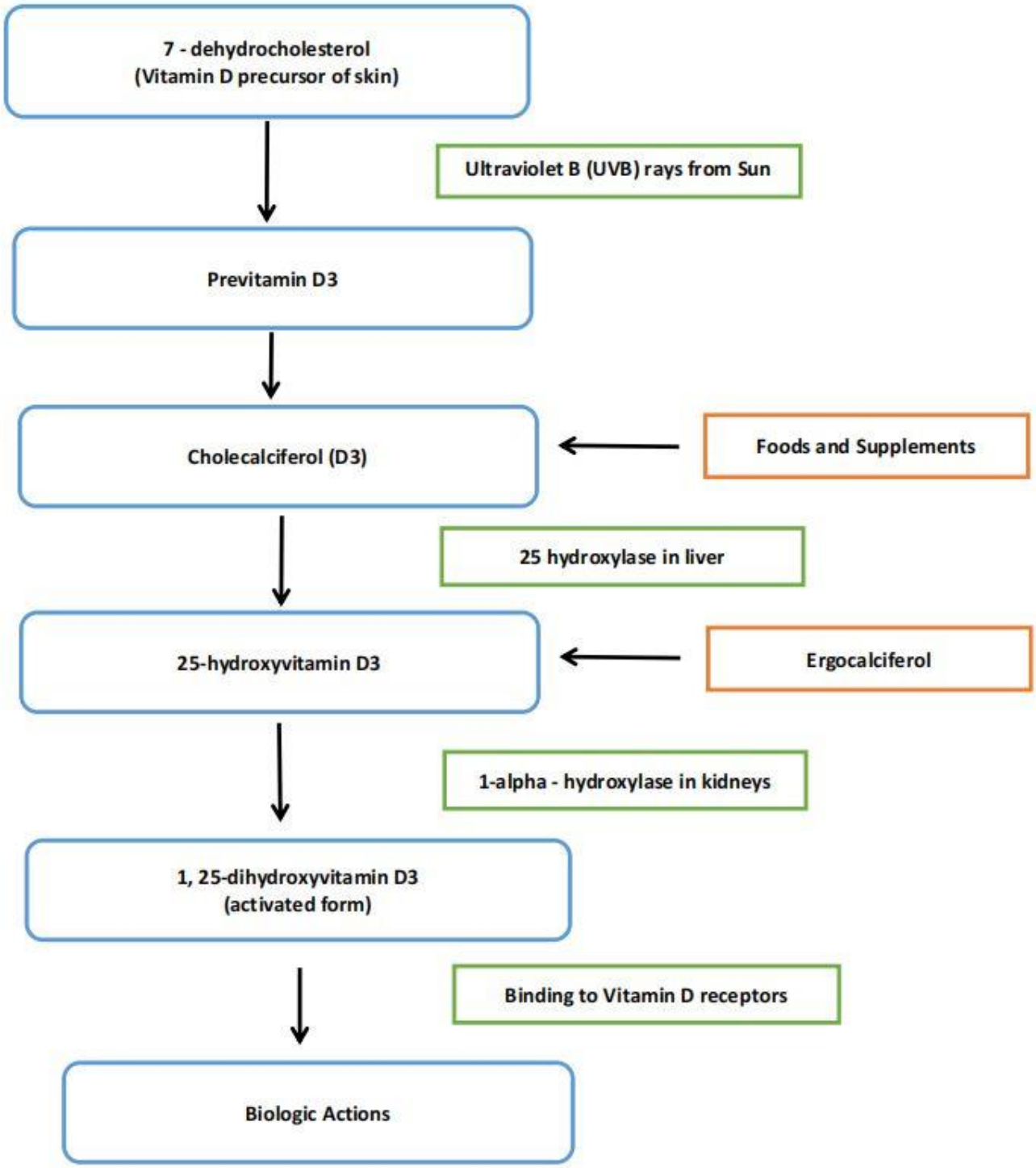


Fig 2.3: Vit D metabolism in body starting from liver to its site of action

2.4 Assay of Vit D

Biggest marker indicator of vit D level, status and storage of level of the 25(OH)D. 25 dihdroxyvit d is one of the most major circulating form of Vit D whose half-life is of two three weeks whereas the half-life of 1,25dihdroxyvit d is almost 4 hours, which circulates in less amounts than 25dihydroxyvit D, and therefore is strictly modulated by Parathyroid hormone, Ca^{+2} and P , so it doesn't decline appreciably unless severe VDD is present. Its concentration is also linked to kidney function rather than insufficiency of Vit D. It is of no use for vit D levels. Because Vit D2 and Vit D3 derivatives of 25-hydroxyvitamin D are biologically active following 1--hydroxylation, the vitamin D assay should assess both.

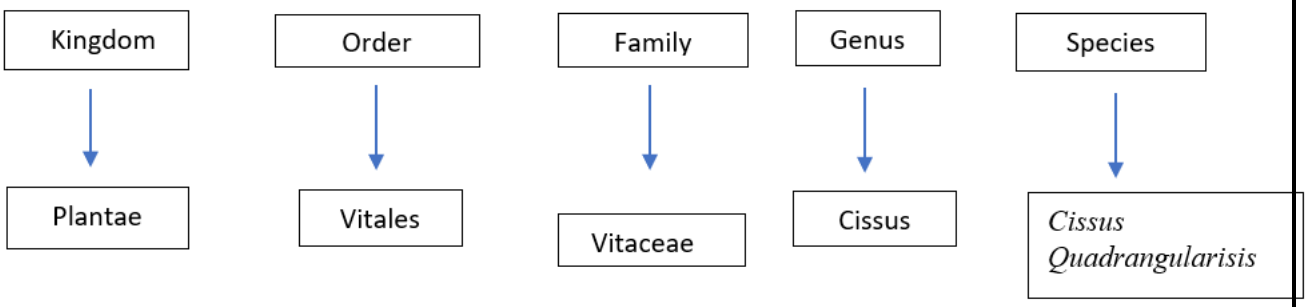
Vitamin D status is determined using a variety of commercially available 25(OH)D tests. In which chemical assays such as HPLC and MS as well as binding assay was performed – immunoassays which include radioimmunoassay (RIA), chemiluminescent immunoassay (CLIA), enzyme immunoassay (EIA). The gold standards for vitamin D analysis are HPLC and liquid chromatography-mass spectroscopy (LC-MS), which provide a greater vitamin D level. Total value is reported by immunoassays and protein-binding tests. Both of the can be measured using chemical tests.

2.5 Cissus quadrangularis plant

2.5.1 Background:

Cissus quadrangularis is a tropical perennial herb with medicinal characteristics found all over the world. It is one of India's most widely utilised medicinal plants. The plant is said to be indigenous to many parts of countries like India, java and all. The phytochemical composition, pharmacological actions, and toxicological evaluation of this plant are all being investigated. It helps to repair bones [46]. This plant is used in Ayurveda to treat a variety of diseases. synonym for *cissus quadrangularis* also referred as horjora in Hindi & pirandai in Tamil, is a member of the Vitaceae family. The plant can be found in Asia and Africa's tropical forest regions [47], [48].

2.5.2 Classification of Plant:



Different Parts of *cissus quadrangularis* plant is used in treating of the diseases.

1) For bone fracture, stems and roots of *cissus Quadrangularisis* uses.

2) Limewater along with stem paste is given to asthma patients.

3) To treat irregular menstruation, epitaxies and otorrhoea stem juice of used.

4) Cattle has been fed by herbs of it to increase the milk flow.

5) To treat some bowel infection and haemorrhoids herb powder is used.

CHAPTER 3

MATERIALS AND METHOD

3.1 Chemicals and reagents used

Plant Material (Cissus), Ethanol, muslin cloth, Soxhlet extractor, isomantle, condenser, Molisch reagent, Ninhydrin reagent, $FeCl_3$, H_2SO_4, Chloroform, Glucose, Anthrone reagent, Bovine Serum Albumin, Sodium carbonate, NaOH, $KNaC_4H_4O_6 \cdot 4H_2O$, $CuSO_4$., Folin Ciocteau , Quercetin, sodium nitrate, Al_2Cl_3, crystal violet, iodine, ethanol, safranine, KOH, H_2O_2, MR broth, methyl red, DPPH, Galic acid, methanol, ABTS, Potassium persulfate, Resazurin, Muller Hilton agar, Muller Hilton broth.

3.2 Process for Plant Extraction

Plant extract was prepared using plant *cissus quadrangularis* and for the extraction procedure two method were used which are Soxhlet extraction and Cold maceration.

3.2.1 General method for Solvent Extraction:

250.0 mL of ethanol (solvent) was put to RB flask attached to an isomantle with a Soxhlet extractor and condenser. The thimble is loaded with 20 gm of plant material (inside the Soxhlet extractor). Solvent is heated in isomantle and vapours are formed which then goes to condenser. The vapours will gets collected into the reservoir which has thimble. After the solvent is reached to syphon, it is then poured backed to the flask and cycle is restarted again. For 2-3 days, the process was conducted for 12 hours. A rotary evaporator was used to evaporate the solvent. After evaporation, the plant material was refrigerated at 4°C for later use. 9) The following equation was used to obtain the plant's % yield.

$$\text{Extraction yield (\%)} = \frac{\text{Weight of extract after evaporating solven and drying}}{\text{Dry weight of the sample}} * 100$$

Fig3.1: Pictorial presentation of Soxhlet Extraction

3.2.2 General method for cold maceration:

4g of plant extract was weighed. Plant extract was dissolved in 20 millilitres of autoclaved H_2O and 20 millilitres ethanol (1: 1 ratio). It was incubated in shaking incubator for 24 h at 37° C. Filter it with Whatman No. 1filter paper. Filtrate was collected at allowed it to dry. The filtrate was further stored in refrigerator at 4 °C.

Fig 3.2 Pictorial representation of cold maceration

3.3 Methods for Plant Extract Characterization

Plant extraction characterization can be done by qualitative and quantitative characterization.

3.3.1 Methods for Qualitative characterization:

3.3.1.1 Analysis of phytochemical in *cissus quadrangularis* plant:

A) Molisch Test or carbohydrates Test:

From 10mg/ml concentration of cissus quadrangularis plant extract 1ml was added in test tube ,2-3 drops of Molisch reagent was added in same test tube. After that, few drops of 2-3 drops of sulphuric acid were added. Occurrence of violet ring indicates the presence of carbohydrate in the extract solution [50], [51].

B) Ninhydrin test or Amino acid test:

From 10mg/ml concentration of cissus quadrangularis plant extract 1ml was added in test tube and then few drops of ninhydrin reagent was added in same test tube. Test tube was placed in hot water bath at 40 degrees Celsius for 7-10 minutes. Occurrence of purple or blue colour indicates the presence of amino acids [50] [51].

C) Foam Test or saponin Test:

From 10mg/ml concentration of cissus quadrangularis plant extract in test tube. Few drops of double distilled water was added in same test tube and shaked vigorously. Emergence of foam indicate the presence of saponin [50], [51].

D) Ferric chloride or Tannin test:

From 10mg/ml concentration of cissus quadrangularis plant extract 1ml was added in test tube to that test tube few drops of $FeCl_3$ solution was added. Occurrence of deep/ dark blue colour indicate the presence of tannin [50], [51].

E) Salkowski test or Glycoside Test:

From 10mg/ml concentration of cissus quadrangularis plant extract 1ml was added in test tube .2 ml of chloroform was added and then mixed properly, 2 ml of $H_2SO_4$was added to the test tube. Occurrence was reddish brown colour ring indicate the presence of glycoside in the extract solution [50], [51].

3.3.2 Method for Quantitative Characterization:

A) General procedure for estimation for carbohydrates:

Different concentration of glucose (0, 2.0, 4.0,6.0,8.0 and 10.0 milligram per millilitre) were prepared from stock (10mg/ml) .10 milligram per millilitre of standard extract of plant was prepared .1 millilitre of test sample which can be plant extract or different concentration of glucose were added and four millilitre of reagent was added (anthrone). Test tube was kept at 100 °C (8- 10 minutes). Absorbance was recorded using spectrophotometer at 630 nm [52].

B) General procedure for estimation of protein

Reagent 1 was prepared by taking 48ml of $NaCO_3$ with 0.1 N NaOH, 1ml $KNaC4H4O6{\cdot}4H2O$,1 ml of $CuSO_4$. Reagent 2 was prepared by taking 1:1ration of Folin reagent and distilled water. Different concentration of BSA (0,2.0, 4.0, 6.0,8.0,10.0 milligram per millilitre) was prepared. 10mg/ml of plant extract was added in test tube. 1ml of test sample was taken and 4 ml of Reagent A was added. It was then incubated for 10 min at 30°C. 0.50 millilitre of B reagent is added to the test tube. The test tubes were incubated for 20 min in dark. Absorbance was measured by spectrophotometer at 750nm [53].

General Procedure for Flavonoid Content:

1 gram of Quercetin is dissolved in 100.0 millilitre of methanol to get final concentration of 1% quercetin(10mg/ml). Different Dilution of Gallic acid was prepared so that standard curve can be plotted (2.0,4.0,6.0,8.0,10.0) in methanol.150.0 microlitre of Al_2Cl_3 was then put and allowed for 5 minutes of incubation, then 200 µl of sodium hydroxide was added. Record the absorbance 510 nm. Content of flavonoid can be calculated from quercetin equivalents [54].

3.4 Methodology for estimation of Biochemical Activity of microbes

3.4.1 General procedure for Gram staining:

Gram staining method is used to differentiate between gram positive and negative bacteria on the basis of peptidoglycan layer. Bacterial suspension was prepared on glass slide and air dried. Crystal violet was added and kept for 30 sec to 1 min and was rinsed with water. Later, iodine stain was added for 1 min and was washed with water. 95 % ethanol was added for 30 sec followed by safranin for 1 min and was washed with water. It was then air dried and observed under microscope.

3.4.1 General procedure for Catalase:

Catalase test is used to differentiate between bacteria that produce enzyme catalase. By using a sterile loop, small colony of bacteria was picked up from agar plate. It was then transferred to clean glass slide and 3% H_2O_2 was added. Evolution of oxygen bubbles was observed.

3.4.3 General procedure for KOH test:

Is used to differentiate between gram positive and negative bacteria on the basis of cell wall lysis. Bacterial specimen was kept on the clean slide.20% KOH was added. It was then observed under microscope.

3.4.4 General procedure for MR test:

Prepare MRVP broth in test tubes. Inoculate the broth aseptically with 2 loopful of respective bacterial culture. Label the test tubes with name of organism inoculated. Incubate the test tubes at 37°C for 48-72 hours. Add few drops of methyl red indicator in the incubated tubes. Observe the results.

3.5 Estimation of biological activity of *cissus quadrangularis*

3.5.1 Method to check anti-oxidant activity of *cissus quadrangularis*:

3.5.1.1 DPPH Assay procedure:

Fresh solution of 0.002 % (2,2-diphenyl-1-picryl-hydrazyl-hydrate) was made by dissolving in solvent methanol and for standard value record the absorbance of it at 515.0 nanometre Different concentration of Galic acid were prepared (0,2,4,6,8,10) in methanol. 50 µl of extract of plant (1.0 milligram per millilitre) were added to 3ml (2,2- diphenyl 1-picryl-hydrazyl-hydrate and was allowed for 15 min incubation at RT [55]. 4. Record the absorbance at 515 nanometres. Percentage inhibition of (2,2-diphenyl-1-picryl-hydrazyl-hydrate) calculated the by formula.

$$\% \text{ Inhibition} = \frac{(A-B)}{A} * 100$$

Where, A= (2,2-diphenyl-1-picryl-hydrazyl-hydrate) in pure form absorbance (oxidised) and B= sample absorbance taken after incubation for 15 minutes. Calibration curve was drawn by % inhibition to Different concentration of Gallic acid to determine the IC50 value of extracts

i.e., the concentration at which 50 % (2,2-diphenyl 1-picryl-hydrazyl-hydrate) is scavenged. All the experiments were performed in triplicate [55].

3.5.1.2 ABTS Assay procedure

7mM of (2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid)) with 2.450 millimolar of potassium persulfate was mixed and added in 100 ml of methanol and was kept in dark for 16 hours. Different dilution of galic acid was prepared (0.0,2.0,4.0,6.0,8.0,10.0) mg/ml. Absorbance was taken at 745 nm (it should be around 0.7) . Test sample was added with 3.0 millilitre of ABTS and incubate it for 6 mins 5) Record the absorbance at 745 nm (Galic acid was positive control). % ABTS scavenging potential was calculated by the formula [56].

$$\frac{\text{Control absorbance} - \text{sample absorbance}}{\text{Control absorbance}} * 100$$

3.6 Method for anti-microbial activity of *cissus quadrangularis*

3.6.1 Well diffusion method:

Used to assess if plant or microorganism extracts have antibacterial properties. Bacteria was grown in Muller Hilton broth and turbidity was matched up to 0.5 McFarland standard so that it should have 10^6cells It was then inoculated on Muller Hilton agar and plates were allowed to dry and wells were punched by sterile cork borers. 50µl of plant extract was added to the well of different concentration (positive control was antibiotic and blank was DMSO) Plates were kept for 24h at thirty-seven degrees Celsius in incubator and was allowed to diffuse to form zone of inhibition. Zone of inhibition was measured of different bacteria [57].

3.6.2 MIC:

Stands for minimum inhibitory concentration in which the lowest dose of the any anti-microbial agent that might prevent the test organism under investigation from growing. In 96 well plate, 100 µl of Muller Hilton broth. 100 ul of plant extract was added to each well and 10 µl of bacterial ($3 * 10^6$cells) cells were added. 96 well plate was incubated for 24h at 37°C. Resazurin dye was added to each well. Observed for colour change from blue to pink. MIC was calculated [58].

3.6.3 MTT assay:

The MTT assay uses colorimetry to evaluate the metabolic activity of cells, which is a sign of their viability, proliferative potential, and cytotoxicity. The assay relies on metabolically active cells converting a yellow tetrazolium salt (MTT) into purple formazan crystals. The cells were suspended in the T- flask and trypsin EDTA was added to the flask to loosen the cells. After that, the T-flask was put inside the incubator for 2 minutes at a temperature of 37 °C to improve the enzyme's activity. The cells were centrifuged for 4-5 secs at 300 *g and was washed twice with PBS and centrifuged to form a pellet. It was then suspended into growth media. In 96-well culture plates, seed the cells at a density of 7000 cells/well and incubate for 24 hours at 37°C and 5 percent CO_2 was maintained. Cell viability was assessed after a 24-hour incubation period using the readily available MTT dye. Each well received 10 l of the produced 5 mg/ml MTT solution, which was then incubated for 4 hours at 37 °C and 5% CO_2 concentration. Following incubation, the media was withdrawn, 100 ul of DMSO was added to each well, and the wells were then incubated at a temperature of 37°C and a CO_2 concentration of 5% for 30 minutes. Cell viability was calculated and the OD at 570 nm was obtained using a spectrophotometer.

Cell death % was calculated by the given formula:

$$\% \text{ Cell death } = \frac{\text{OD control} - \text{OD sample}}{\text{ODcontrol}} * 100$$

3.7 Isolation of Peripheral Blood Mononuclear cells (PBMC)

4 ml of blood was collected into isocitrate tubes and was mixed gently by inverting the tubes 2- 3 times. In a fresh tube, 4 ml of Ficol histopaque was added and then slowly 4 ml of blood was added to the tube so that the layer is not disturbed. The tube was then centrifuged for 30 min at 4000 RPM. White buffy layer formed between plasma and ficol interphase was then isolated and stored for future use. PBMC; s was then stained with Giemsa stain and observed under the microscope. Giesma stains the leucocytes purple in colour.

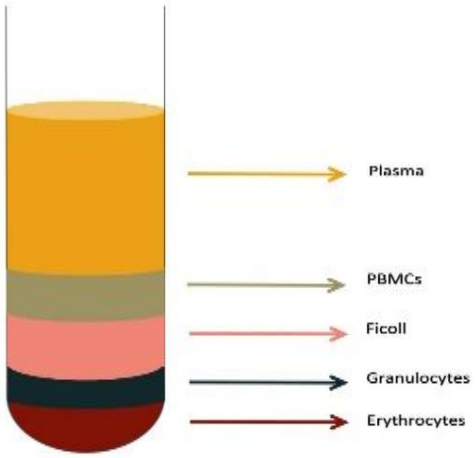


Fig 3.3 Blood separation by Ficol histopaque

3.8 Evaluation of Calcium deposition

PBMC's were seeded in Eppendorf tubes in PBS and incubated overnight at 37^0C. After 24 hours, PBMC's were treated with various concentrations of Vitamin D2, Vitamin D3 and different combinations of Vitamin D2, D3 and plant extract Cissus quadrangularis. This treatment lasted for 24 hours. Then media was aspirated with PBS and cells were fixed with formalin. Then the cells were stained with Alizarin red dye, incubated for 20 minutes, followed by washing with PBS. The stained cells were then observed under microscope.

CHAPTER 4

RESULTS AND DISCUSSION

4.1 Result of qualitative test

Qualitative test was done for the *cissus quadrangularis* plant extract and the result obtained for carbohydrate, amino acid, saponin, glycoside was positive and negative result was observed for tannin.

Table 4.1 Result of phytochemical analysis

Phytochemical test	**Result**	**Observance**
Carbohydrate (Molisch Test)	Positive	Violet ring was observed
Amino Test (Ninhydrin)	Positive	Dark purple colour was observed
Saponin test	Positive	Foam formation was observed
Tannin Test (Ferric chloride test)	Negative	Deep blue colour was not observed
Glycoside Test (Salkowski Test)	Positive	Reddish colour was observed

4.2 Result of Quantitative analysis

On performing different quantitative analysis on plant *cissus quadrangularis* extract different results were obtained and analysed. The result obtained where 5% glucose was found in plant cissus *quadrangularis* extract in 10 mg/ml concentration and 4.7% protein was found from 10 mg/ml concentration of extract and the flavonoid content is 75 ± 0.74 QE/g DW.

4.3 Result of Antioxidant activity

On performing antioxidant activity on plant *cissus quadrangularis*, the result was obtained:

For DPPH assay:

Table 4.2: DPPH scavenging activity of hydrolytic extracts of *cissus quadrangularis*

Concentration (ug/ml)	**% Inhibition *On cissus quadrangularis***
0	0
250	19.18
500	38.95
750	54.06
1000	70.06

From this IC50 Value was calculated from equation, (IC_{50} = MX+N) where, IC_{50} = 0.0707x + 1.2791 and the value obtained is 0.689 mg.

For ABTS assay:

Table 4.3: ABTS activity of hydrolytic extracts of *Cissus quadrangularis*

Concentration (ug/ml)	**% Inhibition On *cissus quadrangularis***
0	0
250	93.58

500	94.70
750	95.71
1000	96.8

from this IC50 Value was calculated from equation (IC_{50} = MX+N)

IC_{50} = 0.0043x + 92.506 and the value obtained is 0.166 mg.

4.4 Result of extraction Yield

The extract yield was obtained from *cissus quadrangularis* plant was 20 percent and it was calculated using formula:

$$\text{Extraction yield (\%)} = \frac{\text{Weight of extract after evaporating solven and drying}}{\text{Dry weight of the sample}} * 100$$

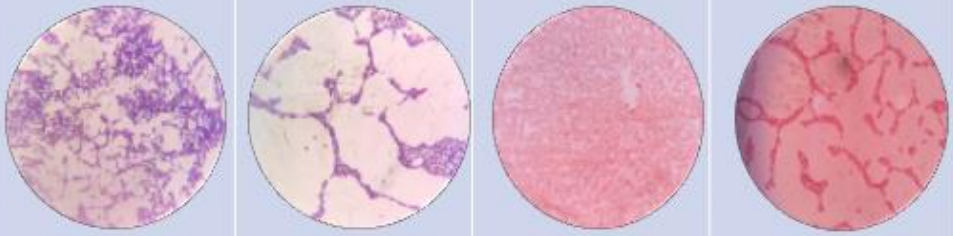
$$= \frac{4}{20} * 100$$

$$= 20\ \%$$

4.5 Activity of different bacterial strains

On the different strain of bacteria biochemical test was performed and in gram staining S. aureus and B. subtilis were tested gram positive and S.typhi and E.coli were tested gram negative. All the strains were tested positive for catalase test. S. aureus and B. subtilis were negative for KOH test and positive for S.typhi and E.coli. In Mr test, S.typhi and E.coliwere positive and S. aureus and B. subtilis were negative.

Table 4.5 Biochemical activity of different organism

	S. aureus	*B. subtilis*	*S. typhi*	*E. coli*
Gram Staining				
Catalase	+ve	+ve	+ve	+ve
KOH	-ve	-ve	+ve	+ve
MR	-ve	-ve	+ve	+ve

4.6 Result of Minimum Inhibitory concentration

When MIC was calculated for different bacterial strain, the result obtained are:

Table 4.6.1: Minimum Inhibitory concentration by *Bacillus Subtilis*

Sample	**Concentration (ug/ml)**
Cissus	6.25
Antibiotic	0.78

Table 4.6.2: Minimum Inhibitory concentration by *S. aureus*

Sample	**Concentration (ug/ml)**
Cissus	6.25
Antibiotic	0.39

Table 4.6.3: Minimum Inhibitory concentration by *E. coli*

Sample	Concentration (ug/ml)
Cissus	3.12
Antibiotic	0.390

Table 4.6.4: Minimum Inhibitory concentration by *S.typhii*

Sample	Concentration (ug/ml)
Cissus	3.13
Antibiotic	1.56

4. 7 MTT assay

MTT assay was performed to evaluate the effect of *Cissus Quadrangularisis* on Neuro 2A cells when treated with different concentrations. The increasing concentrations of plant extract were found to be cytotoxic to the N2a cells. The maximum cell death was observed when cells were treated with 200µg/ml of plant extract. Thus, the results of MTT assay revealed that the extract of *Cissus Quadrangularisis* is having antiproliferative effects on N2a cells.

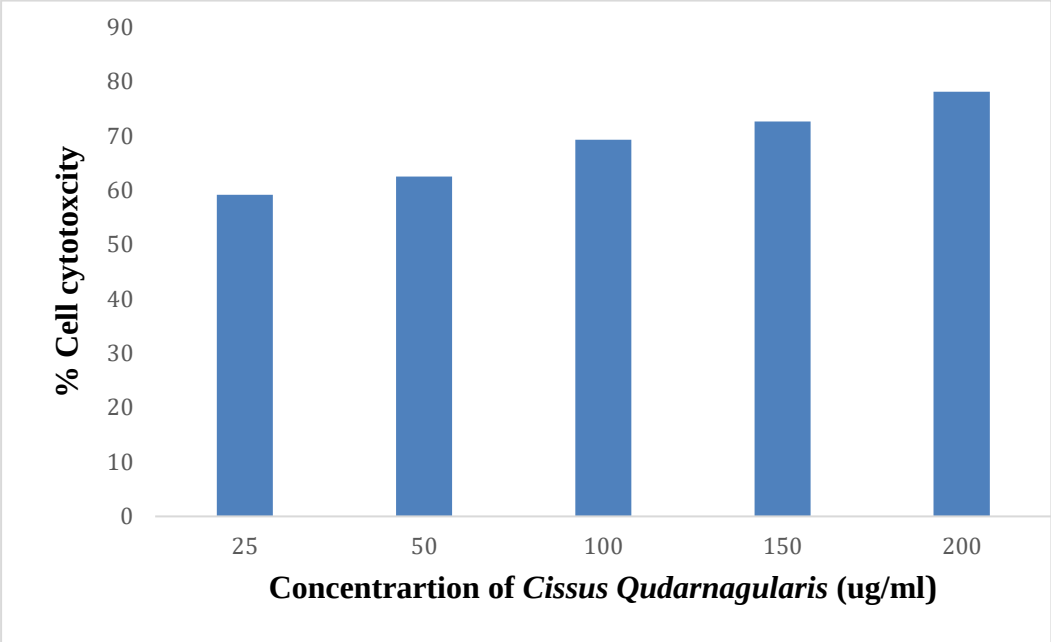


Fig 4.1 Concentration of Cissus quadrangularis vs % cell cytotoxicity which demonstrates that with increasing concentration of plant extract on N2A cells, the percentage of cell death increased.

4.8 Calcium deposition

Vitamin D2 is associated with calcium levels in vertebrates. It increases 1,2-4(OH) levels in kidney which increases the calcium deposition. PBMC's when treated with Vitamin D2 and D3 along with Cissus, the calcium precipitates were observed by staining it with alizarin red. In control cells, devoid of any treatment, very less calcium precipitates were observed. Whereas, the cells treated with D2 in combination with Cissus extract, the maximum calcium deposition was seen, followed by the cells treated with D3 along with Cissus extract.

Hence, it could be concluded that Cissus increases the efficacy of Vitamin D2. Further experimentation for evident study could be done in future.

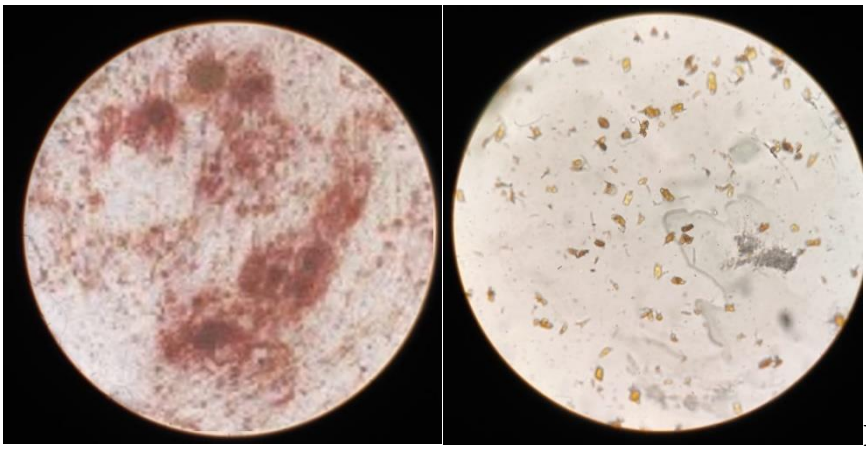
 F

Fig 4.2: A) Vitamin d2 with plant extract B) Control (PBCs)

CHAPTER 5

CONCLUSION

In this thesis research work, we worked on *cissus quadrangularis* plant extract and find different qualitative and quantitative analysis in which protein, carbohydrate and flavonoid were found. This plant has shown antioxidant activity which was proven by assays like DPPH and ABTS method. Later, PBMCs were isolated from blood by ficol histopaque and cells were treated with Vitamin D2 and D3 along with Cissus, the calcium precipitates were observed by staining it with alizarin red. It was observed that in control cells, devoid of any treatment, very less calcium precipitates were observed. Whereas, the cells treated with D2 in combination with Cissus extract, the maximum calcium deposition was seen, followed by the cells treated with D3 along with Cissus extract.

CHAPTER 6

REFERENCES

[1] L. Tripkovic *et al.*, "Comparison of vitamin D2 and vitamin D3 supplementation in raising serum 25-hydroxyvitamin D status: a systematic review and meta-analysis," *Am. J. Clin. Nutr.*, vol. 95, no. 6, pp. 1357–1364, Jun. 2012, doi: 10.3945/ajcn.111.031070.

[2] "Calman: Nutrition and bone health: with particular reference to calcium and vitamin D. Bernan Assoc; 1998.

[3] G. Jones, "Extrarenal Vitamin D Activation and Interactions Between Vitamin D_2, Vitamin D_3, and Vitamin D Analogs," *Annu. Rev. Nutr.*, vol. 33, no. 1, pp. 23–44, Jul. 2013, doi: 10.1146/annurev-nutr-071812-161203.

[4] "Utiger: The need for more vitamin D - ," *Annu. Rev. Nutr.*, vol. 55, no. 1, pp. 23, Jul. 2017, doi: 10.1146/annurev-nutr-071812-161204.

[5] R.-J. H. Keegan, Z. Lu, J. M. Bogusz, J. E. Williams, and M. F. Holick, "Photobiology of vitamin D in mushrooms and its bioavailability in humans," *Dermatoendocrinol.*, vol. 5, no. 1, pp. 165–176, Jan. 2013, doi: 10.4161/derm.23321.

[6] A. C. Ross, "The 2011 report on dietary reference intakes for calcium and vitamin D," *Public Health Nutr.*, vol. 14, no. 5, pp. 938–939, Apr. 2011, doi: 10.1017/S1368980011000565.

[7] S. Faghih, M. Abdolahzadeh, M. Mohammadi, and J. Hasanzadeh, "Prevalence of Vitamin D Deficiency and Its Related Factors Among University Students in Shiraz, Iran," *Int. J. Prev. Med.*, vol. 5, no. 6, pp. 796–799, Jun. 2014.

[8] M. F. Holick, "Vitamin D Status: Measurement, Interpretation, and Clinical Application," *Ann. Epidemiol.*, vol. 19, no. 2, pp. 73–78, Feb. 2009, doi: 10.1016/j.annepidem.2007.12.001.

[9] A. Hossein-nezhad and M. F. Holick, "Vitamin D for Health: A Global Perspective," *Mayo Clin. Proc.*, vol. 88, no. 7, pp. 720–755, Jul. 2013, doi: 10.1016/j.mayocp.2013.05.011.

[10] H. F. DeLuca, "History of the discovery of vitamin D and its active metabolites," *BoneKEy Rep.*, vol. 3, p. 479, Jan. 2014, doi: 10.1038/bonekey.2013.213.

[11] P. Lips *et al.*, "The prevalence of vitamin D inadequacy amongst women with osteoporosis: an international epidemiological investigation," *J. Intern. Med.*, vol. 260, no. 3, pp. 245–254, Sep. 2006, doi: 10.1111/j.1365-2796.2006.01685.x.

[12] M. F. Holick, P. K. T. Pang, and M. P. Schreibman, “Phylogenetic and evolutionary aspects of vitamin D from phytoplankton to humans,” *Vertebr. Endocrinol. Fundam. Biomed. Implic.*, vol. 3, pp. 7–43, 1989.

[13] M. F. Holick, “Resurrection of vitamin D deficiency and rickets,” *J. Clin. Invest.*, vol. 116, no. 8, pp. 2062–2072, Aug. 2006, doi: 10.1172/JCI29449.

[14] “Mellanby: The part played by an accessory factor... - Google Scholar.” https://scholar.google.com/scholar_lookup?journal=J+Physiol&title=The+part+played+by+an+%E2%80%98accessory+factor%E2%80%99+in+the+production+of+experimental+rickets&author=T+Mellanby&volume=52&publication_year=1918&pages=11-4& (accessed May 22, 2022).

[15] H. Steenbock, “THE INDUCTION OF GROWTH PROMOTING AND CALCIFYING PROPERTIES IN A RATION BY EXPOSURE TO LIGHT,” *Science*, vol. 60, no. 1549, pp. 224–225, Sep. 1924, doi: 10.1126/science.60.1549.224.

[16] S. J. Cowell, “IRRADIATION OF MILK AND THE HEALING OF RICKETS,” *Br. Med. J.*, vol. 1, no. 3352, pp. 594–595, Mar. 1925.

[17] Institute of Medicine (US) Committee to Review Dietary Reference Intakes for Vitamin D and Calcium, *Dietary Reference Intakes for Calcium and Vitamin D*. Washington (DC): National Academies Press (US), 2011. Accessed: May 16, 2022. [Online]. Available: http://www.ncbi.nlm.nih.gov/books/NBK56070/

[18] “Office of Dietary Supplements - Vitamin D.” https://ods.od.nih.gov/factsheets/VitaminD-HealthProfessional/ (accessed May 16, 2022).

[19] M. F. Holick, “Vitamin D deficiency,” *N. Engl. J. Med.*, vol. 357, no. 3, pp. 266–281, Jul. 2007, doi: 10.1056/NEJMra070553.

[20] R. Nair and A. Maseeh, “Vitamin D: The ‘sunshine’ vitamin,” *J. Pharmacol. Pharmacother.*, vol. 3, no. 2, pp. 118–126, 2012, doi: 10.4103/0976-500X.95506.

[21] E. Murayama, K. Miyamoto, N. Kubodera, T. Mori, and I. Matsunaga, “Synthetic studies of vitamin D3 analogues. VIII. Synthesis of 22-oxavitamin D3 analogues,” *Chem. Pharm. Bull. (Tokyo)*, vol. 34, no. 10, pp. 4410–4413, Oct. 1986, doi: 10.1248/cpb.34.4410.

[22] A. J. Brown and E. Slatopolsky, “Vitamin D analogs: therapeutic applications and mechanisms for selectivity,” *Mol. Aspects Med.*, vol. 29, no. 6, pp. 433–452, Dec. 2008, doi: 10.1016/j.mam.2008.04.001.

[23] K. Langlois, L. Greene-Finestone, J. Little, N. Hidiroglou, and S. Whiting, “Vitamin D status of Canadians as measured in the 2007 to 2009 Canadian Health Measures Survey,” *Health Rep.*, vol. 21, no. 1, pp. 47–55, Mar. 2010.

[24] R. P. Heaney, “Functional indices of vitamin D status and ramifications of vitamin D deficiency,” *Am. J. Clin. Nutr.*, vol. 80, no. 6 Suppl, pp. 1706S–9S, Dec. 2004, doi: 10.1093/ajcn/80.6.1706S.

[25] W. G. Tsiaras and M. A. Weinstock, “Factors influencing vitamin D status,” *Acta Derm. Venereol.*, vol. 91, no. 2, pp. 115–124, Mar. 2011, doi: 10.2340/00015555-0980.

[26] S. Salekzamani *et al.*, “Is vitamin D status a determining factor for metabolic syndrome? A case-control study,” *Diabetes Metab. Syndr. Obes. Targets Ther.*, vol. 4, pp. 205– 212, 2011, doi: 10.2147/DMSO.S21061.

[27] J. E. Aaron *et al.*, “Frequency of osteomalacia and osteoporosis in fractures of the proximal femur,” *Lancet Lond. Engl.*, vol. 1, no. 7851, pp. 229–233, Feb. 1974, doi: 10.1016/s0140-6736(74)92545-8.

[28] M. F. Holick, “High prevalence of vitamin D inadequacy and implications for health,” *Mayo Clin. Proc.*, vol. 81, no. 3, pp. 353–373, Mar. 2006, doi: 10.4065/81.3.353.

[29] H. A. Bischoff-Ferrari, W. C. Willett, J. B. Wong, E. Giovannucci, T. Dietrich, and B. Dawson-Hughes, “Fracture prevention with vitamin D supplementation: a meta-analysis of randomized controlled trials,” *JAMA*, vol. 293, no. 18, pp. 2257–2264, May 2005, doi: 10.1001/jama.293.18.2257.

[30] H. A. Bischoff-Ferrari *et al.*, “Fall prevention with supplemental and active forms of vitamin D: a meta-analysis of randomised controlled trials,” *The BMJ*, vol. 339, p. b3692, Oct. 2009, doi: 10.1136/bmj.b3692.

[31] L. L. Brown, B. Cohen, D. Tabor, G. Zappalà, P. Maruvada, and P. M. Coates, “The vitamin D paradox in Black Americans: a systems-based approach to investigating clinical practice, research, and public health - expert panel meeting report,” *BMC Proc.*, vol. 12, no.

Suppl 6, p. 6, 2018, doi: 10.1186/s12919-018-0102-4.

[32] C. L. Wagner, F. R. Greer, American Academy of Pediatrics Section on Breastfeeding, and American Academy of Pediatrics Committee on Nutrition, “Prevention of rickets and vitamin D deficiency in infants, children, and adolescents,” *Pediatrics*, vol. 122, no. 5, pp. 1142–1152, Nov. 2008, doi: 10.1542/peds.2008-1862.

[33] M. C. Silva and T. W. Furlanetto, “Intestinal absorption of vitamin D: a systematic review,” *Nutr. Rev.*, vol. 76, no. 1, pp. 60–76, Jan. 2018, doi: 10.1093/nutrit/nux034.

[34] H. M. Pappa, E. Bern, D. Kamin, and R. J. Grand, “Vitamin D status in gastrointestinal and liver disease,” *Curr. Opin. Gastroenterol.*, vol. 24, no. 2, pp. 176–183, Mar. 2008, doi: 10.1097/MOG.0b013e3282f4d2f3.

[35] “Ahn: Serum vitamin D concentration and prostate cancer... - Google Scholar.” https://scholar.google.com/scholar_lookup?journal=J+Natl+Cancer+Inst&title=Serum+vitamin+D+concentration+and+prostate+cancer+risk:+A+nested+case

control+study&author=J+Ahn&author=U+Peters&author=D+Albanes&author=MP+Purdue &author=CC+Abnet&volume=100&publication_year=2008&pages=796-804&pmid=18505967& (accessed May 17, 2022).

[36] X. Li, Y. Liu, Y. Zheng, P. Wang, and Y. Zhang, “The Effect of Vitamin D Supplementation on Glycemic Control in Type 2 Diabetes Patients: A Systematic Review and Meta-Analysis,” *Nutrients*, vol. 10, no. 3, p. E375, Mar. 2018, doi: 10.3390/nu10030375.

[37] E. Kassi, C. Adamopoulos, E. K. Basdra, and A. G. Papavassiliou, “Role of vitamin D in atherosclerosis,” *Circulation*, vol. 128, no. 23, pp. 2517–2531, Dec. 2013, doi: 10.1161/CIRCULATIONAHA.113.002654.

[38] I. Al Mheid and A. A. Quyyumi, “Vitamin D and Cardiovascular Disease: Controversy Unresolved,” *J. Am. Coll. Cardiol.*, vol. 70, no. 1, pp. 89–100, Jul. 2017, doi: 10.1016/j.jacc.2017.05.031.

[39] M. Sahay and R. Sahay, “Rickets–vitamin D deficiency and dependency,” *Indian J. Endocrinol. Metab.*, vol. 16, no. 2, pp. 164–176, 2012, doi: 10.4103/2230-8210.93732.

[40] J. B. Dibble, P. Sheridan, and M. S. Losowsky, “A survey of vitamin D deficiency in

gastrointestinal and liver disorders," *Q. J. Med.*, vol. 53, no. 209, pp. 119–134, 1984.

[41] "Serum vitamin D and the risk of Parkinson's disease - PMC." https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3091074/ (accessed May 18, 2022).

[42] R. E. S. Anglin, Z. Samaan, S. D. Walter, and S. D. McDonald, "Vitamin D deficiency and depression in adults: systematic review and meta-analysis," *Br. J. Psychiatry J. Ment. Sci.*, vol. 202, pp. 100–107, Feb. 2013, doi: 10.1192/bjp.bp.111.106666.

[43] M. F. Holick *et al.*, "Photosynthesis of previtamin D3 in human skin and the physiologic consequences," *Science*, vol. 210, no. 4466, pp. 203–205, Oct. 1980, doi: 10.1126/science.6251551.

[44] V. Tangpricha, C. Spina, M. Yao, T. C. Chen, M. M. Wolfe, and M. F. Holick, "Vitamin D Deficiency Enhances the Growth of MC-26 Colon Cancer Xenografts in Balb/c Mice," *J. Nutr.*, vol. 135, no. 10, pp. 2350–2354, Oct. 2005, doi: 10.1093/jn/135.10.2350.

[45] C. Zhou *et al.*, "Steroid and xenobiotic receptor and vitamin D receptor crosstalk mediates CYP24 expression and drug-induced osteomalacia," *J. Clin. Invest.*, vol. 116, no. 6, pp. 1703–1712, Jun. 2006, doi: 10.1172/JCI27793.

[46] N. Singh *et al.*, "Osteogenic potential of cissus quadrangularisqudrangularis assessed with osteopontin expression," *Natl. J. Maxillofac. Surg.*, vol. 4, no. 1, pp. 52–56, 2013, doi: 10.4103/0975-5950.117884.

[47] S. S. Chopra, M. R. Patel, L. P. Gupta, and I. C. Datta, "Studies on Cissus quadrangularisquadrangularis in experimental fracture repair: effect on chemical parameters in blood," *Indian J. Med. Res.*, vol. 63, no. 6, pp. 824–828, Jun. 1975.

[48] "Determination of marker constituents from Cissus quadrangularisquadrangularis Linn. and their quantitation by HPTLC and HPLC - PubMed." https://pubmed.ncbi.nlm.nih.gov/11705245/ (accessed May 25, 2022).

[49] J. Redfern, M. Kinninmonth, D. Burdass, and J. Verran, "Using Soxhlet Ethanol Extraction to Produce and Test Plant Material (Essential Oils) for Their Antimicrobial Properties†," *J. Microbiol. Biol. Educ.*, vol. 15, no. 1, pp. 45–46, May 2014, doi: 10.1128/jmbe.v15i1.656.

[50] Bhardwaj, K., & Dubey, W. (2019). Quantitative analysis of primary and secondary metabolites of ethanol seed extract of Origanum majorana (Marjoram). *Journal of Pharmacognosy and Phytochemistry*, *8*(1), 1251-1255.

[51] Fahal, M. E., Rani, B. M. A., Aklakur, M. D., Chanu, T. I., & Saharan, N. (2018). Qualitative and quantitative phytochemical analysis of Moringa oleifera (Lam) Pods. *International Journal of Current Microbiology and Applied Sciences*, *7*(5), 657-665.

[52] Hedge, J. E., Hofreiter, B. T., & Whistler, R. L. (1962). Carbohydrate chemistry. *Academic Press, New York*, 17.

[53] Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951). Protein measurement with the Folin phenol reagent. *Journal of biological chemistry*, *193*, 265-275.

[54] Woisky, R. G., & Salatino, A. (1998). Analysis of propolis: some parameters and procedures for chemical quality control. *Journal of apicultural research*, *37*(2), 99-105.

[55] Öztürk, H., Kolak, U., & Meric, C. (2011). Antioxidant, anticholinesterase and antibacterial activities of Jurinea consanguinea DC. *Records of Natural Products*, *5*(1), 43

[56] Re, R., Pellegrini, N., Proteggente, A., Pannala, A., Yang, M., & Rice-Evans, C. (1999). Antioxidant activity applying an improved ABTS radical cation decolorization assay. *Free radical biology and medicine*, *26*(9-10), 1231-1237.

[57] Chand, B. (2021). Antibacterial effect of garlic (allium sativum) and ginger (zingiber officinale) against staphylococcus aureus, salmonella typhi, escherichia coli and bacillus cereus. *Journal of Microbiology, Biotechnology and Food Sciences*, *2021*, 2481-2491.

[58] Chadha, J., Gupta, M., Nagpal, N., Sharma, M., Adarsh, T., Joshi, V., ... & Diwan, P. (2021). Antibacterial potential of indigenous plant extracts against multidrug-resistant bacterial strains isolated from New Delhi region. *GSC Biological and Pharmaceutical Sciences*, *14*(2), 185-196.