Phytochemical Screening and Pharmacological Evaluation of *Fagonia arabica*

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By

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Roll No: MSPC-F21-004

Session: 2021-2023

FACULTY OF PHARMACY

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Ethical Approval Committee of the Faculty of Pharmacy

Dated: May, 20, 2023

SUBJECT: ETHICAL APPROVAL CERTIFICATE

This is to certify that the research project titled "*Phytochemical Screening and Pharmacological Evaluation of Fagonia Arabica*" conducted by Muhammad Sultan Salahuddin Khan under the supervision of Dr. Muhammad Zaeem Ahsan at Faculty of Pharmacy, Superior University Lahore, Pakistan has been reviewed and approved by the Ethics Committee.

The committee requires the following under mentioned guidelines:

1. You are advised to follow the WHO guidelines while conducting the research. In case you feel unusual, you must report to the healthcare department within 24 hours.
2. The researcher must have knowledge of relevant laws for their work as well as institutional policies and comply with them. Protect confidential communication such as papers, patient records, and consents, etc.
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Thank you for your attention to these guidelines.

Prof. Dr. Abubakar Munir
Dean, Faculty of Pharmacy
Convener, Ethical Approval Committee
The Superior University, Lahore, Pakistan.

PLANT AUTHENTICATION CERTIFICATE

Fagonia Arabica
Synonyms: Zygophyllum arabicum

Botanical Name:	Fagonia Arabica
Common Name:	**Dhamasa**
Family:	Zygophyllaceae
Identified by:	Prof Dr Rahmatullah Qureshi
Designation:	Professor
Signature:	16/2/23
Voucher Number:	1003 Arid Agriculture University Rawalpindi

CERTIFICATE OF APPROVAL

This is to certify that the research work presented in this thesis, entitled "Phytochemical Screening and Pharmacological Evaluation of *Fagonia arabica*" was conducted Muhammad Sultan Salahuddin Khan Under the supervision of "Dr. Muhammad Zaeem Ahsan" No Part of this thesis has been submitted anywhere else for any other degree. This thesis is submitted to the Faculty of Pharmacy, Superior University, Lahore in partial fulfilment of the requirements for the degree of Master of Philosophy in field of Pharmacology in Faculty of Pharmacy at Superior University, Lahore.

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DEDICATION

Dedicated to my respected Parents especially my Mother and my whole family for their endless support, appreciation and encouragement.

ACKNOWLEDGEMENT

Foremost, I express by bowing myself before Almighty Allah (Subhanahu-Wa-Taalaa), the Most Gracious and the Most Merciful, who gave me courage and patience to accomplish my work. All respects are for The Prophet, Hazrat Muhammad (Peace Be upon Him), who enabled us to recognize our Creator and taught us our duties and responsibilities for ultimate success in life.

First of all, I would like to express my deepest gratitude to my respectable supervisor, **Dr. Muhammad Zaeem Ahsan** for his encouragement, professional guidance and helpful advice throughout my research work. I am extremely grateful for his support, valuable time and scientific approach and find no words to express my thanks to him for providing me excellent laboratory environment and research facilities.

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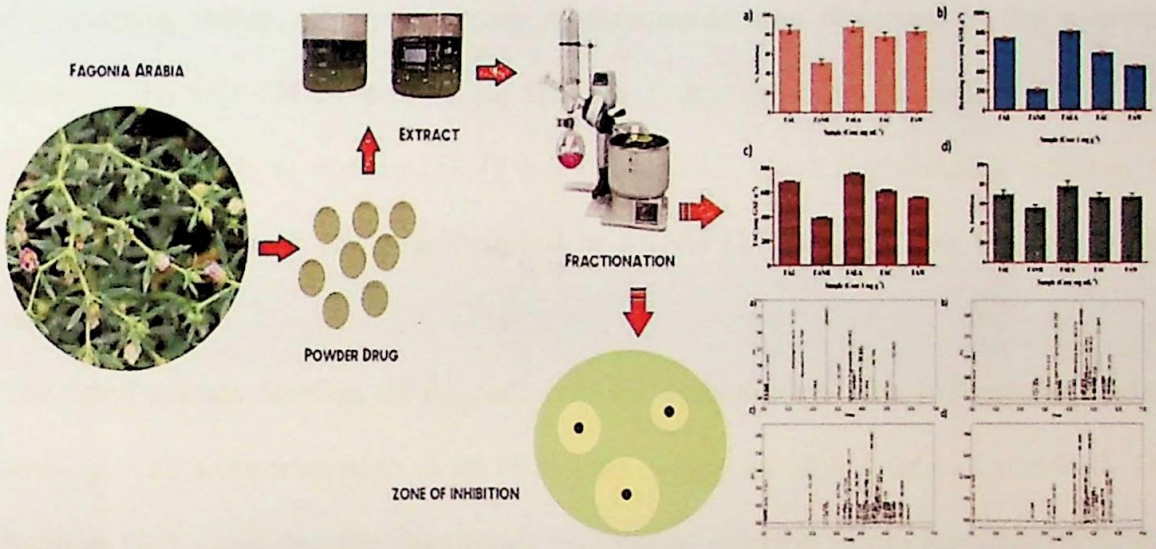
Muhammad Sultan Salahuddin Khan

Fagonia arabica confirmed the presence of nine peaks closely aligned with the standard retention times for various phenolic compounds, including Gallic acid (1.85 min), Ferulic acid (19.6 min), Rutin at (30.66 minutes), Fisetin at (33.66 mintes), Apigenin-7-glucoside at (35.102 minutes), Naringenin at (39.03 minutes), Benzene-triol at (43.00 minutes), Apigenin at (46.86 minutes), and Chrysin at (57.00 minutes). In contrast, the fraction represented by FAC contained a lower quantity of phenolic compounds, with five peaks identified, including Gallic acid (1.85 min), Fisetin (33.66 min), Benzene-triol (43.00 min), Apigenin (46.86 min), and Chrysin (57.00 min).

Keywords: *Fagonia arabica,* Antioxidant, HPLC, TPC, Tyrosinase inhibition

GRAPHICAL ABSTRACT



ABSTRACT

The research aimed to assess the antioxidant potential, phytochemical composition, tyrosinase enzyme inhibition capacity, and antimicrobial activity of significant members of the Zygophyllaceae family, particularly the hydroalcoholic extract of Fagonia arabica (FA) and its fractions obtained using solvents ranging from non-polar to polar. The extraction and fractionation processes were carried out, and various qualitative and quantitative parameters were evaluated. Data were then compared and correlated to investigate potential relationships among different parameters, including antioxidant activity, Total Phenolic Contents (TPC), Total Flavonoids Contents (TFC), tyrosinase enzyme inhibition, and the antimicrobial zone of inhibition.

Qualitative analysis of the plant material indicated the presence of tannins, phenols, and flavonoids in all fractions and extracts of Fagonia arabica. Specifically, the ethyl acetate fraction (FAE) was found to be rich in flavonoids (82.12 ± 1.56 µg QE mg-1 dry sample), while a lower quantity of flavonoids was observed in the n-hexane fraction (FANH) (54.64 ± 0.30 µg QE mg-1 dry sample). Regarding TPC, FAE exhibited the highest amount (72.77 ± 2.78 µg GAE mg-1 dry sample), whereas the minimum quantity of TPC was detected in FANH (27.14 ± 2.03 µg GAE mg-1 dry sample).

The ethyl acetate fraction of Fagonia arabica demonstrated the highest free radical activity, with a concentration of 88.16 mg/mL, using ascorbic acid as a standard. This fraction also exhibited the maximum tyrosinase enzyme inhibition activity (78.23 mg/mL), likely attributable to its high phenolic and DPPH (2,2-diphenyl-1-picrylhydrazyl) potential. Notably, *Fagonia arabica* ethyl acetate fraction in the study exhibits the maximum reducing power, quantified as 814 mg GAE per gram of dry sample.Furthermore, HPLC-DAD analysis of the ethyl acetate fraction (FAEA) of

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LIST OF ABBREVIATION AND ACRONYMS

Abbreviations	Meanings
P.O	Per oral
FAEA	Ethyl acetate fraction of *Fagonia arabica*
FAW	Water fraction of *Fagonia arabica*
FAM	Methanolic extract of *Fagonia arabica*
FANH	n-Hexane fraction of *Fagonia arabica*
FAC	Chloroform fraction of *Fagonia arabica*
SEM	Standard error of mean
STDEV	Standard deviation
ANOVA	Analysis of variance
β	Beta
α	Alpha
µm	*Micro gram*
Mg	Mile gram
LSD	Least significant difference
ALT	Alanine aminotransferase
CCL_4	Carbon tetra chloride
PDA	Potato dextrose agar
Kg	Kilogram
ALP	Alanine phosphatase
UI	Ulcer index
ROS	Reactive oxygen species

RH	Relative humidity
UV	Ultra violet

CHAPTER: 1

1. INTRODUCTION

Historical records bear testament to the utilization of botanical extracts, seeds, fungi, and herbs across a wide spectrum of disorders, thereby establishing a profound correlation with contemporary medical practices (Nobili, Lippi *et al.* 2009). In the present era, scientists worldwide exhibit significant interest in the traditional system of medicine. They are actively involved in conducting research on medicinal plants, specifically in the field of phytochemistry. These plants are carefully examined and isolated to evaluate their potential pharmacological and therapeutic effects (Harsha, Hebbar *et al.* 2002).

Phytochemicals are harmless than synthetic chemicals owing to their abundant availability and broader range of tolerability (Gullett, Amin *et al.* 2010). The compounds obtained from medicinal plants are valuable resources for potential drugs. As compare to synthetic compounds natural compounds have less side effects, making therapies safer and more cost-effective. Throughout history, plants have been used as therapeutic agents against various diseases, serving as a foundation for isolating phytochemicals responsible for their therapeutic effectiveness. This isolation process is the initial step in the development of innovative drugs (Yadav and Agarwala 2011).

Currently, the contemporary transdermal drug delivery system holds immense significance and allure, as this avenue of drug administration proves suitable for both localized and systemic delivery of pharmaceuticals. The direct application of potent therapeutic agents onto the human epidermis not only serves as an efficacious treatment modality but is also regarded as a superior remedy for addressing localized dermatological conditions (Mitkari, Korde *et al.* 2010). Topical application has advantage of no first pass metabolism, so increased availability of drugs at site of

action. In topical delivery system, the drug moieties diffuse from the delivery system and well absorbed by the skin (Zi, Yang *et al.* 2008).

Botanical specimens serve as the progenitors of a multitude of antioxidant compounds boasting extraordinary antioxidative process. Among these compounds are noteworthy entities such as flavonoids, isoflavones, catechins, isocathechins, and lignans (Sanjukta and Ghosh 2012), and large number of other important phytochemicals like coumarins, and most important nitrogen containing compound e.g. alkaloids (Kumar and Kumar 2009) These compounds function as inherent to decease the concentration of free radicals such as the H_2O_2, O^{-2}, and hydroxyl nitric oxide, effectively mitigating their deleterious effects. Moreover, they exhibit a prophylactic influence on the aging process (Khalaf, Shakya *et al.* 2008).

Phytochemicals are defined as biologically active moieties or secondary metabolites produced by plants and existing in its various parts (Abbasi, Khan *et al.* 2010). Among these valuable secondary metabolites most common are polyphenols and flavonoids, ascorbic acid, tocopherol, carotenoids and benzoic acid (Ghasemzadeh, Jaafar *et al.* 2010).

The skin, which is the largest organ in the human body, acts as a protective barrier against various pollutants and hazards. Environmental factors such as radiation, microbial attacks, and aging processes can cause damage and deterioration to facial skin collagen and elastin fiber, as well as alter the structure of cell membranes. These factors can also lead to changes in the DNA of skin cells, which are essential for maintaining the integrity and health of our skin (Gray, Gummer *et al.* 2000). The occurrence of spots and melasma on the skin is often attributed to an overproduction or uneven distribution of the melanin pigment. To address these hyperpigmentation disorders, hydroquinone stands out as the optimal treatment option (Draelos 2011).

Acne is a skin disorder that arises due to an excessive production of sebum. Sebum is an oily substance naturally produced by the skin's sebaceous glands. When the production of sebum becomes excessive, it can clog the hair follicles and lead to the formation of acne. This condition often manifests as pimples, blackheads, or whiteheads on the surface of the skin. The excess sebum provides an environment suitable for the growth of bacteria, particularly Propionibacterium acnes, which further contributes to the development of inflammation and acne lesions (Hutter, Salman *et al.* 1996).

Antioxidants are compounds that shield our skin and body from oxidative stress that cause high degree of damages. Oxidative stress occurs due to imbalance and high amount of production of harmful species like free radicals in the body and body's defense mechanism unable to neutralize them. Free radicals are generated as byproducts of chemical reactions in our body and have the ability to modify the structure of proteins. This can lead to various undesirable effects such as skin aging, inflammatory diseases, and even cancer. Antioxidants help counteract the harmful effects of free radicals by donating electrons to neutralize them, thereby safeguarding our skin and body from their destructive impact.(Khan, Akhtar *et al.* 2013). The utilization of antioxidants in cosmeceutical products is gaining quick recognition in the cosmetic market, thanks to their beneficial effects in treating various skin disorders. Antioxidants have the ability to counteract the harmful effects of free radicals, which are molecules that can cause damage to our skin cells. By neutralizing these free radicals, antioxidants help protect the skin from oxidative stress, which is known to contribute to skin issues such as aging, inflammation, and certain skin disorders. Incorporating antioxidants in cosmeceutical preparations can aid in the recovery and improvement of these skin conditions, promoting healthier

and more vibrant skin. (Aburjai and Natsheh 2003). Polyphenols and flavonoids, which are derived from natural sources, possess significant potential as antioxidants that can help mitigate oxidative stress. Oxidative stress occurs when there is an imbalance between harmful molecules called free radicals and the body's ability to neutralize them. These natural compounds have the ability to donate electrons and neutralize free radicals, thereby reducing oxidative stress and its damaging effects on the body. By incorporating polyphenols and flavonoids from natural sources into our diets or utilizing them in therapeutic interventions, we can harness their antioxidant properties to support overall health and well-being (Lin, Tseng *et al.* 2008) The antioxidant activity of various extracts obtained from plants and herbs is evaluated using the DPPH (2,2-diphenyl-1-picrylhydrazyl) method. This method is commonly employed in scientific research to assess the ability of different compounds to scavenge free radicals and exhibit antioxidant properties. The DPPH assay involves measuring the reduction of a stable free radical, DPPH, by the tested extracts and fractions (Rangkadilok, Sitthimonchai *et al.* 2007).

CHAPTER: 2

2. LITERATURE REVIEW

2.1 *Fagonia arabica*

Fagonia arabica, also known as Dhamasa Booti, is a tropical plant that has garnered scientific interest due to its various medicinal properties (Dilshad and Batool 2022), *Fagonia arabica* is a shrub and versatile plant of Zygophyllaceae family. It is commonly known as 'Kharasan' thorn in English and referred to as "Dhamasa" in India. This green shrub typically grows to a height of 1 to 3 feet and can be found on calcareous rocks in various regions including the Mediterranean, South Africa, Afghanistan, India (Rajasthan, northwest Punjab, and Western India), and Pakistan (Sindh, Punjab, KPK). The entire plant is used for medicinal purposes, and it is particularly recognized as a remedy for fevers in hilly areas.

The plant's infusion is known for its cooling effects in treating stomatitis (inflammation of the mouth). It is believed to cleanse the blood and act as deobstruent, aiding in the removal of obstructions in the body. Fagonia arabica is also used to treat skin diseases, smallpox, and as an application for snake bites. Additionally, a paste made from the twigs of the plant is applied externally to treat tumors and reduce swelling in the neck (Prasad, Kashyap *et al.* 2007). (Iftikhar, Chatha *et al.* 2022). It is an Ayurvedic herb with significant ethno-pharmacological importance, as it possesses a wide range of medicinal properties. This herb is known for its anti-inflammatory properties, Additionally, *Fagonia arabica* has analgesic and antipyretic effects. These medicinal properties make Fagonia arabica a valuable herb in traditional medicine and contribute to its use in various therapeutic applications (Ravindra, Rahul *et al.* 2012).



Figure 2. 1 *Fagonia arabica* plant description of various parts (Kanwal, Fatima *et al.* 2021).

2.1.1 Description and Constituents

The Fagonia genus is made up of plants that can be classified as both herbs and shrubs. The flowers of this plant species have petals that are purplish pink in color, with pointed structures and a pleasant scent. The fruit of the Fagonia plant is in the form of a capsule, which contains multiple chambers called locules (Alamami, Elshibani *et al.* 2022).

Numerous pharmacologically active compounds have been extracted from the Fagonia arabica plant, encompassing glycosides, flavonoids, terpenoids, saponins, alkaloids, and trace elements. The scientific literature also documents the presence of two specific flavonoid glycosides known as kaempferol-7-O-rhamnoside and acacetin-7-O-rhamnoside within Fagonia arabica. (Iftikhar, Chatha *et al.* 2022). Previous scientific investigations on various species of Fagonia have revealed their potential in treating a wide range of diseases. Different parts of this herb have been traditionally utilized to address hematological (related to blood), neurological (related to the nervous system), endocrinological (related to hormonal imbalances), and inflammatory disorders.

The herb has also shown antioxidant activities, meaning it can combat the free radicals which cause damaging effects in the body. Additionally, Fagonia has demonstrated antibacterial and antifungal effects, making it effective against certain

types of bacteria and fungi. It has even displayed insecticidal properties, proving useful in repelling or eliminating insects. Furthermore, Fagonia exhibits cytotoxic effects, meaning it can cause cell death in certain circumstances. These beneficial effects are attributed to the high levels of phenolic compounds found in the herb, which contribute to its efficient scavenging of free radicals (El-Amier and Aisha 2019).

Table 2. 1 Botanical classification of *Fagonia arabica*

Kingdom	**Plantae**
Phylum	Tracheophyta
Class	Magnoliopsida
Order	Zygophyllales
Family	Zygophyllaceae
Genus	Fagonia
Species	*Fagonia arabica*

2.1.2 Ethnopharmacological Analysis

2.1.2.1 Antioxidant activity

Several studies have indicated that the plant contains phenolic compounds that exhibit antioxidant properties. Additionally, previous research has highlighted the plant's ability to provide relief from pain, reduce inflammation, and lower fever, in addition to its antioxidant effects. The researchers have also investigated the mechanisms through which the plant exerts its beneficial effects. Moreover, the plant has been found to aid in the restoration of antioxidant enzymes and decrease lipid peroxidation, which is the process of oxidative damage to lipids (Satpute, Kashyap *et al.* 2009).

2.1.2.2 Antibacterial activity

Fagonia arabica exhibits a wide range of activity against various types of bacteria. It shows effectiveness against both Gram-positive bacteria, such as Staphylococcus aureus, Bacillis subtillis, and Streptococcus pyogenes, as well as Gram-negative bacteria, including Pseudomonas aeruginosa, Escherichia coli, and Klebsiella pneumonia (El-Amier and Aisha 2019).

2.1.2.3 Antidiabetic activity

The alcoholic extract derived from Fagonia arabica demonstrated anti-diabetic effects when tested on Albino Wistar rats with Alloxan monohydrate-induced diabetes. Through dose fixation studies, it was determined that a dosage of 250 mg per kilogram of body weight was the most effective in reducing fasting glucose levels (Al-Ziaydi 2015).

2.1.2.4 Antifungal activity

Fagonia arabica, possess antifungal properties against *Candida albicans*. The study conducted involved using methanolic extracts of *Fagonia arabica* at a concentration of 50 mg/ml. The results indicated a significant antifungal effect, as evidenced by a zone of inhibition measuring 9 mm against *Candida albicans*. This suggests that the plant has the potential to combat fungal infections caused by *Candida albicans* (El-Amier and Aisha 2019).

2.1.2.5 Allelopathic activity

The aerial parts crude extract obtained from *Fagonia arabica* exhibited a notable phytotoxic impact on the germination and growth of P. oleracea seeds at various concentrations (5-40 mg/ml) compared to the control group. The findings suggest that the root system of P. oleracea experienced a greater impact than the shoot system due

to the inhibitory allelopathic activity caused by *Fagonia arabic* (El-Amier and Aisha 2019).

2.1.2.6 Thrombolytic activity

The study evaluated the clot lysis activity of Streptokinase and *Fagonia arabica* herbal preparations. The results showed that the clot lysis percentage obtained after treating the clots with *Fagonia arabica* extract was significantly higher (75.6%; p value < 0.0001) compared to the control group. This suggests that *Fagonia arabica* exhibits a promising thrombolytic activity *invitro*, meaning it has the ability to dissolve blood clots. However, further research is needed to determine the active component(s) responsible for the clot-dissolving property of *Fagonia arabica* and to investigate its efficacy *invivo* (within a living organism) (Prasad, Kashyap *et al.* 2007).

2.2 Antioxidant

Antioxidants play a crucial role in protecting against auto-oxidation, which is the process of oxidation caused by reactive oxygen species (free radicals). They achieve this through several mechanisms, including:

1. Decreasing local O_2 concentration: Antioxidants can reduce the concentration of oxygen (O_2) in specific areas, limiting the availability of oxygen for oxidation reactions. By lowering the local O_2 concentration, antioxidants help prevent the initiation and progression of auto-oxidation.
2. Curbing peroxide production: Antioxidants can inhibit the production of peroxides, which are highly reactive compounds involved in oxidative reactions. By limiting peroxide formation, antioxidants hinder the propagation of auto-oxidation reactions, thus protecting cells and tissues from damage (Brewer 2011).

3. Inhibiting free radical formation and chelating with metal ions: Certain antioxidants, such as selenium and zinc, act as essential nutrient antioxidants. They can interfere with the formation of free radicals and also chelate or bind with metal ions. By binding to metal ions, these antioxidants prevent their involvement in oxidative reactions and subsequent auto-oxidation (Sirisha, Sreenivasulu *et al.* 2010).
4. Breaking the auto-oxidative chain reaction: Antioxidants disrupt the phases involved in auto-oxidative chain reactions, namely induction, propagation, and termination. They can interrupt these steps by interacting with free radicals and preventing their destructive effects. This interruption helps to halt the progression of auto-oxidation and mitigate its damaging consequences (Brand-Williams, Cuvelier *et al.* 1995)

Various forms of natural antioxidants can be found in fruits, barks, and leaves. These sources provide a rich array of antioxidant compounds that help protect against oxidative damage (Kähkönen, Hopia *et al.* 1999). In various sources bioactive compounds can be found, such as vegetables, fruits and plant extracts which include important antioxidants like vitamin C, vitamin A, vitamin E, phenolic compounds, carotenoids, and flavonoids. These compounds possess significant antioxidant activity, meaning they have the ability to counteract the harmful effects of oxidative stress in the body (Sanjukta and Ghosh 2012).

2.2.1 Phytoantioxidants

Historical records indicate the utilization of pharmacognostic plant extracts, fungi, seeds, and herbs in the treatment of various disorders, establishing a connection to modern medicine. These traditional remedies have been employed for centuries,

offering insights into the development of contemporary medical practices (Nobili, Lippi *et al.* 2009).

In the present times, the scientists worldwide have shown significant interest in the traditional system of medicine. They are actively conducting research on medicinal plants through phytochemical analysis to identify and isolate bioactive compounds. These compounds are then evaluated to determine their therapeutic action (Harsha, Hebbar *et al.* 2002).

Natural compounds are more abundant in nature and they are also highly tolerable by the human, that's why they considered safer than other synthetic compounds. Natural compounds can be derived from different sources i.e. plants, animals, and minerals, and they have evolved alongside living organisms over time. As a result, these compounds often possess a higher level of compatibility with biological systems, reducing the risk of adverse effects or toxicity. Additionally, natural compounds tend to exhibit a greater diversity of chemical structures, allowing for a wider range of interactions and potential therapeutic benefits.

Furthermore, the human body has evolved to metabolize and eliminate natural compounds more efficiently, resulting in a decreased likelihood of accumulation or harmful interactions. These factors contribute to the perception that natural compounds are generally safer and more suitable for use in various applications, including pharmaceuticals, dietary supplements, and personal care products (Gullett, Amin *et al.* 2010). The compounds obtained from medicinal plants have emerged as a valuable resource for potential drug development. These compounds, when compared to synthetic compounds, often exhibit fewer side effects, making therapy safer and more cost-effective. Throughout history, plants have been utilized as therapeutic agents to address various diseases. This traditional knowledge has paved the way for

the isolation of specific phytochemicals responsible for the therapeutic effects observed. Isolating these phytochemicals is the initial step in the development of new drugs. By harnessing the therapeutic potential of natural compounds derived from plants, researchers can explore novel avenues for drug discovery and contribute to safer and more economical treatment options for a wide range of medical conditions (Yadav and Agarwala 2011).

Phytochemicals are bioactive compounds that naturally occur in plants and are essential for the human body. The term "phyto" originates from the Greek word for plants. Scientists have identified approximately 5,000 different phytochemicals found in vegetables, fruits, and herbs. These phytochemicals are categorized into various classes, including phenolics (such as flavonoids), alkaloids, carotenoids, vitamins, organosulphur compounds, and nitrogen-containing compounds. Out of these classes, the polyphenols have garnered significant interest because to their diverse range of therapeutic activities and their importance in the human diet. Polyphenols can be found in various plant-based foods and have been associated with potential health benefits. Exploring the properties and effects of phytochemicals, particularly polyphenols, provides insights into their potential roles in promoting human health and well-being (Huang, Cai *et al.* 2009).

Polyphenolic compounds are a diverse group of natural compounds found in plants that contain multiple phenolic functionalities. They have various applications in synthesis, medicine, and industry. These compounds possess biological activities and are being explored as potential drug candidates for diseases like AIDS, heart ailments, ulcers, bacterial infections, mutagenesis, and neural disorders. Additionally, polyphenols play a role in flower coloration through supramolecular interactions, such as aromatic nucleus stacking and keto-enol tautomerism, which can be

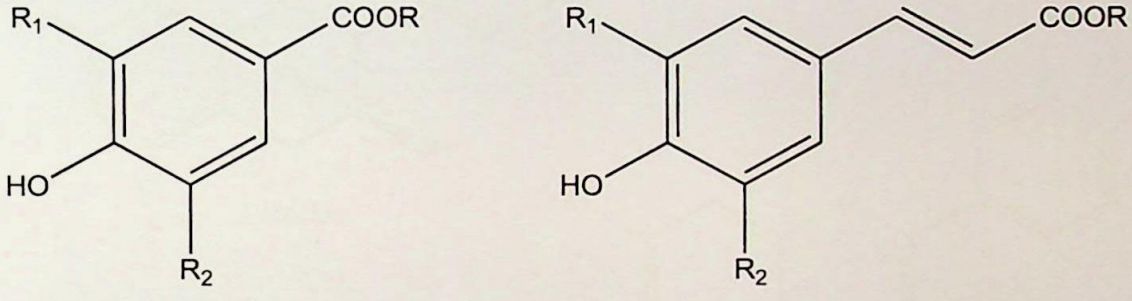
influenced by pH. Polyphenols can react with one-electron oxidants, preventing the formation of free radicals in biological systems. Their ability to interact with metal ions like Fe^{2+} is also significant in their biological activity, as these ions can generate free radicals. However, under certain conditions, some polyphenols can act as pro-oxidants. Another important aspect is the interaction of polyphenols' multiple polar functional groups with biologically important molecules like proteins, leading to the formation of protein-polyphenol complexes. These complexes often precipitate and are used in the tanning process of leather due to their strong binding affinity with proteins (Handique and Baruah 2002).

Polyphenols can be further classified into two main groups: flavonoids and phenolic acids. Flavonoids and phenolic acids are abundant in various plants and are responsible for their vibrant colors and distinct flavors. These polyphenols come in a staggering variety, with approximately 8,000 different known structures identified so far. Each structure has unique properties and potential health benefits. Studying and understanding the diverse range of polyphenols allows researchers to explore their specific roles in plant physiology and their potential effects on human health (Fresco, Borges *et al.* 2010).

Phenolic compounds found in plants possess powerful antioxidant properties and play a crucial role in preventing and treating diseases. These compounds can be classified into various groups based on their structures and attached elements. The major categories include phenolic acids, flavonoids, tannins, stilbenes, curcuminoids, coumarins, and lignans. Phenolic compounds derived from plants exhibit a wide range of health benefits. For example, phenolic acids like rosmarinic acid and carnosic acid, hydroxybenzoic acids like vanillic acid, and hydroxycinnamic acids like ferulic acid and chlorogenic acid are commonly found in foods such as herbs,

fruits, spices, grains and seeds. Other phenolic compounds include essential oils like carvacrol, limonene, and eugenol, as well as flavonoids like apigenin, quercetin, catechin, and rutin. These compounds are known for their ability to scavenge harmful free radicals in the body and contribute to overall health and well-being. The diverse range of phenolic compounds found in plants can be grouped into four main categories: essential oils, phenolic acids, flavonoids, and phenolic diterpenes.

Benzoic acid derivatives and cinnamic acid derivatives are two major classes of Phenolic acids. Different levels of hydroxylation in these compounds further contribute to their varied effects and functions in plants (Fresco, Borges *et al.* 2006)



Benzoic Acid Cinnamic Acid

Figure 2. 2 Structure of Benzoic and Cinnamic Acid

Hydroxybenzoic acids, such as gallic acid, ellagic acid, protocatechuic acid, and 4-hydroxybenzoic acid, are typically found in fruits but in very small amounts. On the other hand, hydroxycinnamic acids like caffeic acid, ferulic acid, sinapic acid, and p-coumaric acid are present in fruits at much higher concentrations compared to hydroxybenzoic acids. These hydroxycinnamic acids play a significant role in the overall intake of polyphenols from fruits, surpassing the contributions of flavonoids or benzoic acid derivatives like flavones and flavanols (Lafay and Gil-Izquierdo 2008).

According to a study conducted in Germany, it was found that the average daily intake of hydroxycinnamic acids is 211 mg per day, while the intake of hydroxybenzoic acids is 11 mg per day. These values represent the amount of these specific compounds that individuals in the study were consuming on a daily basis. The study aimed to understand the dietary habits and intake of these acids in the population, providing valuable information about their contribution to the overall dietary polyphenol intake (Radtke, Linseisen *et al.* 1998).

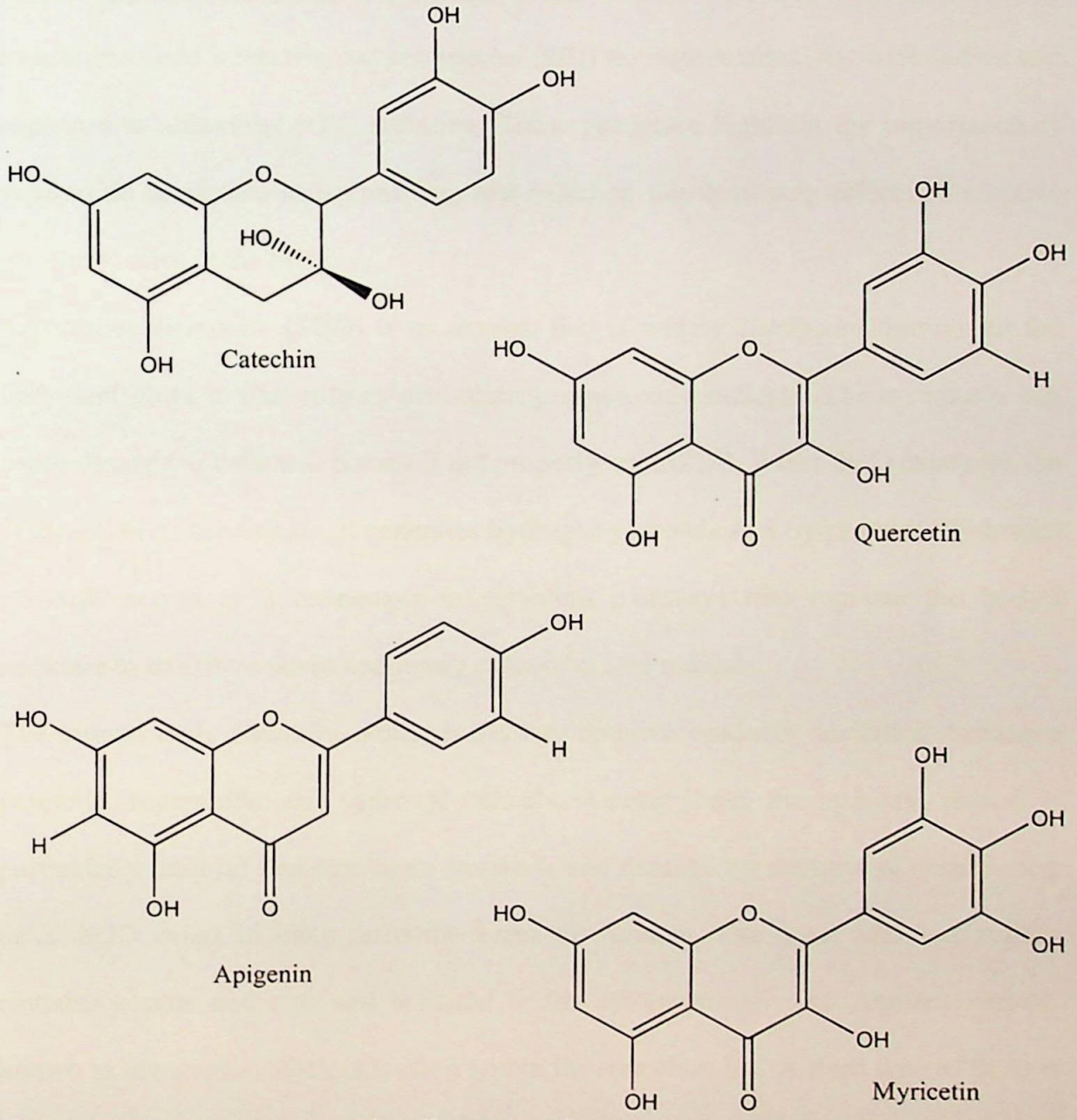


Figure 2. 3 Structure of various phenolic compounds

2.2.2. Anti-oxidant Enzymes

Human body has three important antioxidant enzymes like superoxide dismutase, glutathione peroxidase and catalase. Superoxide dismutase (SOD) plays a crucial role in protecting the skin from oxidative damage caused by the free radical of superoxide. This radical is considered slighter reactive as compared to other harmful radicals like the hydroperoxyl radical (O_2H) and hydroxyl radical (OH). The superoxide dismutase enzyme helps to convert the superoxide free radical into the hydrogen peroxide. Unlike superoxide, hydrogen peroxide is not a free radical itself, but it can be transformed into a reactive oxygen species (OH) through interactions with metals and exposure to ultraviolet (UV) radiation. These processes highlight the importance of superoxide dismutase in neutralizing and reducing the damaging effects of reactive oxygen species in the body.

Superoxide dismutase (SOD) is an enzyme that is widely distributed throughout the body and plays a vital role in neutralizing superoxide radicals. These radicals can cause damage to cells and tissues if not properly controlled. When SOD catalyzes the dismutation of superoxide, it generates hydrogen peroxide as a by-product. Hydrogen peroxide serves as a messenger in signaling pathways that regulate the body's response to oxidative stress and injury caused by free radicals.

The human body naturally produces various reactive oxidants, including hydrogen peroxide, superoxide, and hydroxyl radicals. Among these, the hydroxyl radical is particularly harmful and can cause severe tissue damage by destroying neighboring cells. SOD exists in three different forms or variants. The most common variant contains copper and zinc and is found in the cytoplasm of cells. Another variant, known as manganese SOD, is located within the mitochondria. A third type of SOD is present outside of cells, in the extracellular space. These different forms of SOD

contribute to the overall defense against oxidative stress and help maintain cellular health (Jeeva, Sunitha *et al.* 2015).

Within the body, there are two different forms of the superoxide dismutase (SOD) enzyme: copper/zinc-dependent SOD, which is found in the cytosol (the fluid inside cells), and manganese-dependent SOD (MnSOD), which is located within the mitochondria, the energy-producing structures within cells. MnSOD is a particularly important antioxidant enzyme because it plays a crucial role in neutralizing and removing a majority of the reactive oxygen species (ROS) present in the mitochondria. ROS are highly reactive molecules that can cause damage to cells and tissues if their levels become too high. By acting as a scavenger, MnSOD helps to maintain the balance of ROS and protects the mitochondria from oxidative stress and potential harm (Girotti 1998).

Catalase (E.C. 1.11.1.6) is an essential antioxidant enzyme found in nearly all organisms that require oxygen to survive. Its primary function is to neutralize harmful hydrogen peroxide molecules by converting them into oxygen and water through a two-step reaction. This process helps protect cells from oxidative damage and maintains their overall health and integrity. The presence of catalase deficiency or impaired function has been linked to various medical conditions, including diabetes mellitus, vitiligo, cardiovascular diseases, Wilson disease, hypertension, anemia, certain dermatological disorders, Alzheimer's disease, bipolar disorder, and schizophrenia. In these diseases, the inadequate activity or dysfunction of the catalase enzyme can contribute to the development or progression of the associated symptoms and complications. Proper functioning of catalase is crucial for maintaining cellular balance and protecting against oxidative stress, and its dysfunction can have far-reaching effects on overall health (Nandi, Yan *et al.* 2019). Catalase is an enzyme that

contains a heme group, a special molecule that binds to iron. It plays an important role in the breakdown of hydrogen peroxide, a chemical compound that can be harmful to cells. When hydrogen peroxide is present in high concentrations, catalase steps in and facilitates a reaction that converts it into water and molecular oxygen. This process helps to eliminate the toxic effects of hydrogen peroxide and maintains a safe environment within the cells. By reducing hydrogen peroxide to water and oxygen, catalase acts as a natural defense mechanism against oxidative stress, which can damage cells and lead to various health problems.

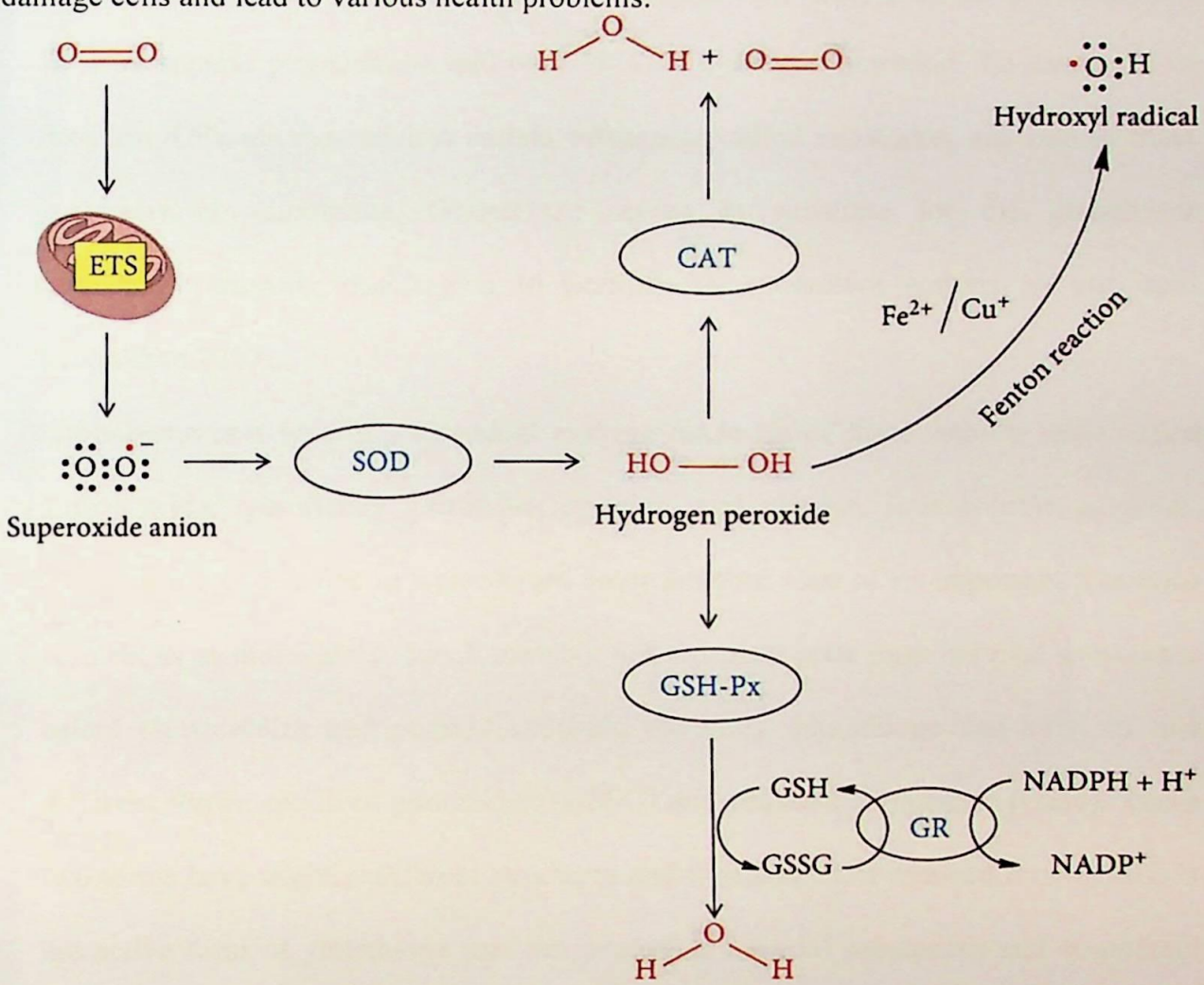


Figure 2. 4 Role of Antioxidant enzyme

Glutathione Peroxidase (GPx) is a special type of enzyme that helps protect our cells from damage caused by harmful substances called oxidants. This enzyme contains a

mineral called selenium (Se) on its active side, which is an essential component for its function. GPx primarily works on a molecule called reduced glutathione (GSH) and a compound called hydrogen peroxide (H_2O_2). When these substances interact with GPx, they undergo a chemical reaction that results in the formation of oxidized glutathione (GSSH) and a molecule called hydroxyl radical (HO). One of the important roles of GPx is to reduce lipid peroxidation in cell membranes. It can help prevent the damage caused by oxidants to the fats present in our cell membranes. The activity of glutathione peroxidase enzyme is quite remarkable. It can reduce around 70% of organic peroxidation and over 90% of hydrogen peroxide. To carry out its function, GPx always requires certain substances called substrates, and one of these substrates is glutathione. Glutathione serves as substrate for the glutathione peroxidase enzyme, enabling it to perform its protective actions in our cells (Zulaikhah 2017).

Glutathione is a type of antioxidant enzyme made up of three smaller units called amino acids, specifically glutamine, cysteine, and glycine. It is soluble in water, meaning it can dissolve in water-based environments. One of its important functions is to act as an antioxidant, which means it helps protect cells from harmful substances called electrophiles and peroxides. Inside the cells, glutathione can exist in two different forms: oxidized glutathione (GSSG) and reduced glutathione (GSH). These two forms have slightly different structures and functions. The reduced form (GSH) is the active form of glutathione that can neutralize harmful substances and counteract oxidative stress. On the other hand, the oxidized form (GSSG) is the result of glutathione's reaction with harmful substances and serves as an indicator of the body's exposure to oxidative stress. Overall, glutathione plays a crucial role in maintaining cellular health and protecting against oxidative damage (Townsend, Tew *et al.* 2003).

In many living organisms, there is an enzyme called glutathione peroxidase that acts as an antioxidant. This enzyme is responsible for deactivating hydrogen peroxide and lipid peroxides, which can be harmful to cells. Glutathione peroxidase enzyme active site contain selenium ion and reduced glutathione (GSH), which work together to convert hydrogen peroxide and lipid hydroperoxides into water. The enzyme essentially helps to break down these harmful substances, reducing their potential damage to cells. The presence of selenium and reduced glutathione in the active site allows for the efficient reduction of hydrogen peroxide and lipid peroxides, ensuring the protection of biological systems from oxidative stress. In biological systems, there is a compound called oxidized glutathione (GSSH) that is formed when glutathione (GSH) undergoes oxidation. This oxidized form of glutathione plays a role in various cellular processes and serves as an indicator of oxidative stress. Additionally, when fatty acids in the body undergo hydroxylation, they form hydroxylated fatty acids (LOH). These hydroxylated fatty acids can react with reactive oxygen species to form lipid hydroperoxides (LOOH). Lipid hydroperoxides are unstable and can contribute to oxidative damage in cells (Charles 2012).

$H_2O_2 + 2GSH \rightarrow 2H_2O + GSSG$

$LOOH + 2GSH \rightarrow LOH + H_2O + GSSG$

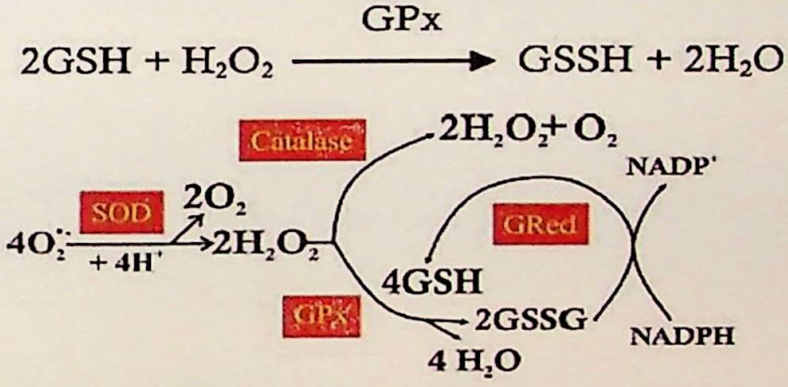


Figure 2.5 Antioxidant role of antioxidant enzymes

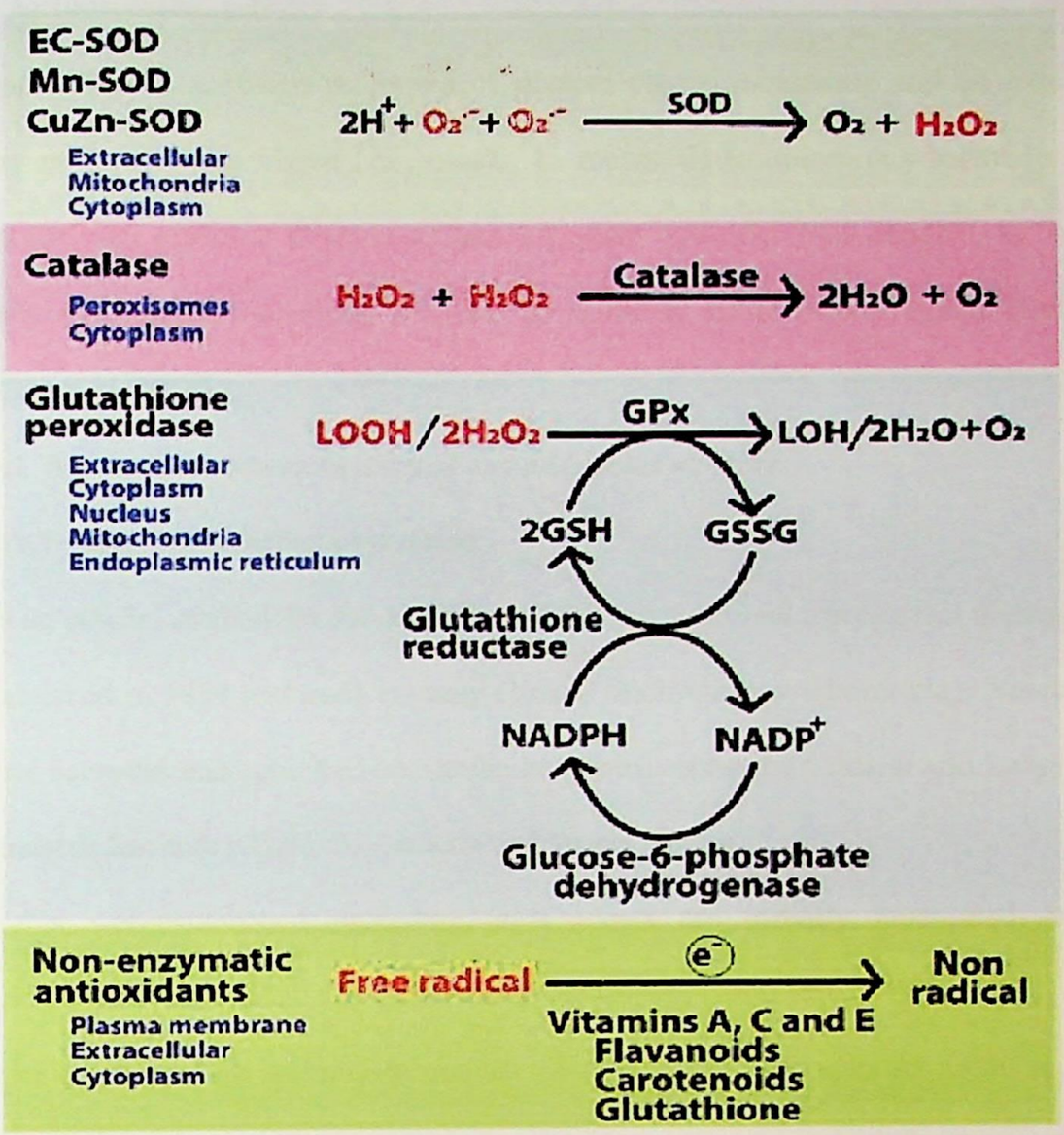


Figure 2.6 Mechanism of action and free radical asavanging action of various antioxidant enzyme

2.3 Antimicrobial activity

Plants serve as abundant reservoirs of diverse phytoantioxidants and an extensive array of structurally varied compounds. In recent times, numerous scientists have directed their attention towards scrutinizing plant and microbial extracts, as well as newly synthesized molecules, for their potential as antimicrobial agents (Balouiri, Sadiki *et al.* 2016).

2.3.1 Methods of determination of antimicrobial activity

2.3.1.1 Agar Disc-Diffusion method

It is an official method for the determination of antimicrobial activity this method was established in 1954 and used in many clinical microbiology laboratories. Nowadays, many accepted and approved standards are published by the Clinical and Laboratory Standards Institute (CLSI) for bacteria and yeasts testing.

In this well-established procedure, agar plates are initially inoculated with a standardized amount of the target microorganism. Next, small filter paper discs, typically measuring around 6 mm in diameter, are impregnated with the test compound at the desired concentration and delicately positioned on the agar's surface. These prepared Petri dishes are then carefully incubated under suitable environmental conditions. Normally, the antimicrobial substance diffuses into the agar medium, hindering the germination and proliferation of the test microorganism. Following incubation, the dimensions of the zones in which growth inhibition is observed are measured and evaluated.

2.3.1.1.1 Limitations in agar disc diffusion methods

Various limitations of agar disc diffusion methods are given below:

- Cannot differentiate between bacteriostatic and bactericidal bacteria
- Minimum inhibitory concentration cannot measured by using this methods

2.3.1.1.2 Advantages of disc-diffusion methods

Advantages of disc diffusion method are given below:

- It is simple methods
- Method has low cost
- Interpretations of results are easy
- Method is suitable for wide range of microorganism
- Verity on antimicrobial agent can be analyzed by using this method

The advantages mentioned earlier, particularly the method's clarity and cost-effectiveness, have a great contribution to its extensive use in screening the antimicrobial properties of plant essential oils, extracts, and various pharmaceutical compounds (Balouiri, Sadiki *et al.* 2016).

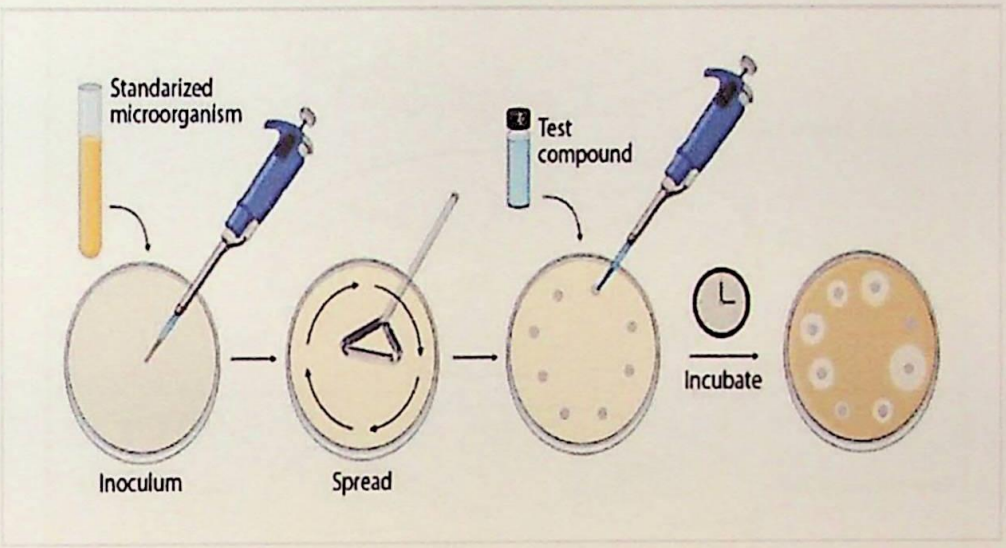


Figure 2. 7 Method of determination of agar disc diffusion method.

2.3.1.2 Gradient method

This method is mainly use to determine MIC value and base on the combined principle of dilution method and agar diffusion method and also on creating concentration gradient in agar medium. In this technique, a strip impregnated with an antimicrobial agent, featuring a concentration gradient that varies from lower to higher concentrations, is placed onto the pre-inoculated agar surface containing the

microorganism being studied. MIC values of antibiotic, antifungal and antimycobacterial can be calculated by using this method.

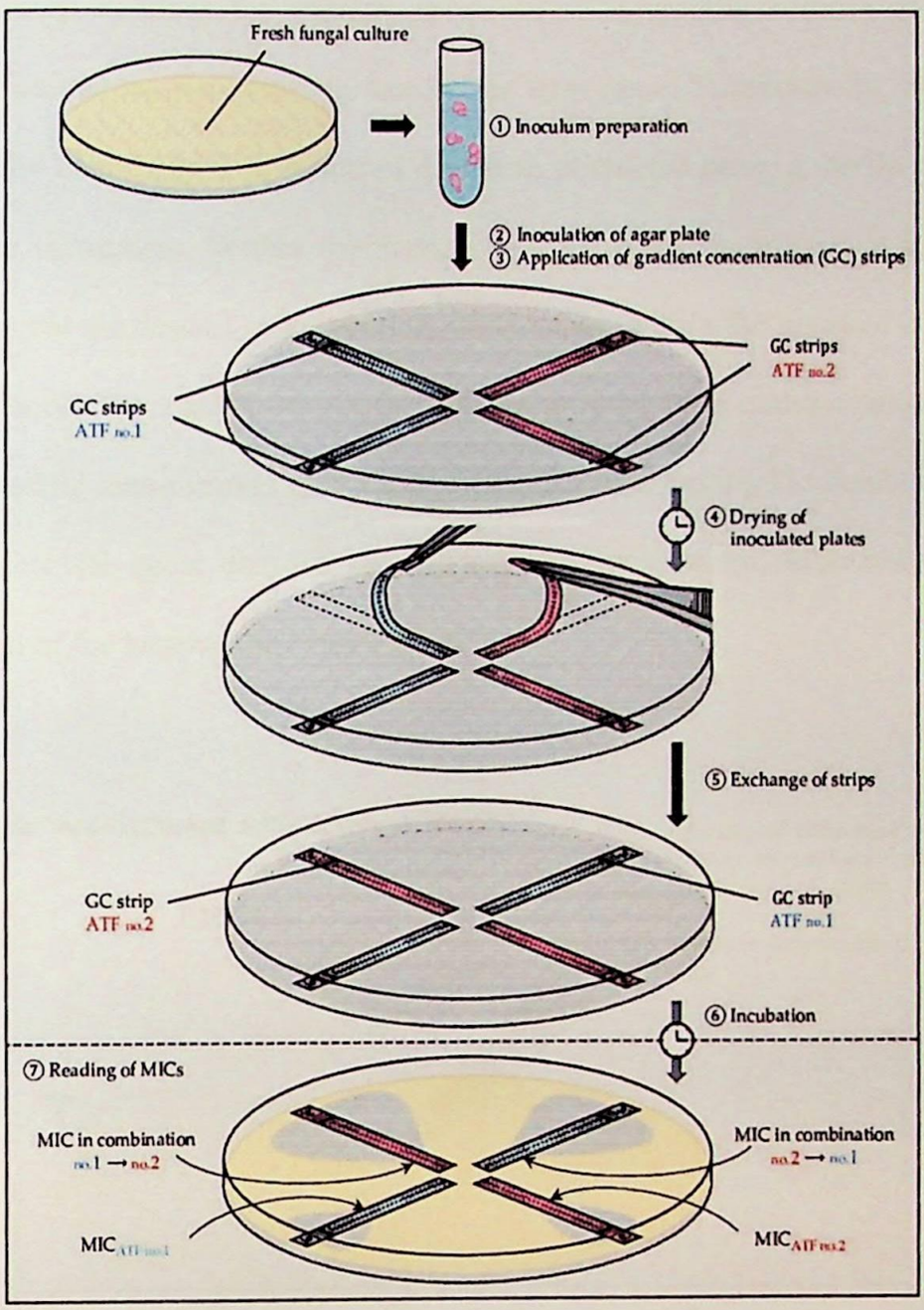


Figure 2. 8 Antimocrobial gradient method for determination of MIC value of antimicrobial agent

2.3.1.3 Agar well diffusion

This method is widely used for the determination of antimicrobial activity plant extract. Following a process akin to the one used in the disk-diffusion method, the initial step involves the uniform spreading of a specific volume of the microbial inoculum across the entire surface of the agar plate. Subsequently, an aseptic well, typically measuring 6 to 8 mm in diameter, is created using a sterile cork borer or a similar instrument. Within this well, a volume of the antimicrobial agent or extract solution at the desired concentration, often falling within the range of 20 to 100 mL, is introduced. The agar plates are then placed in incubation under conditions tailored to the specific requirements of the test microorganism. During the incubation period, the antimicrobial agent diffuses throughout the agar medium, ultimately impeding the growth of the targeted microbial strain.

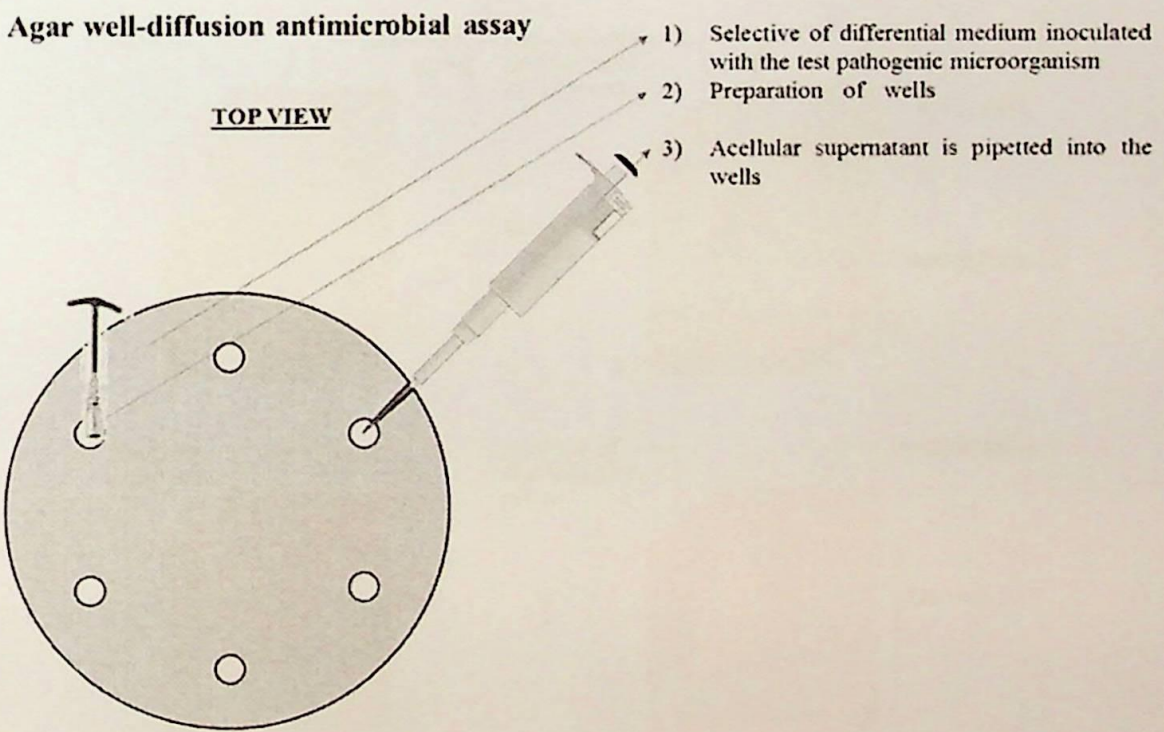


Figure 2. 9 Agar well diffusion methods

2.4 Propionibacterium acnes

The microbial community present on the surface of the skin is predominantly composed of bacteria from three primary genera: *Corynebacteria*, *Propionibacteria*, and *Staphylococci*. These bacteria play a crucial role in shaping the skin's ecosystem and maintaining its overall health (Dréno, Pécastaings *et al.* 2018).

Cutaneous propionibacteria, specifically *Propionibacterium acnes*, are commonly present on human skin and other keratinized epithelial surfaces as harmless inhabitants. These bacteria are gram-positive, which means they retain a violet stain during the Gram staining process. They are nonmotile, lacking the ability to move on their own. When observed under a microscope after isolation, they typically display a distinctive coryneform morphology, characterized by irregular and short branching patterns (Bojar and Holland 2004).

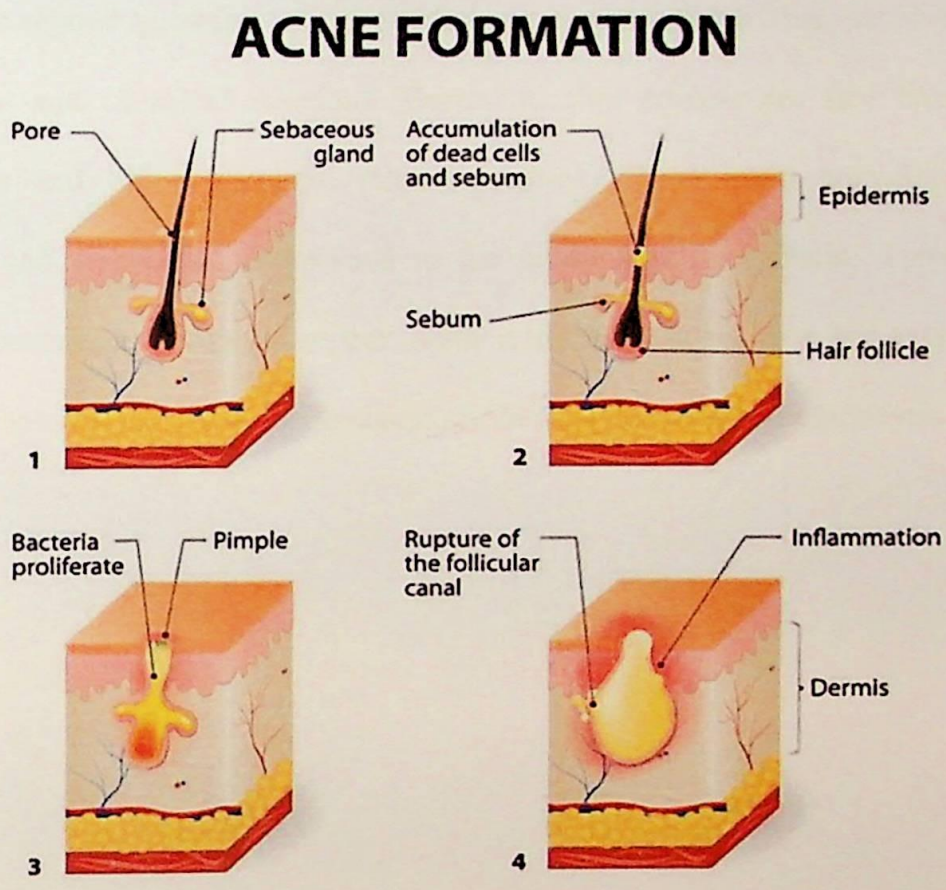


Figure 2. 10 Formation of acne due bacterial infection in the skin

2.5 Tyrosinase enzyme

Indeed, Tyrosinase is the crucial enzyme responsible for enzymatic browning and melanogenesis in mammals (Kilimnik and Dembitsky 2016). Melanins, the dominant pigments accountable for the coloring of human skin, hair, and eyes, are produced by melanocytes in a process known as melanogenesis. Melanogenesis and skin pigmentation serve as pivotal defenses safeguarding the skin against the detrimental impact of ultraviolet radiation from the sun, thus lowering the risk of skin photocarcinogenesis. When there is an abnormal reduction of melanin or depigmentation, it can introduce substantial aesthetic and dermatological concerns for individuals, impacting their facial appearance and overall skin health (Brenner and Hearing 2008). Melanogenesis is a complex process involving numerous enzymatic and chemical reactions. Key enzymes in this process include tyrosinase and other tyrosinase-related proteins. Melanogenesis is a mechanism that involves various enzymatic and chemical reactions. Central to this process are key enzymes like tyrosinase and other tyrosinase-related proteins. Three main enzymes, TYRP1, TYRP2, and tyrosinase, are pivotal in the production of melanin. Tyrosinase, an enzyme containing dinuclear copper, holds a multifunctional role and acts as a rate-limiting factor in the process of melanin synthesis in epidermal melanocytic cells.

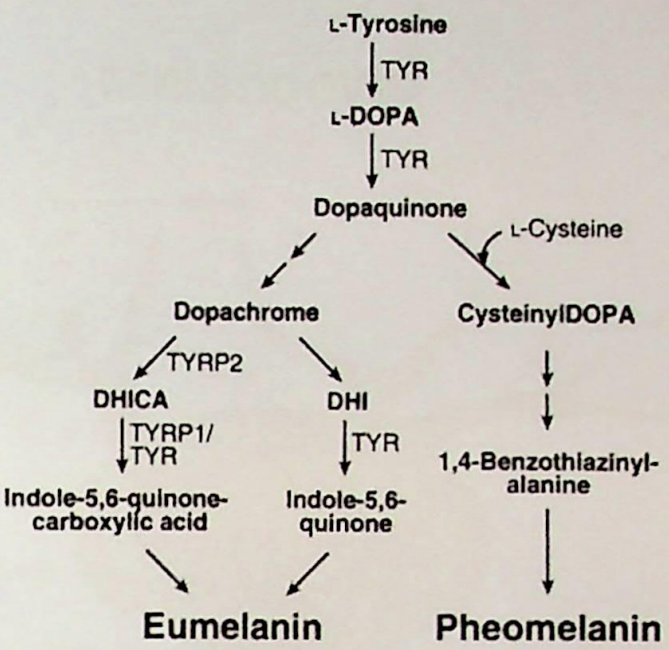


Figure 2. 11 Role of Tyrosinas enzyme in production of melanin

2.5.1 Melanocyte

Melanocytes, as described by Quevedo Jr, Fitzpatrick *et al.* in 1975, are the primary cells having oval shape which contains a number of melanosomes, that is essential place for melanin synthesis. Melanin, recognized as a key pigment influencing variations in skin color (Burkitt, Young *et al.* 1993) produced within these cells. These melanocytes are dispersed randomly in the lower part of the epidermis and do not form direct connections with futher keratinocytes (Ortonne and Bissett 2008). Moreover, differences in the color of skin are influenced by the number and varying sizes of these cells.

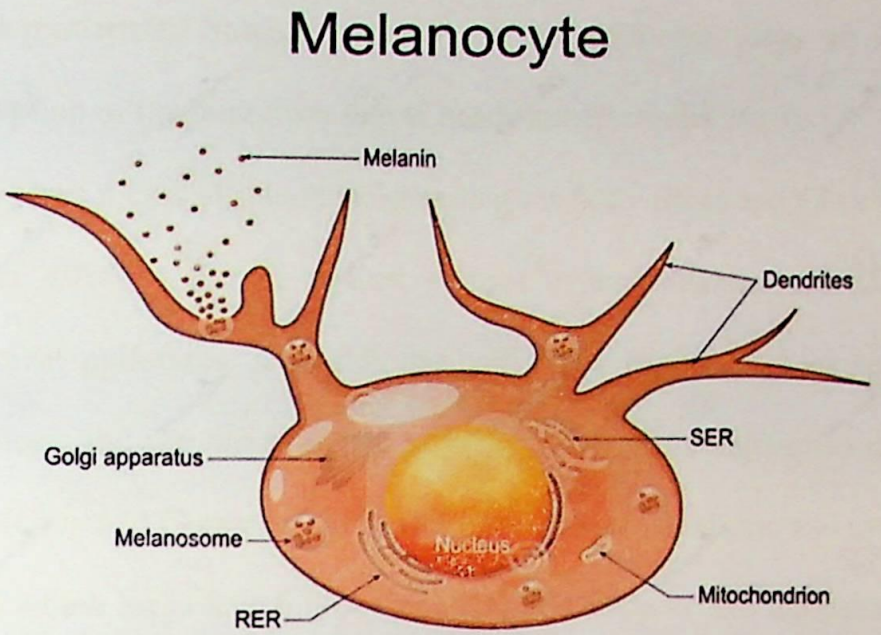


Figure 2. 12 Melanin producing cells (Melanocytes) structure and functions

Additionally, two distinct forms of melanin, phaeomelanin and eumelanin, are produced by irregularly shaped and round-shaped melanocytes, present in the skin (Gray and Gummer 2000). The skin, being the body's largest organ, serves as the primary interface connecting the human body to the external environment (Ross and Christiano 2006). It is a crucial component of the integumentary system (Gray and Gummer 2000) and acts as an outer protective barrier against adverse weather conditions and environmental exposures (Ross and Christiano 2006). In different individuals the thickness of skin can vary, ranging between 1.5 mm-4 mm (Veena 2005). Gender and age differences often result in variations in skin thickness, with males typically having thicker skin than females (Gray and Gummer 2000). The skin covers an area of approximately 1.6 m^2 (Mitsui 1997) and has an average weight of around 3 kg for women and 5 kg for men (El-Amier and Aisha 2019).

The skin consists of three essential protective layers: the dermis, the epidermis, and the hypodermis. Stratum corneum of the epidermis plays an important role in absorption of the drug from skin (Chanda and Baravalia 2010).

Skin plays a crucial role in safeguarding the body (Ross and Christiano 2006). It acts as an effective barrier against various external threats, including physical and chemical pollutants. It shields the body from factors such as radiation, microbial invaders, and the natural aging process, which can compromise the integrity of the skin (Gray and Gummer 2000). Skin can be categorized into two main types: glabrous skin, which lacks hair follicles and is found in areas like the palms, and hairy skin, where multiple hair follicles are present on other parts of the body (Hendriks 2005).

Skin color is determined by melanin, the pigment responsible for imparting color, and erythema, which causes redness due to increased hemoglobin levels. However, repeated exposure and varying environmental stresses can significantly influence skin color changes. The comprehensive process that explains these variations in skin color is referred to as ethnicity (Chan, Ehrlich *et al.* 2005). The anatomy of the skin reveals it to be a multi-layered structural and components (Silver, Freeman *et al.* 2001). However, differentiation in the functions and anatomy of various structures was observed in various races (Chan, Ehrlich *et al.* 2005). Our skin is composed of three important layers: the epidermis, the dermis, and the hypodermis (Igarashi, Nishino *et al.* 2007). The overall structure of the skin is depicted. The epidermis is a outermost covering of skin which is thick, and multi-layered structure composed of stratified epithelia. It serves as barrier that control loss of water from the skin and provides protective coverage for the body (Grabe and Neuber 2005). The thickness of the epidermis varies within the range of 35-50 μm, and it is considered a translucent

structure as it allows light to pass through. It relies on the deeper layers for oxygen and nutritional requirements (Gray and Gummer 2000).

The primary cells that make up the epidermis are called keratinocytes, and their composition varies as they progress through different layers. These cells originate in the deeper layers of the epidermis and gradually migrate towards the upper layers (Hendriks 2005). The epidermis plays a crucial role in generating all the essential lipids necessary for its barrier function, often utilizing glycosyl ceramide as a source (Ponec, Weerheim *et al.* 1997). The epidermis is structured into five layers or strata.

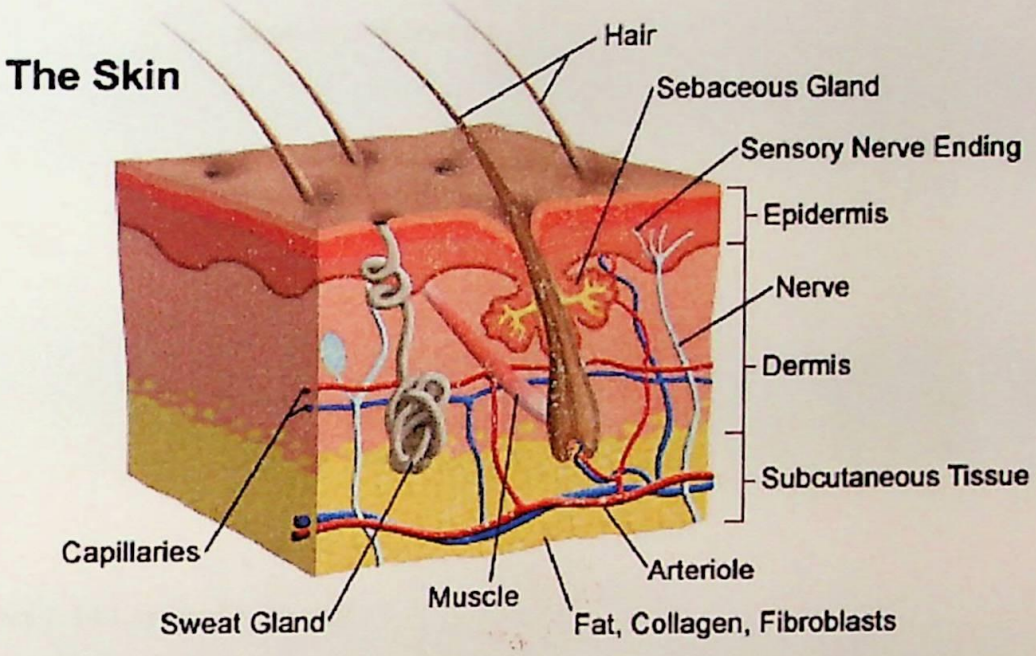


Figure 2.13 Structure of skin

The epidermis is a superficial, thick, and multi-layered structure composed of stratified epithelia. It serves as an excellent barrier against water loss and provides protective coverage for the body (Grabe and Neuber 2005). The thickness of the epidermis varies within the range of 35-50 μm, and it is considered a translucent structure as it allows light to pass through. It relies on the deeper layers for oxygen and nutritional requirements (Gray and Gummer 2000).

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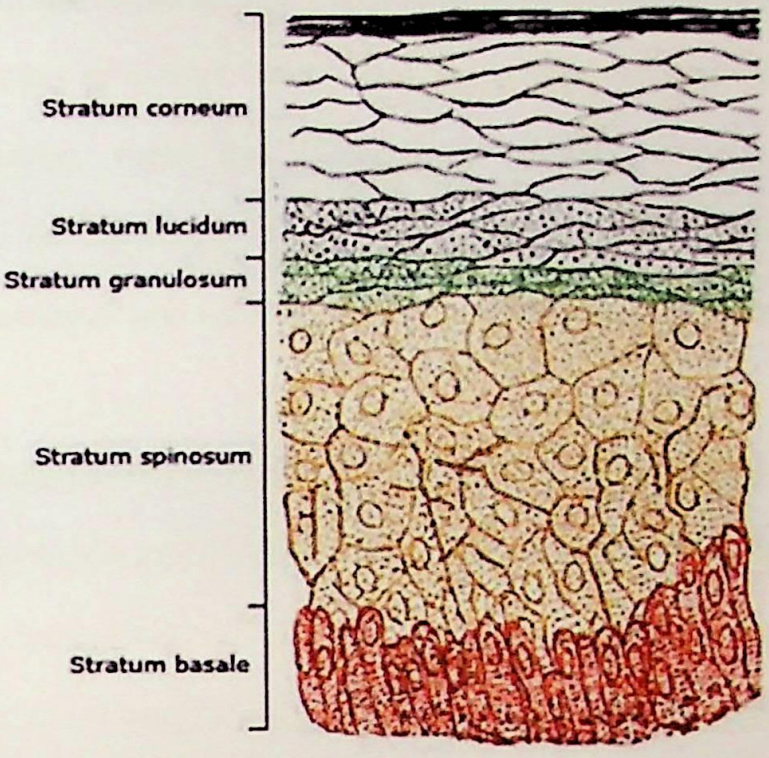

Figure 2. 14 Layers of epidermis

Basal layer of skin contain keratinocytes that are typically found in different shapes. Mostly exhibit cuboidal or columnar shape, organized in a single layer, with oval-shaped nuclei. It's important to note that desmosomes at the cell surfaces play a crucial role in connecting adjacent cells. This basal layer exhibits significant mitotic activity, particularly in the generation of keratinocytes, which collectively form the stratum germinativum. These newly formed cells gradually move towards the uppermost layers, displacing the older cells (Hendriks 2005). The basal layer plays a

vital role in the keratinization process and contributes to water retention, becoming firmer with advancing age (Igarashi, Nishino *et al.* 2007). Additionally, basal layer also contain melanocytes and Merkel cells. Merkel cells are involved in sensory functions, while melanocytes produce melanin (McLafferty, Hendry *et al.* 2012). Stratum Lucidum is a distinctive, thin layer consisting of translucent or lucid bands found exclusively in thick skin areas, such as the soles of the feet or the palms of the hands. It is made up of eosinophilic cells that lack nuclei and are neatly organized in two to three layers (Hendriks 2005). This layer also enhances the skin's barrier function, preventing water loss (McLafferty, Hendry *et al.* 2012). Stratum Granulosum: As the name suggests, this layer of the epidermis is composed of flat, granular, and rhomboid skin cells with elongated axes, organized into 3 to 5 distinct layers.

This layer has huge number of keratinocytes that produce a protein known as keratin. It contains multiple granules of keratohyalin, which can bind and shape these keratin filaments (Piepkorn, Predd *et al.* 2003). In the Stratum Spinosum layer, the keratinocytes have a distinctive polyhedral shape, with round or spherical nuclei on their surfaces. These cells also display well-defined spines on their cell surfaces, which enable them to connect and bond with neighboring cells through desmosomes. Furthermore, these spines play important role in initiating the process of keratinization (Ross and Christiano 2006).

The Stratum Corneum serves as the outermost and most significant epidermal layer, consisting of cornified, dead cells typically organized into 25-30 distinct layers, which are primarily keratinocytes (Hendriks 2005). These cells are rich in lipids, which optimize cells barrier function and help in protective mechanism of skin. These lipids are essential for retaining water, accounting for as much as 98% of this layer

(Plasencia, Norlén *et al.* 2007). Additionally, they play a crucial role in the absorption of drug compounds. The dermis, located beneath the epidermis, is the second important layer of the skin and has a thickness ranging from 1mm to 4mm (Igarashi, Nishino *et al.* 2007). The main structural component of this layer is abundant collagen fibers, constituting approximately 75% of its composition, along with the presence of elastin fibers. Consequently, the dermis has more fibers than cells, in contrast to the epidermis. The dermis is further divided into two layers:

- Papillary Layer
- Reticular Layer

Papillary layer is the dermis first significant part, which is present beneath the upper epidermis (Igarashi, Nishino *et al.* 2007). This layer is also known as a sheet of loose connective tissue. Its primary components include fibroblasts, different types of nerve fibers, capillary vessels, and prominent sensory receptors. Additionally, this layer also contain high amount of collagen fibers. The second most important layers reticular layer is, thicker part of the dermis and is characterized by randomly planned connective tissue and a reduced quantity of nerve fibers (Helfrich, Sachs *et al.* 2008). Its increased thickness is primarily due to the accumulation of collagen fibers in huge packages (Cotton and Claridge 1996). These packages are join together with the assistance of elastic fibers composed of protein i.e. elastin.

The hypodermis, situated beneath the dermis layer, is abundant in fatty tissues that provide essential support to the human skin. It is also referred to as the subcutaneous layer and constitutes approximately 10% of the total body. This layer acts as a crucial role in drug absorption and is primarily composed of adipose tissue, with smaller amounts of loose connective tissues. The thickness of the hypodermis can vary among individuals, influenced by factors such as nutrition, gender, and age (Hendriks 2005).

The level of fatty tissue in the hypodermis is mainly depending on age, gender, and the distribution of fats in various parts of the body. The hypodermis is also essential for providing insulation to the body, regulating heat, and serving as an energy reservoir (Cottam 1969).

2.5.2 Other Epidermal cells

Langerhans cells are well-known for their star-shaped appearance and distinctive surface antigens. They are also characterized by the presence of special Birbeck-type granules. Due to their numerous phagocytic activities,

Langerhans cells play important role in the immune defense of the skin (Wickett and Visscher 2006). These cells are primarily found in various layers of the epidermis, with a higher concentration in the spinosum and granulosum layers (Gartner 2020). Merkel Cells are distinctive for their spine-like projections and the presence of multiple granules that give their cytoplasm a darker color.

These cells are primarily located in the basale layer of the epidermis. They are often connected to keratinocytes through desmosomes. Merkel cells are believed to have interactions with Langerhans cells, suggesting a role in neuro-immunology, likely due to their association with free nerve endings.

The most abundant and essential epidermal cells are, specialized in the protein production know as keratin. These keratinocytes play a vital role in protecting the skin from various environmental elements and retaining moisture, thereby contributing to skin health. As new cells continually replace older ones, the entire process is referred to as desquamation (Gartner 2020).

2.5.3 Other skin appendages

Sebaceous glands are crucial, round-shaped glands distributed throughout the body, except soles and palms. They are abundant in the dermal layer but open into hair

follicles through ducts. These glands are known for their synthesis of sebum (Youn, Na *et al.* 2005). This intricate mixture serves to protect the skin against microbes and prevent fungal growth (Gray and Gummer 2000). Additionally, it helps maintain the skin's normal hydration levels and moisture content (Celleno and Tamburi 2009). Sweat glands can be further categorized into two distinct types based on their structural variability and the nature of their secretions: Eccrine Sweat Glands are generally the most common type of tubular glands which are found in the dermal layer, but they are not present in external genitalia and our lips.

These glands consist of two important components: dark cells and clear cells, each with distinct structures and involved in the release of different types of secretion. Dark cells release a mucoid secretion, while clear cells release a watery secretion. The sweat produced by eccrine sweat glands is a clear discharge containing urea, water, sodium chloride, and some other salts. These glands play an important role in regulating the temperature of body (Youn, Na *et al.* 2005). The location of Apocrine Sweat Glands are predominantly found in areas such as the axilla (armpits), internal pubic area and the breast parts (areola) (Gray and Gummer 2000). These glands release a clear secretion that is initially odorless but can develop a musky odor due to the involvement of bacteria (Kolarsick, Kolarsick *et al.* 2011). Despite their presence, the exact functions of apocrine sweat glands remain unknown (Junqueira, Barros *et al.* 2004). Nails are significant structures composed of hard cells densely packed with the keratin proteins. They continually grow throughout a person's life. Nails consist of a broad nail plate and other associated structures, including the nail bed and the extensive nail matrix (Uitto, Bruckner-Tuderman *et al.* 2018). Their primary functions include protecting the tips of the fingers and being useful for scratching, applying cosmetics, and other daily activities related to the skin (De Berker, André *et al.* 2007).

Hairs are distributed throughout the body, except for areas such as the palms, soles, and lips. The length of a hair can vary widely, ranging from 1mm to 1m, and they also come in different widths. The number and distribution of hairs on different parts of the body can vary. For instance, the face has approximately 600cm² of hair, while the body has around 60cm² (Standring 2021).

Hairs consist of important components, including a distinct hair bulb, a deep root, and a long hair shaft (Landois 1905). Hairs play essential roles in providing protection and serving significant sensory functions (Buffoli, Rinaldi *et al.* 2014). The skin performs numerous functions, with its barrier function being one of the most prominent, primarily relying on the lipids secreted by various glands. The Stratum Corneum, in particular, plays a significant role in these barrier functions (Plasencia, Norlén *et al.* 2007). In addition to its barrier function, the skin plays a crucial role in the following four functions (Young, O'Dowd *et al.* 2013):

The outer layers of the epidermis play a crucial role in shielding the skin from exposure to free radicals and extreme thermal conditions (Jablonski and Chaplin 2010). Additionally, the skin acts as a protective barrier, preventing injuries to internal organs and underlying muscles (The stratum corneum, in addition to its role as a barrier, significantly contributes to preventing the loss of moisture from the body. While the basal layer of the epidermis contains about 70-75% of the skin's moisture, the stratum corneum retains just 10-15% of the total water content (Standring, Ellis *et al.* 2005).

Maintaining the right levels of water content is essential for healthy skin. Adequate water content ensures the pliability of the stratum corneum, and proper hydration enhances its permeability to various substances (Varani, Warner *et al.* 2000).

Our skin functions as the primary barrier against various microbes, such as bacteria and fungi, especially when it is coated with an oil-in-water emulsion (Young, Woodford *et al.* 2013). Additionally, the naturally acidic nature of our skin serves as an antibacterial agent (Nolen-Hoeksema, Larson *et al.* 1999). The skin also plays a significant role in the process of thermoregulation. The regulation of heat is often influenced by the presence of fatty tissue deposits in the skin and hair. Additionally, the sweat glands within the skin are responsible for facilitating heat loss.

2.6 Mushroom tyrosinase

Indeed, tyrosinase enzymes can be found in various sources, including plants, animals, and microorganisms. While the sequences of many tyrosinases, including the human variant, have been identified; only a limited number of them have undergone comprehensive characterization. Mushroom tyrosinase from Agaricus bisporus is a notable and cost-effective source of tyrosinase among various sources. It exhibits a high degree of homogeneity and similarity to human tyrosinase.

CHAPTER: 3

3. MATERIALS AND METHODS

3.1 Apparatus and Chemicals

The research work involved the use of various chemicals and apparatus for experimentation and analysis. These items may include, but are not limited to:

Table 3 1 List of chemicals and apparatus used during study

Chemicals And Reagents	**Origin**
2,2-diphenyl-1-picrylhydrazyl (DPPH)	Sigma Aldrich (St. Louis, MO, USA)
Caffeic acid	Sigma Aldrich (St. Louis, MO, USA)
Distilled Water	Department of Pharmacy, ONIMS
Electric Analytical Balance	Shimadzo (AUX 220), Japan
Electrical Balance	Precisa BJ-210, Switzerland
Ethanol	Merk (Germany)
Ethyl Acetate	Merk (Germany)
Gallic acid	Sigma Aldrich (St. Louis, MO, USA)
Glassware	Pyrex, France
Hot Incubator	Sanyo MIR-162, Japan
HPLC-DAD	(Agilent Germany)
Instruments	
Kojic acid	Sigma Aldrich (St. Louis, MO, USA)
L-DOPA	Sigma Aldrich (St. Louis, MO, USA)
Micropipette	Zhejiang Huawei Scientific Instrument Co., LTD. China
Microplate reader	(Biotek synergy ht, USA)

Mushroom tyrosinase	Sigma Aldrich (St. Louis, MO, USA)
n-Hexane	Merk (Germany)
pH-Meter	WTW pH-197i, Germany
Refrigerator	OREINT, Pakistan
Rotary evaporator	(Heidolph, Co.Ltd. Japan)
Sorbex RXC8	(Agilent USA)
UV spectrophotometer	(UV 4000 ORI, Germany)
Water Bath	HH .$S_{214,}$ China

3.2 Plant Sample Collection and Identification

The *Fagonia Arabica* whole plant was collected from Mianwali. Sample was identified from Peer Mehr Ali Shah (PMAS) University, Rawalpindi with Voucher No: 1003.

3.3 Extraction and Fractionation of Plants

The plant material was collected and washed in running tap water shade dried until complete water loss and moisture content was determined (loss on drying). 500 g of plant material was extracted with 1500 mL ethanol. After maceration for 72 hrs the extract was pass through muslin cloth and filter through whatman filter paper No 1. Filtrate was dried using a rotary evaporator. The fractionations was done with various polar and non-polar organic solvents (n-hexane, ethyl acetate, chloroform and water), was made.

3.4 Phytochemical Analysis

3.4.1 Qualitative Analysis

The methadology of Hossain *et al.* (Hossain, AL-Raqmi *et al.* 2013) was implemented for revealing of phenols, coumarin and saponins in extract/fractions of *Fagonia*

Arabica. The methodology adopted by Ramu *et al.* (Ramu, Mohan *et al.* 2012) was used to spot flavonoids, tannins, terpenoids and alkaloids.

3.4.2. Polyphenolics contents determination

The analysis of flavonoids and phenolic contents in natural plants or herbs is very important to understand their medicinal value. In order to determine the amount of total flavonoid contents (TFC) in different parts of the Fagonia arabica plant, a specific method is followed.

First, a small amount (0.3 mL) of each plant sample is mixed with a solution containing aluminum chloride ($AlCl_3 \times 6H_2O$) in a concentration of 0.3 mol L^{-1}. Then, 0.1 mL of sodium nitrite solution in a concentration of 0.5 mol L^{-1} is added to the mixture. After that, 3.4 mL of methanol (30%) is added. (Park, Jung *et al.* 2008) . The resulting mixture is analyzed using a spectrophotometer, which measures the change in absorbance at a wavelength of 506 nm. The TFC values are usually expressed as micrograms of quercetin equivalents (QE) per milligram of dry plant extract.

The quantity of phenolic contents (TPC) in the plant fraction is determined using a similar procedure. In a mixture of 1 mg of sample in 9 mL of water, 1 mL of Folin-Ciocalteu reagent is added. Properly mix the solution 5 minutes. To this mixture, 10 mL of sodium carbonate (Na_2CO_3) solution in a concentration of 7% is added. After incorporating all the contents quantity sufficient water is added to make the final volume up to 25 mL. For 90 minutes the mixture was incubated at 25°C, and the absorbance was noted at 750 nm. The TPC values are expressed as micrograms of gallic acid equivalents per milligram of dry plant extract (Kim, Chun *et al.* 2003).

3.5 DPPH Method for Antioxidant Activity.

The antioxidant activity of the different fractions of *Fagonia arabica* were determined by 2,2-diphenyl-1-picryl-hydrazyl (DPPH) method. In this method, 24 mg of DPPH reagent is mixed with 100 mL of methanol for the preparation of stock solution and stored at a temperature of 20 degrees Celsius, were subjected to an antioxidant assay. In separate test tubes, 3 mL of a DPPH (2,2-diphenyl-1-picrylhydrazyl) reagent from the stock solution were combined with samples prepared at a concentration of 1 milligram per milliliter (mg mL^{-1}). These test tubes were then incubated for a period of 15 minutes at room temperature. Subsequently, the absorbance of each sample was measured at a wavelength of 517 nanometers (nm). This assay is commonly used to assess the antioxidant activity of samples by measuring the reduction of the DPPH radical, with a lower absorbance indicating higher antioxidant capacity.(Majid, Khan *et al.* 2015).

3.6. Total antioxidant capacity (Phosphomolybdenum assay)

Antioxidant species reduce molybdenum (VI) to molybdenum (V) which basic principle of the phosphomolybdate assay; the reduced form in acidic medium and at high temperature make a complex with phosphate. (Prieto, Pineda *et al.* 1999). The protocol of Umamaheswari and Chatterjee was used to investigate the total antioxidant activity of the plant samples, (Umamaheswari and Chatterjee 2008). According to the protocol, a 0.1 mL aliquot of the plant sample is combined with 1 mL of a reagent solution. This solution of reagent was prepared by dissolving 28 millimoles per liter (mmol L^{-1}) of sodium phosphate (Na_3PO_4) in a solution containing 0.6 mmol L^{-1} of sulfuric acid (H_2SO_4) and 4 mmol L^{-1} of ammonium molybdate. The resulting mixture is then incubated at a temperature of 95 degrees Celsius for a duration of 90 minutes. After the incubation period, the absorbance of

the solution is measured at a wavelength of 760 nanometers (nm), using gallic acid as a standard for reference. This procedure is commonly employed to assess a specific property or concentration in the plant sample, with the absorbance value serving as an indicator.

3.7 Ferric Reducing Ability of Plasma (FRAP)

For reducing potential 2mL of each fraction at 4mg/mL concentration was used by exercising the protocol of Siddhuraju *et al.* (Siddhuraju, Mohan *et al.* 2002) In each sample of every fraction, more than 2 milliliters (mL) of 0.2 molar phosphate buffer with a pH of 6.6 were added, along with 2 mL of potassium ferricyanide at a concentration of 10 milligrams per liter (mg L^{-1}). This mixture was then incubated at a temperature of 50 degrees Celsius for a duration of 20 minutes. After the incubation, the mixture was combined with 2 mL of trichloroacetic acid at a concentration of 100 mg L^{-1}. The sample was subsequently diluted with water, and 0.1% ferric chloride ($FeCl_3$) at a volume of 0.4 mL was added. Measurement of the absorbance of resulting solution was done at 700 nanometers (nm) after 10 minutes, using gallic acid as the standard for comparison. This procedure is typically used to assess the reducing power or antioxidant capacity of the samples, with higher absorbance values indicating stronger reducing or antioxidant properties.

3.8. Inhibitory Effect on Tyrosinase Enzyme Activity

To evaluate tyrosinase inhibition, a series of steps were performed as follows: Initially, 10 microliters (10 µL) of a 0.5 millimolar (mM) test sample were mixed with 40 µL of a 100 mM phosphate buffer at pH 6.8, and then 20 µL of active enzyme (1000 units) were added to reach a total volume of 100 µL for the mixture. This mixture was subsequently incubated for 5 minutes at 37 degrees Celsius. Following this incubation period, 10 µL of a solution containing levo-dopamine at a

concentration of 10 mM were added to the mixture. After all the mixing was completed, the contents were allowed to incubate at room temperature for an additional 10 minutes. To assess the reaction, the absorbance of the sample was measured at 492 nanometers (nm) using a 96-microplate reader. This process is commonly used to assess the inhibition of tyrosinase activity, which is involved in melanin production and can be relevant in studies related to skin pigmentation and related conditions (Kim 2007). .

3.9 HPLC-DAD analysis

HPLC-DAD (High-Performance Liquid Chromatography with Diode Array Detection) analysis was conducted on various fractions of a plant sample using an Agilent Zorbax Rx-C8 analytical column. The column had dimensions of 250 millimeters in length, 4.6 millimeters in internal diameter, an 80 Ångström pore size, and contained particles with a size of 5 micrometers. The analysis followed the method previously established by Majid *et al* (Majid, Khan *et al.* 2015). In this method, a mobile phase comprising two components, Eluent A (acetonitrile-methanol-water-acetic acid, 5/10/85/1) and Eluent B (acetonitrile/methanol/acetic acid, 40/60/1), was employed for chromatographic separation. The analysis was conducted at ambient temperature using an Agilent Zorbax Rx-C8 analytical column (250 × 4.6 mm, pore size 80 Å, particle size 5 μm) following the method established by Majid *et al*. The gradient program consisted of: 0–20 min (0 to 50% B), 20–25 min (50 to 100% B), and then isocratic 100% B (25–40 min), with a flow rate of 1.2 mL/min and a 20 μL injection volume. Prior to injection, samples were filtered using a 0.45 μm pore size membrane filter to remove particulate matter and impurities, ensuring that only dissolved components were introduced into the HPLC-DAD system for analysis.. Among the standards, rutin was analyzed at Various standard

phenolic and flavonoid compounds and thereby eluted at Caffeic acid, Gallic acid, Chlorogenic acid, Apicatechin, Hyperoside, Ferulic acid, Rutin, Luteolin, Fisetin, Benzene-triol, Apigenin-7-glucoside, Naringenin, Chrysin and Apigenin at 1.85 min, 10.07 min, 12.14 min, 15.76 min, 19.6 min, 28.88 min, 30.08 min, 30.66 min, 33.66 min, 35.102 min, 39.03 min, 43.00 min, 46.86 min and 57.00 min respectively.

3.10 Antimicrobial Assay

The antibacterial potential of the utilized extracts was investigated using the agar well diffusion method. To achieve a concentration of 500 µg/mL and 250 µg/mL, each extract/fraction was dissolved in 1 mL of 99.9% dimethyl sulfoxide (DMSO) from Sigma-Aldrich USA. As a positive control, doxycycline (30 µg/mL) in DMSO was prepared. Pure DMSO (99.9%) was employed as the negative control.

3.11 Statistical and Mathematical Analysis

Two-way analysis of variance (ANOVA) and Paired sample "t" test was used to analyzed the data using SPSS 16.0 at 0.05 level of significance. Graphical representation was done using GraphPad Prism.

CHAPTER: 4

4 RESULTS

4.1 Phytochemistry

4.1.1 Phytocompound grouping of *Fagonia arabica* plant extract and fractions

Screening and costing of variety of medicinal plants to discover multiple antioxidant compounds and unique phytochemicals may lead to the unearthing of more heady and safer substances, as more side effects may be associated with other synthetic compounds. According to new research/ advancements as mentioned in different publications of "*Food Chemistry*" it is well established that expenditure of variety of *crude extracts* from many plants/ cereals /fruits and many other bioactive moieties in pure form exhibit potential antioxidant activity (Gundogdu, Duran *et al.* 2012). Results and outcomes from the study is to support the rational corroboration and to the further scientific declaration that the extracts/fractions of *Fagonia arabica* in traditional system of medicines which is proved to be very economical, safer and effective for humans and their skin.

In this study many different phytochemicals and important bio-actives substances such as flavonoids, saponins, coumarin, important alkaloids, carotenoids, tannins and other anthraquinones were distinguished from the different extracts/ fractions as they were collected from *Fagonia arabica*. It is clearly evident that these extracts were found to have plentiful saponin, tannins, flavonoids, and important alkaloids. Phenols, tannins and flavonoids were seen in all fractions/extracts of *Fagonia arabica* during phytochemical analysis except in n-Hexane fraction (FAH). Coumarins were part of *Fagonia arabica* chloroform (FAC), ethyl acetate fraction (FAEA) and in the plant extract FAE. FAC and FAEA fraction of the plant show presence of all exercised phytochemical groups. *Fagonia arabica* water fraction (FAW) shows the occurrence of Phenols, Flavonoids, Tannins and Terpenoids Table 4.1.

Table 4 1 Phytochemical grouping of various fractions of *Fagonia Arabica*

Constituents	*Fagonia Arabica*				
	FAE	FAH	FAEA	FAC	FAW
Phenols	+++	-	+++	+++	+
Flavonoids	+++	-	+++	+++	+
Coumarin	+	-	+	++	-
Saponins	+	-	+	+	-
Tanins	++	-	++	++	+
Terpenoids	+	+	+	++	+
Alkaloids	++	-	+	+	-

FAE- *Fagonia arabica* Hydro alcoholic extract , FAH- *Fagonia arabica n*-Hexane fraction, FAEA - *Fagonia arabica* ethyl acetate fraction, FAC - *Fagonia arabica* chloroform fraction, FAW - *Fagonia arabica* water fraction

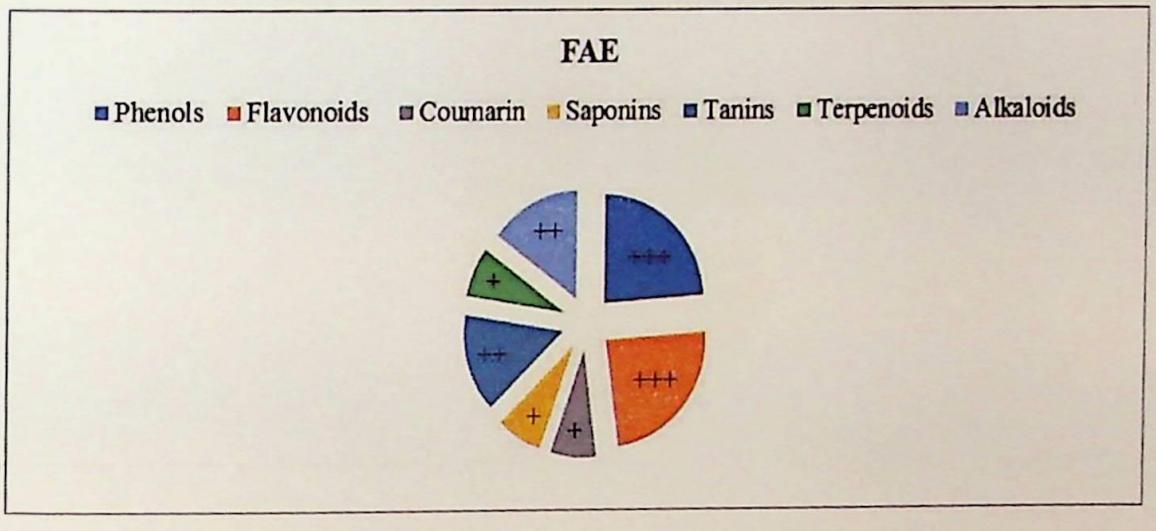

Figure 4. 1 Presence of various phytochemical group in *Fagonia arabica* extract

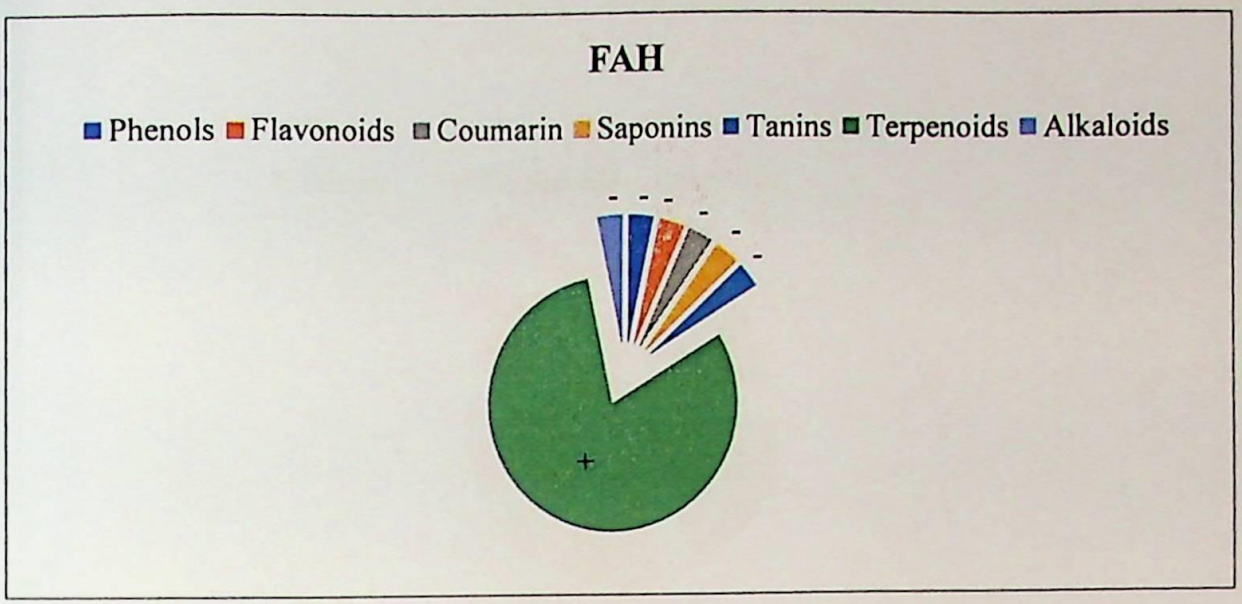

Figure 4.2 Presence of various phytochemical group in *Fagonia arabica* n-hexane fraction.

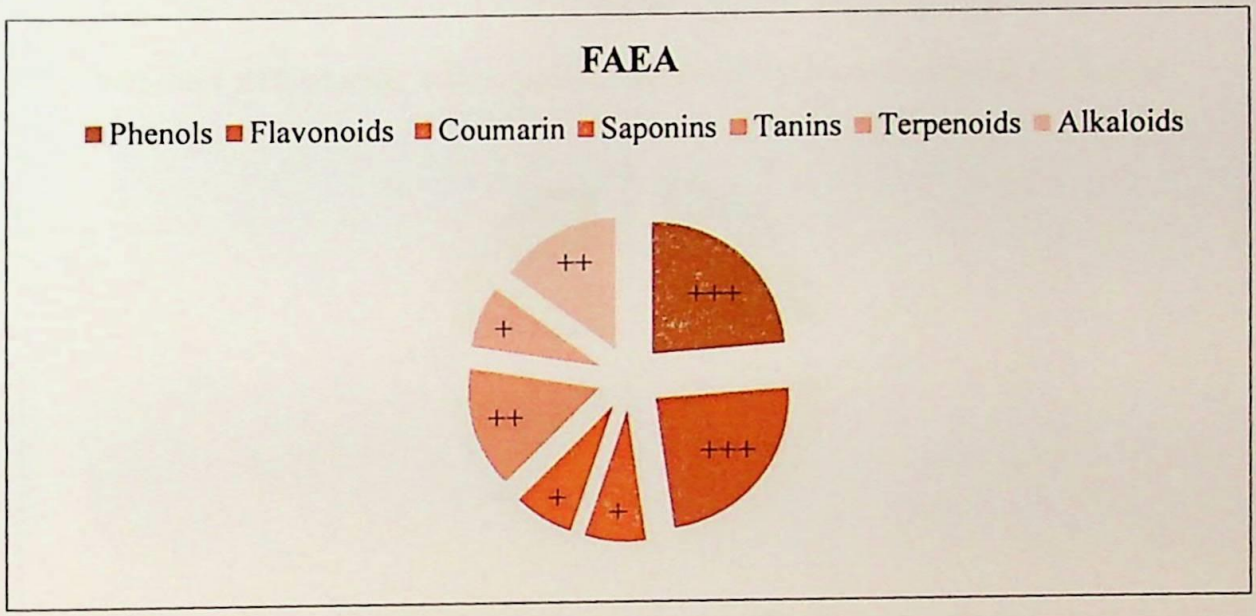

Figure 4.3 Presence of various phytochemical group in *Fagonia arabica* ethyl acetate fraction.

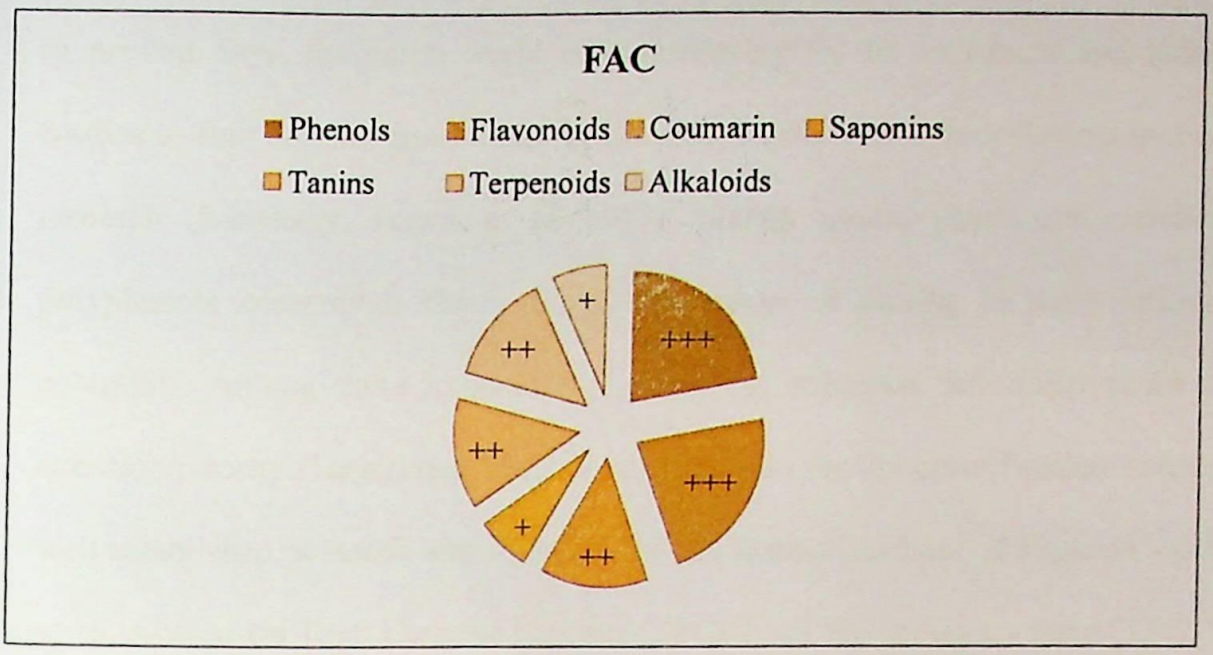

Figure 4. 4 Presence of various phytochemical group in *Fagonia arabica* chloroform fraction.

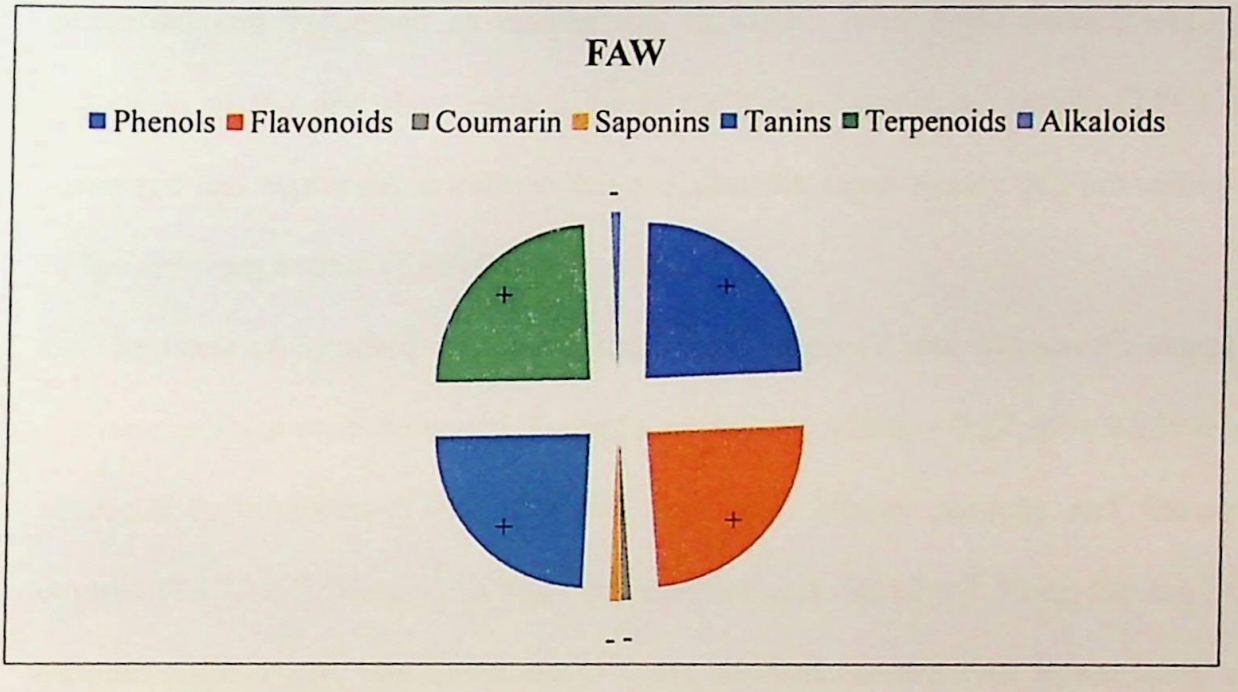

Figure 4. 5 Presence of various phytochemical group in *Fagonia arabica* water fraction

4.1.2 Total phenolic content (TPC) and total flavonoid content (TFC)

In present days, the entire world is concentrating on the on natural and botanical cradles to find out numerous antioxidants components such as polyphenols in modern research (Iranshahy, Javadi *et al.* 2017). Mainly natural plants are enriched in polyphenols compounds that exhibit broad range of owning to their antioxidant potentials. Among these imperative phenols, the important flavonoids attain more attention/priority (Hagerman, Riedl *et al.* 1998). So for the quantification purpose, a well-established protocol was followed for the extract/fractions of *Fagonia arabica*, so as to calculate Total phenolic contents (TPC)/Total flavonoids contents (TFC) by a spectrophotometer. Phenolic content were calculated at 750nm wavelength and flavonoids were measured at 506nm. The Total Phenolic Content (TPC) was quantified and expressed as micrograms of Gallic Acid Equivalents (GAE) per milligram of the dry plant extract, while the Total Flavonoid Content (TFC) was measured and expressed in micrograms of Quercetin Equivalents (QE) per milligram of the dry plant extract (Kim, Jeong *et al.* 2003).

On the basis of standard regression lines Total phenolic and flavonoid content of *Fagonia arabica* were measured, for gallic acid ($y = 0.0197x + 0.27$, $R^2 = 0.9504$) and quercetin ($y = 0.006x+0.395$, $R^2=0.96$) Table 4.2. Higher phenolic and flavonoid content (72.77 ± 2.78 µg GAE mg^{-1} dry sample) and (82.12 ± 1.56 µg QE mg^{-1} dry sample) respectively, was quantified in hydro alcoholic extract FAE in our study and while the lowest quantities of phenolic (27.14 ± 2.03 µg GAE mg^{-1} dry sample) and flavonoids(54.64 ± 0.30 µg QE mg^{-1} dry sample) were found in FANH. Anagnostopoulou *et al.* (Anagnostopoulou, Kefalas *et al.* 2006) documented that ethanolic extract of *Citrus sinensis* was found to contain a higher concentration of phenolic compounds compared to the other fractions..

Table 4 2 Total phenolic and total flavonoid content evaluation in non-polar and polar fractions of *Fagonia arabica*

Extract/fraction	TFC	TPC
	(µg QE mg^{-1} dry sample)	(µg GAE mg^{-1} dry sample)
FAE	82.12088 ± 0.30	72.77157 ± 2.03
FAH	54.64835 ± 1.56	47.39086 ± 2.78
FAEA	76.62637 ± 0.45	58.55838 ± 2.03
FAC	67.28571 ± 0.16	51.95939 ± 1.23
FAW	74.97802 ± 0 .13	67.69543 ± 0.78

GAE stand for gallic acid equivalent, QE stand for quercetin equivalent, TPC stand for total phenolics content, TFC stand for total flavonoids content.

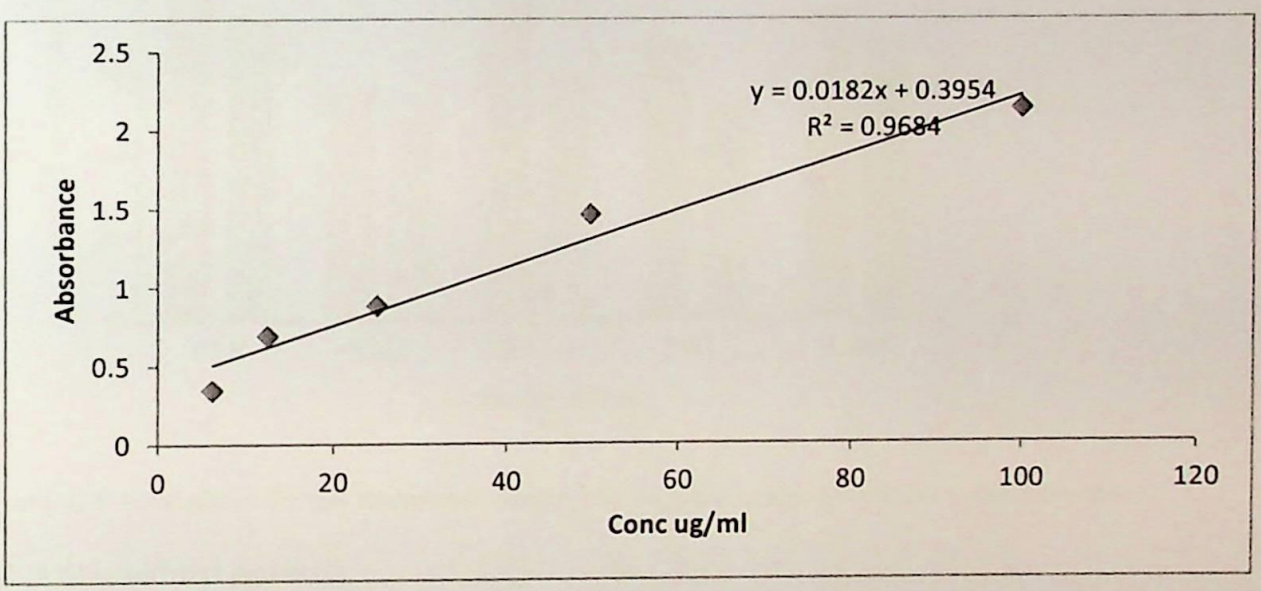

Figure 4. 6 Regression line of Quercetin with Total flavonoid contents

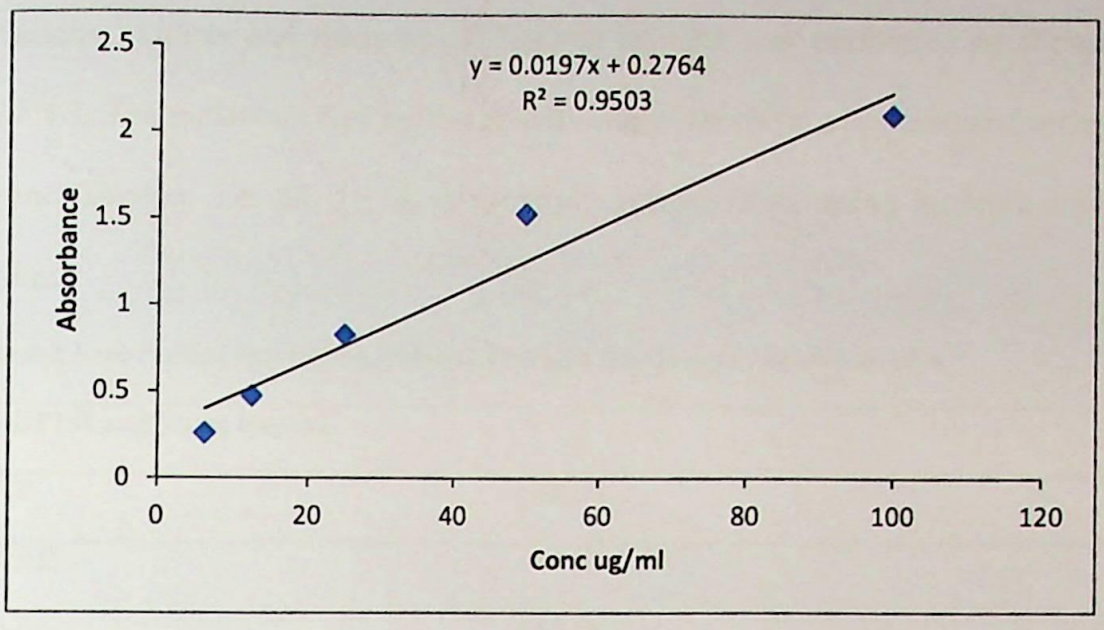

Figure 4. 7 Regression line of Gallic acid with Total phenolic content

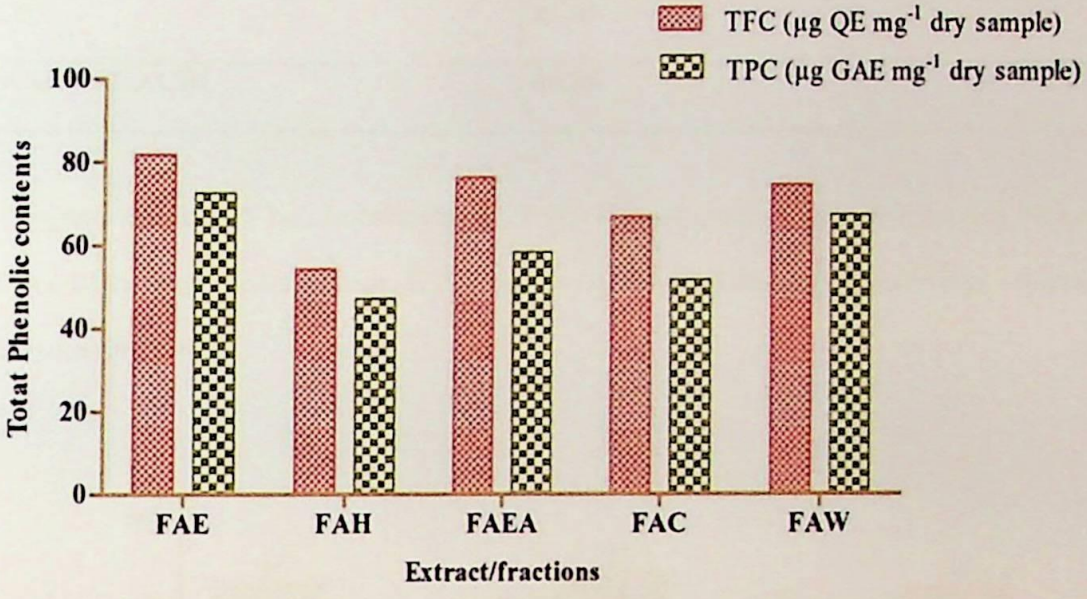

Figure 4. 8 Total phenolic and flavonoids contents in Fagonia arabica various extracts/fractions

4.2 Antioxidant activity

The procedure for the assessment of antioxidant activity is established on scavenging of numerous oxygen species (free). In advanced studies antioxidants from plant/ natural resources such as multiple flavonoids, lycopene, carotenes and ascorbic acid proved them as potential antioxidants (Yilmaz and Toledo 2006). It is understood that extracts are plentiful in these phytochemical moieties therefore calculation/ assessment of their antioxidant activity can be more fruitful. The antioxidant activity

of various extracts and fractions of *Fagonia arabica* was performed as shown in Table 4.3. The maximum free radical activity was observed in ethyl acetate fraction of *Fagonia arabica* i.e. 88. 16 % at 1 mg/mL concentration using ascorbic acid as standard.

Table 4 3 Free radical scavenging activity of various fractions of *Fagonia arabica*

% DPPH activity at Img/mL	
FAE	85.39
FANH	51.68
FAEA	88.16
FAC	78.31
FAW	83.67
ASCORBIC ACID	89.88

FAE- *Fagonia arabica* Hydro alcoholic extract , FAH- *Fagonia arabica n*-Hexane fraction, FAEA *Fagonia arabica* ethyl acetate fraction, FAC - *Fagonia arabica* chloroform fraction, FAW - *Fagonia arabica* water fraction.

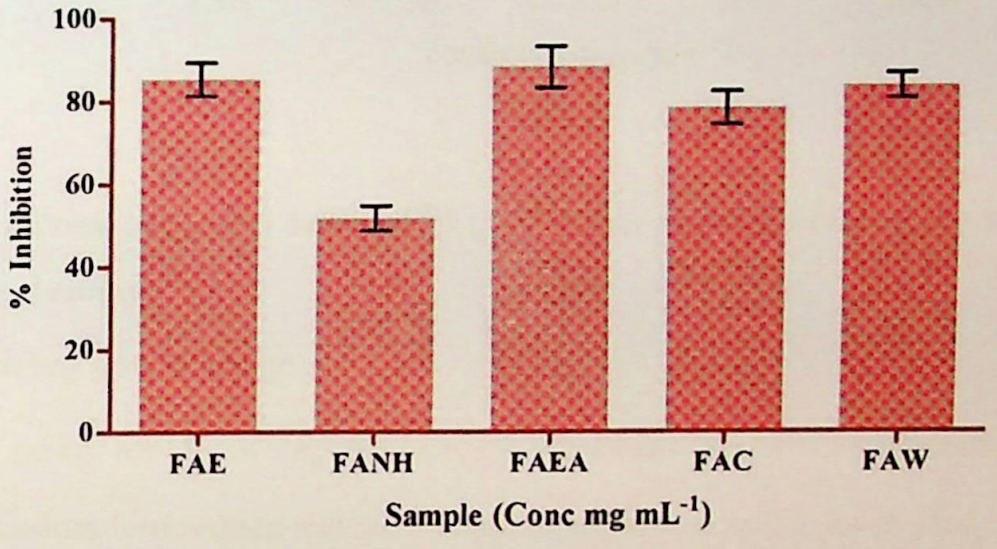

Figure 4. 9 DPPH potential of plants extracts/fractions at 1 mg/mL concentrations.

4.3 Phosphomolybdenum assay

Total antioxidant activity of polar and non-polar fractions was stated as GAE (mg g^{-1} dry sample) Figure 4.10. The increasing order of various extract and fractions of the plant in given as: FANH<FAW <FAC<FAE<FAEA. Total antioxidant activity of the plant has significant correlation with TPC and TFC. Sahreen *et al.* have also documented that, in the phosphomolybdenum assay, the EA (ethyl acetate) fraction; extracted from *Carissa opaca* fruit demonstrated the highest efficacy among the fractions tested (Sahreen, Khan *et al.* 2010).

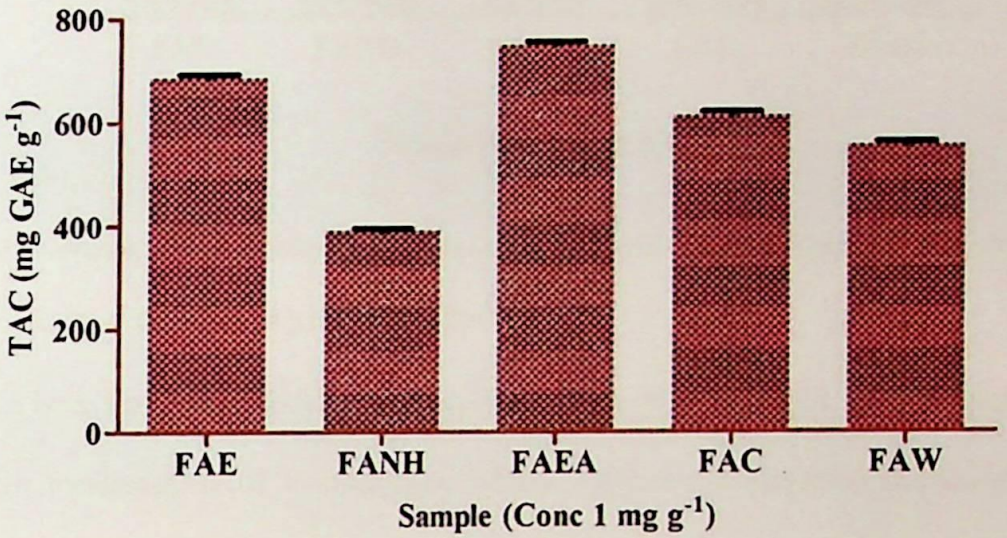

Figure 4. 10 Total antioxidant activity of *Fagonia arabica* extract/fractions (mg Gallic acid Equivalent g^{-1} extract)

4.4 Reducing power assay

Reducing power activity of *Fagonia arabica* extract and fractions was calculated by using Potassium ferricyanide reduction method. Highest reducing power (814 mg g^{-1} dry sample, Figure 4.11) was observe in ethyl acetate fractions of *Fagonia arabica* in our study. Duan *et al.* observed highest reducing power in ethyl acetate fraction of

Polysiphonia urceolata than other reported fractions (Duan, Zhang *et al.* 2006). The observed activity can be attributed to the presence of high levels of phenolic compounds such as caffeic acid, quercetin, rutin, and catechin, all of which exhibit significant ferric ion reduction capabilities (Chen and Ahn 1998).

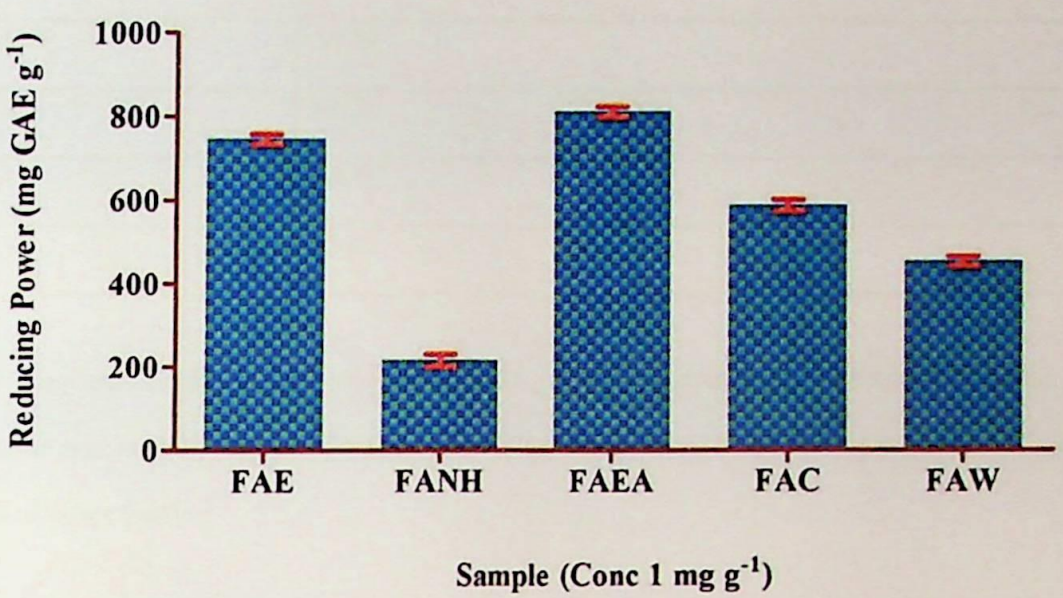

Figure 4. 11 Reducing power activity of *Fagonia arabica* extract/fractions (mg GAE g-1 extract).

4.5 Tyrosinase Enzyme Inhibition effect

Tyrosinase is a renowned oxidizing enzyme; which play a major role in melanin synthesis in melanocytes of human skin. Various hyper-pigmentation conditions of our skin are usually related with enhanced synthesis of this Tyrosinase compound. Past studies shows that such inhibitory activity of Tyrosinase moiety due to the presence of multiple bioactive moieties resulted in wide range application of these substances in daily cosmetic products to treat such hyper-pigmentation ailments (Moon, Yim *et al.* 2010).

DPPH (2,2-diphenyl-1-picrylhydrazyl), antioxidant activity of ethyl acetate fraction of *Fagonia arabica* show 88.16% at I mg/mL Table 4.3 inhibition of oxygen reactive species. Order of inhibitory effect of various fractions for free radicals is as follows; FAEA>FAE>FAW>FAC>FANH. This activity was observed due to presence of high

phenolic in ethyl acetate fraction which also shows maximum tyrosinase enzyme inhibition Table 4.4.

Table 4 4 Enzyme inhibition effect of various fraction of *Fagonia arabica*

Plant samples	% Tyrosinase enzyme inhibitions at Img/mL
FAE	69.65
FANH	55.89
FAEA	78.23
FAC	66.98
FAW	67.43

FAE- *Fagonia arabica* Hydro alcoholic extract , FAH- *Fagonia arabica n*-Hexane fraction, FAEA - *Fagonia arabica* ethyl acetate fraction, FAC - *Fagonia arabica* chloroform fraction, FAW - *Fagonia arabica* water fraction

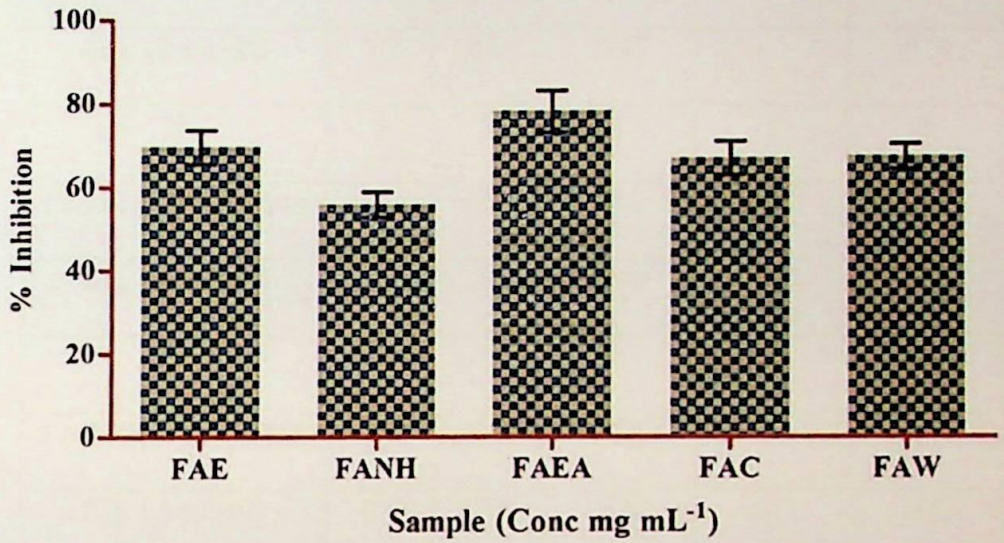

Figure 4. 12 Tyrosinase inhibition of botanical extracts/fractions and standard. Mean ± SD, n= 3

Correlations							
		TPC	TFC	DPPH	TAC	RA	Tyrosinase
TPC	Pearson Correlation	1	.883*	.700	.526	.521	.477
	Sig. (2-tailed)		.047	.188	.363	.368	.417
	N	5	5	5	5	5	5
TFC	Pearson Correlation	.883*	1	.933*	.861	.853	.809
	Sig. (2-tailed)	.047		.021	.061	.066	.097
	N	5	5	5	5	5	5
DPPH	Pearson Correlation	.700	.933*	1	.913*	.881*	.910*
	Sig. (2-tailed)	.188	.021		.030	.048	.032
	N	5	5	5	5	5	5
TAC	Pearson Correlation	.526	.861	.913*	1	.995**	.955*
	Sig. (2-tailed)	.363	.061	.030		.000	.011
	N	5	5	5	5	5	5
RA	Pearson Correlation	.521	.853	.881*	.995**	1	.924*
	Sig. (2-tailed)	.368	.066	.048	.000		.025
	N	5	5	5	5	5	5
Tyrosinase	Pearson Correlation	.477	.809	.910*	.955*	.924*	1
	Sig. (2-tailed)	.417	.097	.032	.011	.025	
	N	5	5	5	5	5	5
*. Correlation is significant at the 0.05 level							
**. Correlation is significant at the 0.01 level							

4.6 HPLC-DAD analysis

For the identification of different types of phenolic and flavonoids phytocompounds the HPLC assay was performed for some phenolic and flavonoid standard. Gallic acid eluted at (1.85 min), caffeic acid at (10.07 min), Chlorogenic acid at (12.14 min), Apicatechin at (15.76 min), Ferulic acid at (19.6 min), Hyperoside at (28.88 min), Luteolin at (30.08 min), Rutin at (30.66 min), Fisetin at (33.66 min), Apigenin-7-glucoside at (35.102 min), Naringenin at (39.03 min), Benzene-triol at (43.00 min), Apigenin at (46.86 min) and Chrysin at (57.00 min) as indicated in standard HPLC chromatogram (Figure. 4.13). The HPLC chromatogram of FAE showed six peaks and the retention time of these peaks coincided closely with the retention time for Gallic acid, Rutin, Apigenin-7-glucoside, Benzene-triol, Apigenin and Chrysin as shown in Figure. 4.14. *Fagonia arabica* ethyl acetate fraction (FAEA) confirmed nine peaks closely aligning with the established retention times for Gallic acid were detected, Ferulic acid, Benzene-triol, Apigenin, Rutin, Fisetin, Apigenin-7-glucoside, Naringenin, and Chrysin (Figure 4.15). Similarly, the FAC fraction represented five peaks of Gallic acid, Fisetin, Benzene-triol, Apigenin and Chrysin (Figure 4.16). Phenolic and flavonoid present in following order Ethyl acetate >Hydroalcoholic extract > Cholroform fraction (Figure. 4.13-4.16).

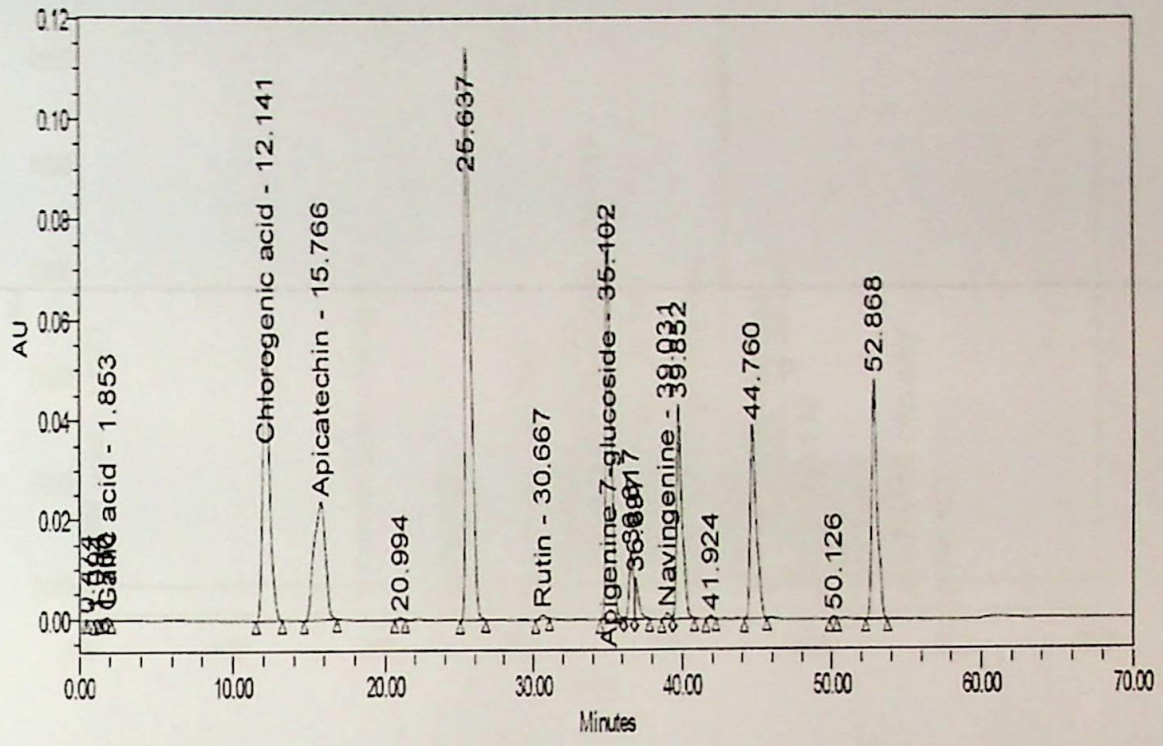

Figure 4. 13 HPLC fingerprint of reference standards.

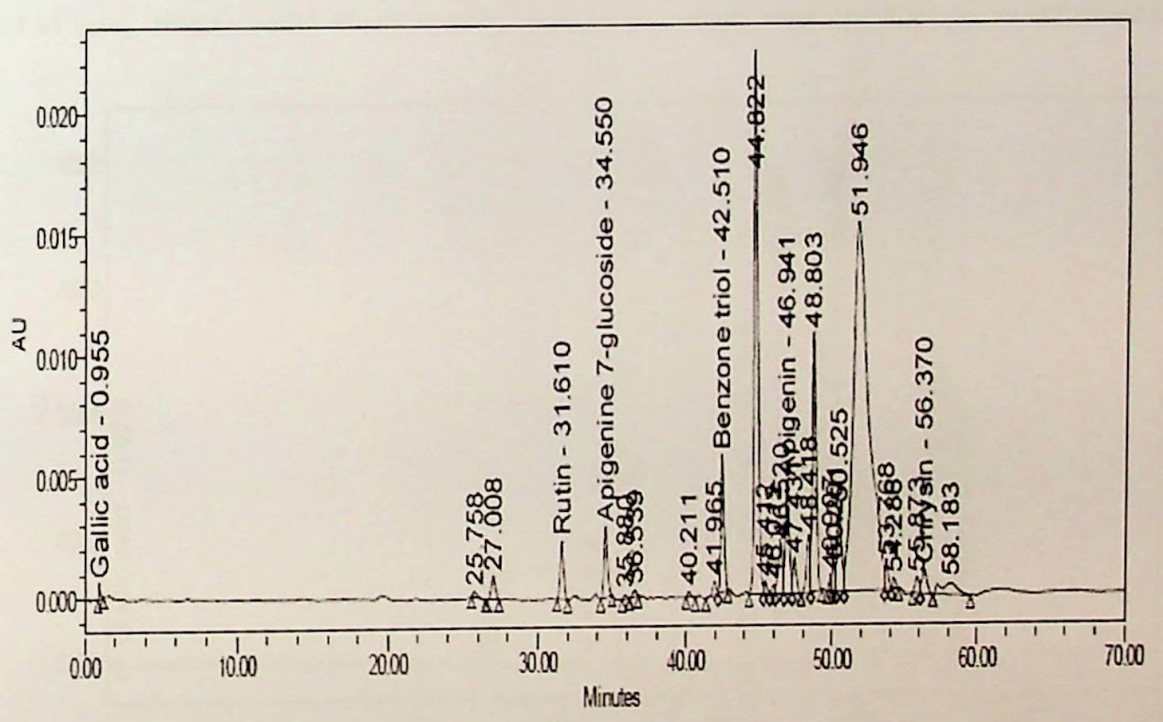

Figure 4. 14 HPLC Chromatogram of FAE. Gallic acid at (1.85 min), Rutin at (30.66 min), Apigenin-7-glucoside at (35.102 min), Benzene-triol at (43.00 min), Apigenin at (46.86 min) and Chrysin at (57.00 min).

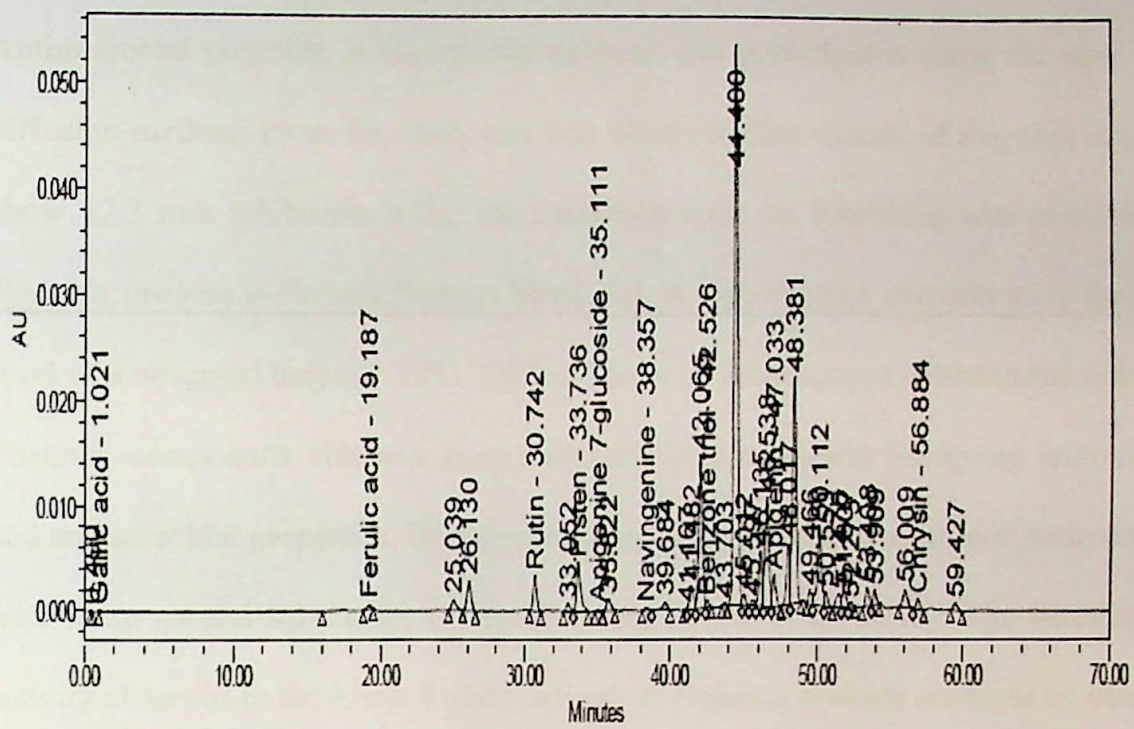

Figure 4. 15 HPLC Chromatogram of FAEA. Gallic acid at (1.85 min), Ferulic acid at (19.6 min), Rutin at (30.66 min), Fisetin at (33.66 min), Apigenin-7-glucoside at (35.102 min), Naringenin (39.03 min), Benzene-triol at (43.00 min), Apigenin at (46.86 min) and Chrysin at (57.00 min).

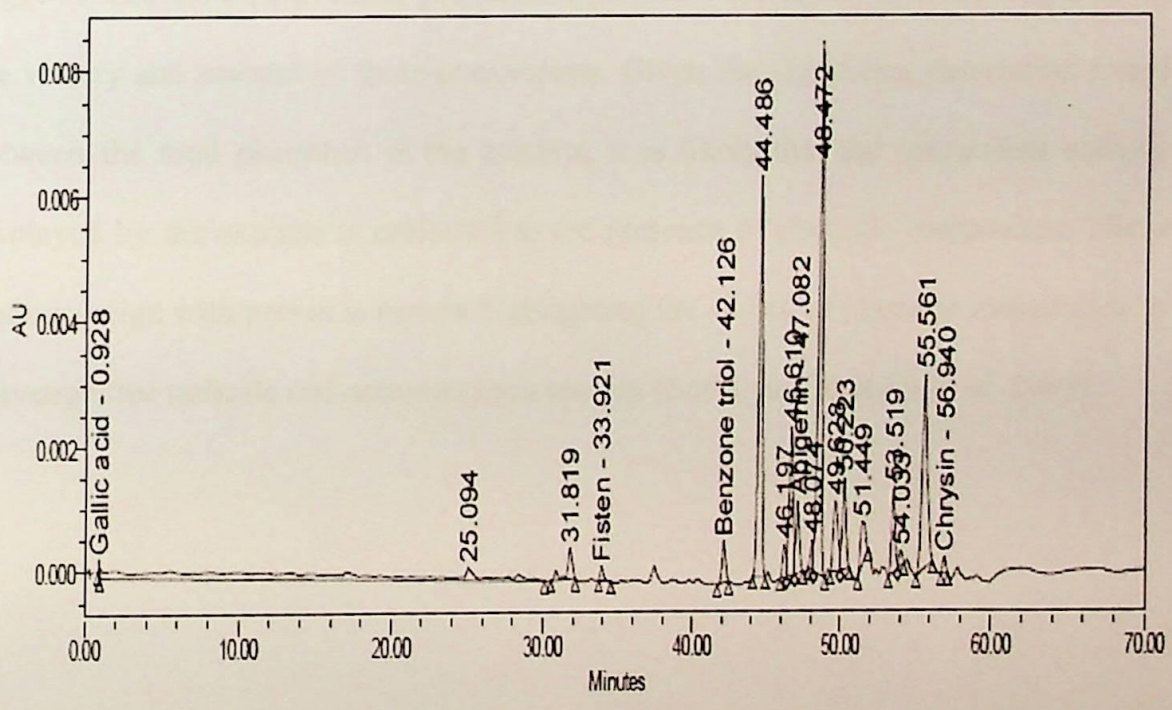

Figure 4. 16 HPLC Chromatogram of FAC. Gallic acid at (1.85 min), Fisetin at (33.66 min), Benzene-triol at (43.00 min), Apigenin at (46.86 min) and Chrysin at (57.00 min).

4.7 Antimicrobial assay

Antimicrobial potential of the utilized extracts was investigated using the agar well diffusion method. From the study this was observed that extract of *Fagonia arabica* show 12.3 mm inhibition, while the minimum zone on inhibition was observed in *Fagonia arabica* n-Hexane fraction Table 4.6. A significance correlation at the 0.05 level was observed between TPC, TFC and zone of inhibition of antibacterial activity. Phenolic compounds obtained from medicinal plants possess intriguing antioxidant and antimicrobial properties. However, it is crucial to substitute synthetic antioxidants with these natural substances (Metrouh-Amir, Duarte *et al.* 2015). The variation in activity observed in the extracts and fractions of *Fagonia arabica* seems to be directly linked to the diversity, both qualitatively and quantitatively, of the compounds present in these extracts. Plants that have a varied range of secondary metabolites, such as tannins and terpenoids, are believed to exhibit stronger biological activities. This suggests that the effectiveness of a natural product's biological activities depends on the variety and amount of these constituents. Given the significant correlation found between the total phenolics in the extracts, it is likely that the antioxidant activity displayed by the extracts is attributed to the presence of phenolic compounds. These findings align with previous reports highlighting the ability of phenolic compounds to scavenge free radicals and active oxygen species (Sofidiya, Odukoya *et al.* 2009).

Table 4 6 Zone of inhibition of various extract and fraction of *Fagonia arabica*

Concentration (µg/ml)	Zone of Inhibition (mm)					
	Extract/fraction					Standard
	FAE	FAH	FAEA	FAC	FAW	
500	12.3	4.2	10.2	5.6	9.2	21.2
250	9.1	2.2	7.7	4.3	6.3	

Table 4 7 Correlation TPC, TFC and DPPH with Antibacterial activity of *Fagonia arabica*

Correlations					
		TPC	TFC	DPPH	Antibacterial
TPC	Pearson Correlation	1	.883*	.706	.914*
	Sig. (2-tailed)		.047	.183	.030
	N	5	5	5	5
TFC	Pearson Correlation	.883*	1	.934*	.956*
	Sig. (2-tailed)	.047		.020	.011
	N	5	5	5	5
DPPH	Pearson Correlation	.706	.934*	1	.813
	Sig. (2-tailed)	.183	.020		.094
	N	5	5	5	5
Antibacterial	Pearson Correlation	.914*	.956*	.813	1
	Sig. (2-tailed)	.030	.011	.094	
	N	5	5	5	5
*. Correlation is significant at the 0.05 level (2-tailed).					

CHAPTER: 05

5 DISCUSSION

5.1 Qualitative and Quantitative analysis

Screening and costing of variety of medicinal plants to discover multiple antioxidant compounds and unique phytochemicals may lead to the unearthing of more heady and safer substances, as more side effects may be associated with other synthetic compounds. According to new research/ advancements as mentioned in different publications of "*Food Chemistry*" it is well established that expenditure of variety of *crude extracts* from many plants/ cereals /fruits and many other bioactive moieties in pure form exhibit potential antioxidant activity (Gundogdu *et al.*, 2012). Results and outcomes from the study is to support the rational corroboration and to the further scientific declaration that the extracts/fractions of *Fagonia arabica* in traditional system of medicines which is proved to be very economical, safer and effective for humans and their skin. In present days, the entire world is concentrating on the on natural and botanical cradles to find out numerous antioxidants components such as polyphenols in modern research (Iranshahy *et al.*, 2017). Mainly natural plants are enriched in polyphenols compounds that exhibit broad range of owning to their antioxidant potentials. Among these imperative phenols, the important flavonoids attain more attention/priority (Hagerman *et al.*, 1998). So for the quantification purpose, a well-established protocol was followed for the extract/fractions of *Fagonia arabica*, so as to calculate Total phenolic contents (TPC)/Total flavonoids contents (TFC) by a spectrophotometer. The Total Phenolic Content (TPC) was quantified and expressed as micrograms of Gallic Acid Equivalents (GAE) per milligram of the dry plant extract, while the Total Flavonoid Content (TFC) was measured and expressed in micrograms of Quercetin Equivalents (QE) per milligram of the dry plant extract (Kim, Jeong, & Lee, 2003).

5.2 Tyrosinae inhibition and Antioxidant potential

The procedure for the assessment of antioxidant activity is established on scavenging of numerous oxygen species (free). In advanced studies antioxidants from plant/ natural resources such as multiple flavonoids, lycopene, carotenes and ascorbic acid proved them as potential antioxidants (Yilmaz & Toledo, 2006). It is understood that extracts are plentiful in these phytochemical moieties therefore calculation/ assessment of their antioxidant activity can be more fruitful. Reducing power activity of *Fagonia arabica* extract and fractions was observed in ethyl acetate fractions of *Fagonia arabica* in our study. Duan *et al.* observed highest reducing power in ethyl acetate fraction of *Polysiphonia urceolata* than other reported fractions (Duan *et al.*, 2006). The observed activity can be attributed to the presence of high levels of phenolic compounds such as caffeic acid, quercetin, rutin, and catechin, all of which exhibit significant ferric ion reduction capabilities (Chen & Ahn, 1998). Tyrosinase is a renowned oxidizing enzyme; which play a major role in melanin synthesis in melanocytes of human skin. Various hyper-pigmentation conditions of our skin are usually related with enhanced synthesis of this Tyrosinase compound. Past studies shows that such inhibitory activity of Tyrosinase moiety due to the presence of multiple bioactive moieties resulted in wide range application of these substances in daily cosmetic products to treat such hyper-pigmentation ailments (Moon, Yim, Song, Lee, & Hyun, 2010).

5.3 Antimicrobial analysis

Phenolic compounds obtained from medicinal plants possess intriguing antioxidant and antimicrobial properties. However, it is crucial to substitute synthetic antioxidants with these natural substances (Metrouh-Amir, Duarte, & Maiza, 2015). The variation in activity observed in the extracts and fractions of *Fagonia arabica* seems to be

directly linked to the diversity, both qualitatively and quantitatively, of the compounds present in these extracts. Plants that have a varied range of secondary metabolites, such as tannins and terpenoids, are believed to exhibit stronger biological activities. This suggests that the effectiveness of a natural product's biological activities depends on the variety and amount of these constituents. Given the significant correlation found between the total phenolics in the extracts, it is likely that the antioxidant activity displayed by the extracts is attributed to the presence of phenolic compounds. These discoveries line up with earlier reports featuring the capacity of phenolic mixtures to rummage free revolutionaries and dynamic oxygen species (Sofidiya, Odukoya, Afolayan, and Familoni, 2009).

CHAPTER: 6

6. Conclusion and Future Research Direction

The allowances drawn from this examination attest the significant supply of phenolic intensifies present inside Fagonia arabica and its consideration of pivotal phenolic components like gallic corrosive and caffeic corrosive. Moreover, the evaluation of its antioxidative potential gives experimental approval of its adequacy in moderating oxidative pressure. The complete phytochemical examination led across different Fagonia arabica concentrates and portions lays out the constant presence of phenols, tannins, and flavonoids, featuring their importance in the organic arrangement.

In rundown, the revelations act as the establishment for future top to bottom logical investigation of Fagonia arabica. Such examinations would dive into the complex instruments of its phenolic parts, unwind the sub-atomic underpinnings of its antioxidative power, and open the herbal's remedial potential outcomes across a range of biomedical and biotechnological domains.

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