Addis Ababa University
Addis Ababa Institute of Technology
School of Chemical and Bioengineering

Extraction and Characterization of Chlorogenic Acid from Coffee Husk and its investigation of Applicability as Preservative in Leather Processing

By:

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June, 2018
Addis Ababa, Ethiopia

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Addis Ababa Institute of Technology
School of Chemical and Bioengineering

Thesis Submitted to The School of Chemical and Bioengineering, Addis Ababa Institute of Technology, Addis Ababa University.

Presented in Partial Fulfilment of the Requirements for the Attainment of the Degree of Masters of Science in Chemical Engineering (Leather Technology)**.**

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This is to certify that the Thesis prepared by Daniel Tesfaye, entitled: Extraction and Characterization of Coffee Husk and its Application as Preservative in Leather Processing and submitted in partial fulfilment of the requirements for the Degree of Masters of Science in Chemical Engineering (Leather Technology) complies with the regulations of the University and meets the accepted standards with respect to originality and quality.

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Abstract

Leather is a perishable material so it has to be preserved from attack of microorganisms. In leather processing steps, skins can be stored at pickle, wet blue, crust and finished stages. Wet blue preservation is prevents the leather from fungal attack and its commercial carried out using TCMTB based chemicals. This TCMTB based commercial chemicals has adverse effect on human health as well as on aquatic lives. Chlorogenic acid, the major active component of coffee husk, extracted by varying solvent, extraction time and solvent to sample ratio using Soxhlet extractor. Twenty Four extractions were randomly done and results were analysed using Design Expert software version 6.0.8. The extraction procedure using Methanol as solvent, extraction time of 4 hours and 10:1 ratio of sample to solvent resulted as the highest value, which is 681 ppm. The minimum yield (468ppm) was obtained at Ethanol solvent, extraction time of 6 hours and 5:1 ratio of sample to solvent.

Staphylococcus aureus, *Pseudomonas aeruginosa*, *Bacillus cereus*, *Escherichia coli* and *Aspergillus niger* were obtained and tested for the susceptibility and it was found to be *Aspergillus niger*, a fungi, is more susceptible or sensitive than both gram positive and gram negative bacteria to Chlorogenic acid extracted from coffee husk. The Minimum Inhibitory Concentration (MIC) for *Aspergillus niger* was found to be 62.5µg/mL.

The wet blue preserved using the extracted Chlorogenic acid was tested for its resistance to fungal attack and resist any attack till 15 days. The wet blues were processed to crusting and tested for its strength properties and found to have no change of those properties upon the use of Chlorogenic acid for preservative. The waste water from the process were tested and showed significant minimization of COD and TSS.

Key word: Coffee Husk, Chlorogenic acid, susceptibility, Wet blue, Microorganism, waste water, Minimum Inhibitory Concentration,

Acknowledgement

First and for the most I would like to praise God for giving me health and patience to accomplish this thesis work successfully and strength on difficult times**.**

I would like to express my heartfelt appreciation **Mr. Wondu Legesse**, Director General of Leather Industry Development Institute for his commitment, direction and leadership in making the happening of all the twining programs successful and kind permission to carry out this work.

I would also like to thank my internal advisor **Dr. Eng. Shegaw Ahmed**-Assistant professor at AAiT-AAU for his constant encouragement, sharing his knowledge and never failing kindness and guidance throughout this period of work.

I would like to thank my external advisors **Dr. J Raghava Rao-**Chief Scientist and **Dr. K. J. Sreeram-**Principal Scientists, Chemical Laboratory, CSIR–CLRI for their sustainable and appreciable guidance, tireless advising, for sharing knowledge, skill and fine-tuning up to the successful completion of thesis.

I am grateful to **Dr. Yasmin K**.-Biotechnology Lab Head of Tannery Division, CSIR–CLRI for her patience, motivation, enthusiasm, and immense knowledge while conducting the anti-microbial tests.

I personally want to thank **Mr. Mishamo Wakaso**, Leather Technology Directorate Director for his valuable support and encouragement during the course work.

Finally, I wish to reveal my thanks to my beloved families and especially to my wife; **Kidist Habte** whose continuous inspiration, unconditional support and sacrifice made me possible to accomplish this study.

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List of Abbreviation and Acronyms

AAiT	Addis Ababa Institute of Technology
AAU	Addis Ababa University
ANOVA	Analysis of Variance
ATCC	American Type Culture Collection
BCS	Basic Chrome Sulphate
BOD	Biological Oxygen Demand
CGA	Chlorogenic acid (caffeoylquinic acid)
CH	Coffee Husks
CLRI	Central Leather Research Institute of India
COD	Chemical Oxygen Demand
CP	Coffee Pulp
FT-IR	Fourier Transform Infrared Spectroscopy
GTP2	Growth and Transformation Plan Two
HOMO	Higher Occupied Molecular Orbital
HPLC	High Pressure Liquid Chromatography
Hr.	Hour
LCMS	Liquid Chromatography Mass Spectroscopy
LOMO	Lower Occupied Molecular Orbital
MIC	Minimum Inhibitory Concentration
MTCC	Microbial Type Culture Collection
PCP	Penta Chloro Phenol
ppm	Parts per Million
TCMTB	2-(thiocyanomethylthio) benzothiazole
TDS	Total Dissolved Solid
TSS	Total Suspended Solid
UV-Vis	Ultra Violate – Visible
SATRA	Shoe and Allied Trades Research Association
WVP	Water Vapor Permeability to sample

Chapter One

Introduction

1.1. Background

Ethiopia is one of the richest countries in live stoke population, having 59.48 million head cattle, 30.7 million head sheep, 30.2 million head goat, and 1.2 million head Camel which made the country 1st in cattle and 3rd in goat and sheep from Africa [FAOSTAT 2016].
Leather industry in Ethiopia is one of a fast growing sub sector, which has been contributing about 10% of the country's income from the manufacturing sector [1]. There are 27 functional tanneries in the country, which have annual installed capacity of producing 70.6 and 204.6 million square feet of cattle hide and skin respectively. Due to several reasons, the actual performance in 2017 was 64% for cattle hide and 47% for skin. Moreover, the sector employed 20,814 people and of which 50% are females [LIDI unpublished].

From the sector government planned to earn $800 million by the end of the Five years Growth and Transformation plan two (GTP2) which is 2020/2021 [1]. To earn this much will not be an easy task by only focusing on export of finished leather, shoe and other leather products. Other programs must be proposed which leads to substitution of import of leather processing chemicals to save the hard currency for procurement of those chemicals.

Raw skins and hides can be preserved by several methods to protect the leather making protein form degradation by microorganisms. The most common way of curing raw hides and skins is to apply common salt on the flesh side to reduce the amount of water and make unfavorable condition for growth of microorganisms. The subsequent processing of salt preserved skins in tanneries results in the generation of large quantities of total dissolved solids(TDS), one of the pollutants that are very difficult to treat [2]. Air drying and sun drying are known to be cleaner drying methods because there is no energy consumption or wastes generated out of these processes. The other ways of drying are freeze drying and Electromagnetic radiation drying which uses high amount of energy as a result of which it is rarely used.

Pickled pelt, wetblue and crust are other process stages where the leather can be preserved and stored for long period of time.

Wetblue preservation is done to protect the leather from fungal attack while preserving it for long period of time. Around 1980's Chloro Phenol derivatives were used as Commercial preservative which were banned due to health and environmental issues [3]. The next generation of fungicides was based on 2-(thiocyanomethylthio) benzothiazole (TCMTB) which was also reported to cause toxicity if inhaled, extremely irritating to skin and eye, has potential to cause cancer and highly toxic to aquatic organisms [4]. In recent years increasing demands for safer and environmentally sound fungicides, microbicide manufacturers continue to search for new products that can meet these demands. The Chlorogenic acid which is a secondary metabolite of plants has antioxidant, antimicrobial and radical scavenging capacities due to its polyphenolic functionality [5].

1.2. Statement of Problem

Tanning industry is considered to be a major source of pollution and tannery wastewater in particular, is a potential environmental pollutant. Tanning industry waste contains chromium, chlorophenols, formaldehydes, oils, resins, biocides, detergents, phthalates, etc. which poses serious environmental impact on water (with its high oxygen demand), discoloration and toxic chemical constituents, terrestrial and atmospheric systems [6, 7].

From pesticides used by tanning industry, Chlorinated phenols like Penta Chloro phenol

(PCP) banned due to their high toxicity to humans, their environmental problems and their aggressiveness in handling. These compounds may cause histopathological alterations, genotoxicity, mutagenicity, and carcinogenicity amongst other abnormalities in humans and animals. Furthermore, the recalcitrant nature of chlorophenolic compounds to degradation constitutes an environmental nuisance [3,8]. The fungicide 2-(thiocyanomethylthio) benzothiazole (TCMTB) became the new standard and remains in use today. The currently commercial fungicides like Busan 30A, Preventol WB, Texcide NT and other are based on TCMTB and is reported to have negative impacts like toxicity if inhaled, extremely irritating to skin and eye, has potential to cause cancer, and highly toxic to aquatic organisms [9,10]. Toxicological report of TCMTB by United States Environmental Protection Agency [4] reported that, it is highly dermal irritant that can cause chronic dermatitis and human carcinogen. The most common symptoms reported for each exposure route are as follows:

- Dermal exposure: skin irritation/burning, rash, itching, skin discoloration/redness and blistering.
- Inhalation exposure: respiratory irritation/burning, irritation to mouth/throat/nose, coughing/chocking, shortness of breath and sore throat.
- Ocular exposure: eye irritation/burning, eye pain and swelling.
- Ingestion/oral exposure: irritation to mouth/throat/nose, abdominal pain, kidney failure, hypothermia and loss of consciousness.

However, with ever increasing demands for safer and environmentally sound fungicides, researches focused on new products that can meet demands.

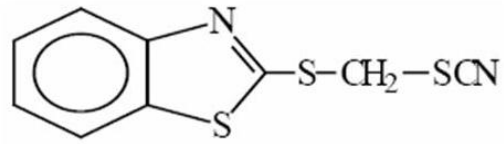


Figure1: 2-(thiocyanomethylthio) benzothiazole (TCMTB)

1.3. Objectives

1.3.1. General Objective

The general objective of this study was to extract active component (Chlorogenic acid) from coffee husk and its evaluation as potential wetblue leather preservative.

1.3.2. Specific Objectives

Specific objective of this study were to:-

- optimize the extraction of active component (Chlorogenic acid) and characterize for effective optimization from coffee husk
- test the anti-fungicidal efficacy under standard conditions in leather
- evaluate the changes in the performance properties of leather and the emission factors upon use of the active component

1.4. Significance of the study

Quantitative and qualitative findings of this research will enhance knowledge and understanding on the potential utilization of coffee husk for auxiliary manufacturing for leather processing. This study should be significant in a way that it will:-

- Show the way to utilize of coffee byproducts such as coffee husk to a highly value added and industrially needed chemicals
- Utilize coffee byproduct to an ecofriendly chemical for tanning industry
- Provides an alternative organic chemical for wet blue preservation
- Indicates way forward to save hard currency by substituting import of leather preservative chemicals
- Benefits the coffee producing society economically if industrial scale utilization coffee husk is going to be applicable

1.5. Scope of the study

The main aim of this study will be extraction, optimization and application of Chlorogenic acid from coffee husk for utilization of coffee waste and substitution of the existing preservative method of wet blue leather. The extraction will be doneusing Soxhlet extractor with varying solvent, temperature, time and sample to solvent ratio to get optimal result. The coffee husk used is only of *coffee Abyssinia*. The fungi (*Aspergillus niger)* and bacteria (*Bacillus cereus, Staphylococcus aureus, P.aeruginosa* and*E.coli*) will be studied in vitro for the application purpose. Moreover, the antifungal efficacy of the extract will be tested on wetblue goat leather and deviation in the performance properties of the leather is eventually reviewed.

Chapter Two

Literature Review

2.1. Leather processing

Leather processing involves mainly four important operations, viz., pre-tanning, tanning, post-tanning and finishing. It includes a combination of single and multi-step processes that employs, as well as expels, various biological, organic and inorganic materials. As the main constituent of the raw skin and hides is protein, they are much susceptible to bacterial degradation. Thus, it is essential to preserve the protein matrix and to arrest microbial attack temporarily prior to processing. Several chemical, biocidal and physical methods have been advocated and adopted, preservation using salts (40-50% based on green weight of the hide/skin) remain popular worldwide due to ease, cost-effectiveness and quality of finished leather produced [11,12,13].

Trimming and sorting

Trimming is done to remove unwanted long Shank, horn, hooves, ears, tails etc. and give a proper shape of hide/ skin. Then sorting is carried out according to size, weight, thickness, grade etc. and formed into batches to maintain the quality of leather. The sorted hide/skin can be weight in order to take the weight of chemicals for the later processes.

Soaking

Soaking is the first among the 'beamhouse' processes, so-called because they traditionally included operations that were conducted over a wooden beam. Prior to the introduction of machinery, hides or skins would be draped over an angled wooden beam, so that they could be hand fleshed, hand dehaired (scudded) and hand shaved to thickness. Nowadays, the term is used to encompass all the processes conducted in the tannery leading to the tanning step. The purpose of the beamhouse is to prepare the pelt for tanning. Another way of putting that would be to say the beamhouse is for purifying the pelt or 'opening up' the pelt structure. Opening up is a generic term that has two components:

I. The removal of non-collagenous skin components: the hyaluronic acid and other glycosaminoglycans, the non-structural proteins, the fats. This is not done to completion (except in the special case of Japanese leather, when removal of non-collagenous components is conducted to extreme), so the

processes in a tannery must be geared to the degree of removing these materials, required to produce the desired properties of the final leather.

II. Splitting the fibre structure at the level of the fibril bundles, to separate them

The objectives of Soaking are

- Rehydration- The function of rehydration is to fill up the fibre structure with water, ensuring that the all elements of the hierarchy of structure are wetted to equilibrium and beyond, with the purpose of facilitating movement of dissolved chemical reagents through the pelt cross section. If this does not happen, chemical processing will be non-uniform and the effect will be reflected in the final leather, giving rise to non-uniform properties.
- Removal of Salt- All salt curing causes dehydration, which means the fibre structure collapses, which consequently hinders movement of solution within the pelt. The removal of salt occurs at the same time as rehydration happens. Therefore, the salt becomes increasingly diluted, reducing the osmotic effect, so the fibre structure is plumped up, eventually beyond the situation in fresh pelt.
- Cleaning the Pelt- Cleaning means the removal of dirt, primarily from the surfaces, i.e. discolouring or abrading agents, such as mud or soil and grit. Cleaning includes removal of blood, urine, body fluids from both surfaces and within the pelt.
- Removal of Non-structural Proteins.
- Removal of Dung- Under conventional condition of soaking, including the use of typical auxiliaries such as alkali and surfactants, dried on dung is not removed. Only in the presence of lignocellulosic enzymes dung removed quickly and efficiently.

Biocides are used at the soaking stage to control the growth of bacteria. There is usually no need for a fungicide at this stage of production, although some bactericides do possess fungicidal activity. The need for bactericide in soaking depends on the totality of all the prevailing conditions and circumstances – the more conditions that would contribute to danger, the greater is the imperative for a bactericide to be used [11, 12].

Figure 2: Modern drum processing

Unhairing

Loosening (depilation) or unhairing may be considered as an extension of soaking. Its purpose is to separate the two structural proteins keratin and collagen. The aim of unhairing (depilation) and liming is to remove the hair, epidermis and to some degree the inter-fibrillary proteins, and to prepare the hide for removal of loose flesh and fat by the fleshing process [11].

In the process of unhairing which follows soaking, the hides and skins are treated with lime, sodium sulphide (sharpener) and other additives in pit/paddle/drum. This is done depending upon the soaked material and final end-product. During this process, hair is loosened/pulled out, flesh loosened and removed, hides/skins suitably plumped for easy fleshing. Hair and flesh are removed by hand/machine to make the material free from any hair and loose flesh presenting a clean surface and the material is taken up for further processing Unhairing method depends upon the raw stock as well as the final leather required to be produced. In case of goat or sheep skins where the wool has some value, a paint unhairing system is to be adopted. In the case of cattle hide, hair pulping method is used in paddle/drum. Short liming is necessary to achieve tightness of leather. For soft leather where good opening up of structure is required, a slightly longer liming is adopted to increase the swelling and splitting of fibres. Such process is dependent on the type of raw stock. Liming with the addition of soda ash/caustic soda is done to adjust the desired degree of plumping. The plumping is also

regulated by the addition of materials like glucose, molasses and sodium thiosulphate. In all cases, it should be ensured that the flesh is sufficiently loosened for easy removal [11,12,13].

Paint Liming

In paint liming, the skins are painted with a paste of lime, sulphide and wetting agent (10% lime, 2% sodium sulphide, 15% water and 0.2% wetting agent) on the flesh side and piled flesh to flesh, kept overnight well covered with wet gunny cloth. Next day they are unhaired either over the beam using unhairing knife or by pulling out the hair by hand and re-limed (using 10% lime and 1-1.5% sodium sulphide for 2 days with occasional running of the paddle for 15' every 2 hrs), taking care to prevent lime blast (Lime blast results when fleshed pelt is exposed to outside air with the formation of calcium carbonate in patches. The presence of calcium carbonate will affect subsequent de-liming and pickling, which in turn influence the tanning) [12, 13].

Drum liming

In Drum liming, the soaked skins are put in the drum with suitable float. The required amount of lime and sodium sulphide is added during the drumming of the skin. The drum is run intermittently and the duration is determined by type of final article. Even here hair is pulped out and the material is sent for fleshing.

The soaked hides after weighing are put in a paddle containing 300-400% water and well agitated for 15 min. 5% lime and 2.5-3% sodium sulphide is added to the paddle while running. Then the paddle is run 10 min every 2 hrs. and left overnight. When the hides are properly plumped they are taken for unhairing and fleshing [12, 13].

De-liming Bating, degreasing and pickling

The functions of de-liming are: removing the lime, lowering the pH in preparation for bating, and suppressing swelling. It can be carried out by using: Weak Acids, Acidic Salts, Ammonium salts (In industry it is common to use either ammonium sulphate or ammonium chloride) and Carbon dioxide.

The degreasing unit operation is primarily important to remove excess fat which otherwise alter uniform chemical distribution and penetration on the next unit operation. The process of degreasing is crucial for sheep skin which has more fat as well as for fatty hides and goat skin from hilly origin. This can be done by using surfactants/emulsifiers such as non-ionic

surfactant example is nonylphenol-etoxylate, solvent such as diesel and, lipase based enzymes also used for hydrolysis of fat.

The pickling process is primarily conducted to adjust the collagen to the conditions required by the chrome tanning reaction to have acidic PH around 2.5-2.8, an ideal PH condition, for the penetration of chrome salt in the skin matrix so as to form coordination covalent bond with the COO- group of collagen during basification. The conventional recipe for pickling based on limed pelt weight is 100% Float, 10% salt, 1% Sulfuric acid [11,12,13].

Tanning

Tanning is the process of converting raw hide and skin, which is unstable and subject to putrefaction, into leather, with adequate strength properties and resistance to various biological and physical agents.

Tanning is a process of introducing a tanning agent in to the hide/skins. This is accompanied by introduction of additional crosslinks into collagen, which binds the active groups of the tanning agents to functional groups of the protein.

A vast number of inorganic compounds are used to treat collagen to increase its shrinkage temperature, among those chromium is very special which is used to obtain light leather of high thermal and bacterial resistance. A usual procedure is to introduce Cr^{3+} into the hide, adjusting a PH of about 3 by pickling, and then make the collagen-chromium complex cross-linking reaction to occur.

The use of chromium (III) salts is currently the commonest method of tanning perhaps 90% of the world's output of leather is tanned in this way. The reasons for the popularity of chrome tanning when compared with vegetable tanning:-

- The process time for the chrome tanning reaction itself is typically less than 24 hours: The vegetable tanning reaction takes several weeks, even in modern process. Chrome tanning confers high hydrothermal stability; a shrinkage temperature of $110^{o}C$ is easily attainable. This opens up new applications, compared with vegetable tanned leather, where the maximum achievable shrinkage temperature is $85^{o}C$, depending on which vegetable tannin type used.
- Chrome tanning alters the structure of the collagen in only a small way: the usual chrome content of fully tanned leather is 4% Cr_2O_3, whereas vegetable tanned leather

may contain up to 30% tannin and hence the handle and physical properties are inevitably modified, restricting applications of the leather.

- Vegetable tanning creates hydrophilic leather, because of the chemical nature of the plant polyphenols that constitute the tanning material, but chrome tanning makes collagen more hydrophobic, so the tannage allows water resistance to be built into the leather.
- Chromium (III) can act as a mordant (fixing agent for dyes) and its pale color allows bright deep and pastel shades (even though the base colour of the leather is pale blue).
- Tanning with plant polyphenols has the effect of making the dyeing effect dull, whichever vegetable tannin or dye types are used.
- Vegetable tanned leather may exhibit poor light fastness, depending on the type of vegetable tannin, but chrome tanned leather is light fast. Hence dyed chrome tanned leather will retain its colour better.
- Versatility is a key characteristic of the process. It is theoretically possible to create any type of leather from any wet blue hide: mens' or ladies' shoe upper, combat upper, soling, clothing, gloving, upholstery, etc.

Retanning

This may be a single chemical process or may be a combination of reactions applied together or more usually consecutively. The purpose is to modify the properties and performance of the leather. These changes include the handle, the chemical and hydrothermal stability or the appearance of the leather. The effects are dependent on both the primary tanning chemistry and the retanning reactions.

Dyeing

This is the coloring step. Almost any color can be struck on any type of leather, despite the background color, although the final effect is influenced by the previous processes.

Fat liquoring

This step is primarily applied to prevent fibre sticking when the leather is dried after completion of the wet processes. A secondary effect is to control the degree of softness conferred to the leather.

Finishing

Finishing is a technique of coatings the grain surface to protect it against dirt, staining, wetting, mechanical stresses like rubbing, scuffing, flexing etc., leveling or evening out the color of the grain surface, hiding grain blemishes and upgrading its quality, improving the aesthetic appeal and the sales value of the product.

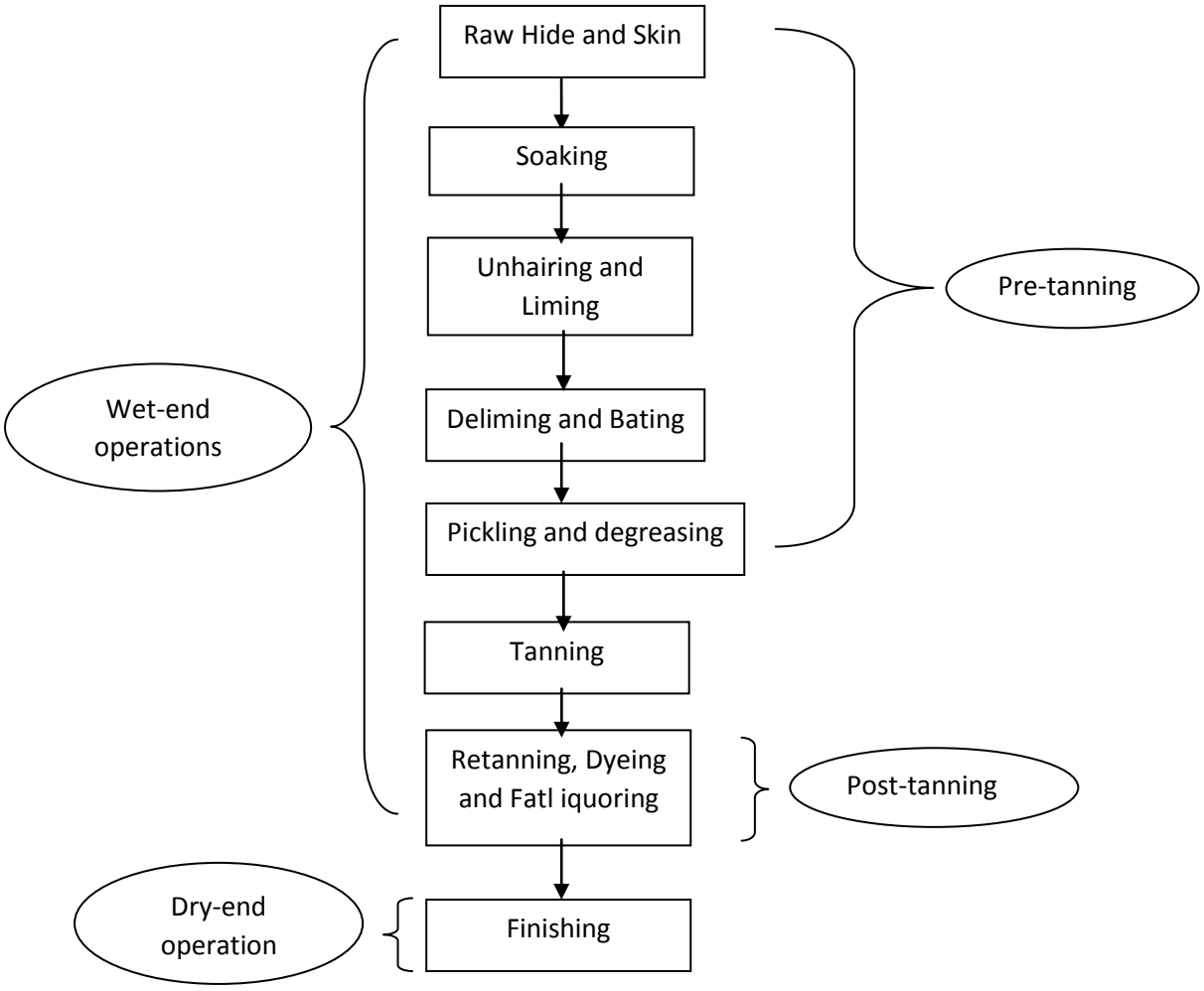


Figure 3: Leather processing steps

Leather is a biological product and very suitable for the growth of microorganisms due to presence of protein and lipids in the form of glycerides. The proteins and fats in the hide present an ideal source of nutrients with a pH of about 4, for fungal growth [8, 14]. It consists of 50% carbon, 25% oxygen, 7% hydrogen, 17.8% nitrogen and 0.2% minerals. Raw leather consists of 64% water, 30% protein, 3% carbohydrate, 2% fat, 0.5% minerals and 0.5% other materials [8, 9].

Bio deterioration is an important factor impairing aesthetic, functional and other properties of leather and other biopolymers or organic materials and also the products made from them. It takes place particularly under conditions of high relative humidity that enable bacteria, actinomycetes or fungi to grow [14].

From the moment it is removed from the animal or flayed and throughout the various preservation processes in the tannery, the raw leather is under threat from micro-organisms. Inspite of such processes as pickling, vegetable tanning and chrome tanning, which are intended to prevent damage from micro-organisms, treated leathers, crust and even finished leathers constitute a source of nutrients for micro-organisms [8].

As illustrated on Figure 3, in the processes before tanning, problems are caused mostly by bacteria, while in tanning and later processes, it is fungi which cause the most problems. In leathers which have been damaged by micro-organisms, removal of such problems as smells, oil leaching, spoiling of the leather and colour irregularities is sometimes impossible [8].

It is universal practice to add a fungicide during any steps in the leather making process where leathers (wetblue, wet white, vegetable tanned, or crust) will be held or stored in a wet or moist condition for a period of time. The fungicide is necessary to protect the leather against attack by fungi as the leather contains adequate nutrition and industry environmental conditions are typically favourable for fungal growth [14]. Airborne spores are everywhere, and fungal growth can occur within days unless wet leathers are adequately protected.

Tanners are well aware that such growth cause damage to the leather surface and can result in significant direct and indirect costs such as:

- Staining
- Non-uniformity in further processing
- Grain damage
- Changes in physical parameters
- Organizational costs related to re-work, and
- Customer complaints and workers' health

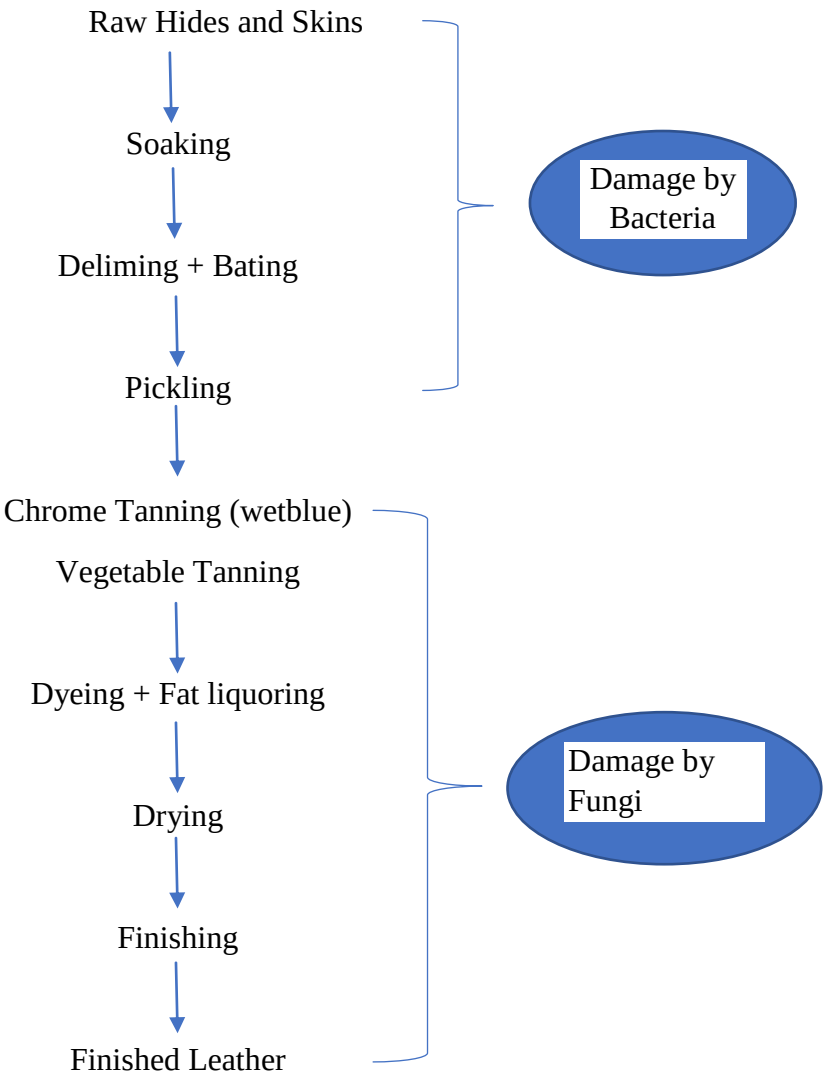


Figure 4: Microbial attack on leather processing steps

Leathers after chrome or vegetable tanning and also finished leathers provide an excellent substratum for microbial growth. The presence of ammonium salts, phosphates, surfactants, fat liquoring agents and other organic agents may tend to promote mold formation on leather matter. On chrome-tanned leather (known technically as wet blue) formation of red spots is a frequent phenomenon. The red spots on the wet blue are not limited to one type of leather, since these fungi attack and cause red coloration in box sides, horse chevreau, pig-skin splits, goat skins, etc. It is possible to avoid this type of damage by using a fungicide in the drum containing the tanning solution or by dipping the chrome tanned leathers in fungicidal solutions [14].

2.2. Bacteria Species Isolated from Skin and Hide

According to the study by Kaygusuz and Isık [9], raw hides and skins may be contaminated with a variety of microorganisms. Bacteria such as *Staphylococcus aureus, Corynebacterium pyogenes, Escherichia coli* and *Pseudomonas aureginosa* are part of the normal flora of leather. Various Gram-positives (such as *Staphylococcus spp.* and *Corynebacterium spp*.) and Gram-negatives (such as *Micrococcus spp., Proteus spp., Agrobacterium spp.* and *Shewanella spp*.) were isolated from cattle hides. It was stated that raw hides and soaking baths offer conditions for abundant bacterial growth such as *Bacillus subtilis, E. coli, Proteus vulgaris* and *P. aeruginosa*.

Curing is carried out by several chemical, biocidal and physical methods. The salt curing system is the most popular animal skin preservation method adopted globally. It was found that the various bacterial species isolated from fresh calf skins had the ability to withstand a high level of salt (NaCI) concentrations (1.5-9% w/v). The isolated bacterial species included *Bacillus coli, B. megatherium, B.mycoides, B. proteus, B. subtilis, Staphylococcus albus, S. aureus, Sarcina lutea* and *Micrococcus roseus. B. subtilis* and *B. mycoides* were found to have survived in a dormant state at a high salt concentration (20% w/v) [15]. Aslan and Birbir [16] reported that a total of 396 Gram-positive bacteria comprising from 12 different genera and 47 bacterial species were isolated and identified from salt cured hides. The most common Gram-positive genera on the salted hides were *Staphylococcus* (115 isolates), *Bacillus* (111 isolates) and *Enterococcus* (75 isolates). A total of 256 Gram-negative bacterial isolates containing 21 different genera and 46 different bacterial species were isolated and identified from the salt-pack cured hide samples. The most common Gram-negative genera on these hides were *Enterobacter* (66), *Pseudomonas* (59) and *Vibrio* (32) [17].

Bacterial types as *Bacillus, Micrococcus* and *Staphylococcus* have been identified not only on salted leathers but also at the liming stage, bacteria with spores have generally been identified. [8].

It has been reported that after the chrome tanning, retanning, drying and finishing processes, the number and variety of bacteria have been observed to decrease, although *Bacillus* species continued to live commonly in the form of spores, and that bacterial development was inhibited because the pH was low [8].

Bacteria such as *B. brevis, B. cereus, B. firmus, B. laterosporus, B. licheniformis, B.megaterium, B. pumilis, B. sphaericus, B.subtilis, S. aureus, S. epidermidis, M. candidus,*

M. luteus, M.rubens, Kurthia variabilis and *P. aureginosa* have been isolated and identified not only from raw leathers but also from leathers at various production stages and even from finished leathers [8].

2.3. Fungi Species Isolated from Skin and Hide

The fat, protein and other materials contained in leather form an ideal nutrient environment for the growth of fungi. Humidity, pH and temperature levels in places where leather is stored and during production are close to the ideal values determined for the growth of fungi, and this is a constant problem for the leather industry.

It is known that humidity levels of 40-60% are sufficient for fungal development in pickled and chromed leathers, while 13-25% is sufficient for finished leathers. During leather production, the stage at which fungal development is most often observed is when semi-finished leathers are stored after tanning [18].

Microbial destruction of tanned leather is mainly caused by the action of filamentous fungi. Moulds can penetrate through the entire thickness of the skin, causing the breakdown of fat and other substances. This interferes of the dyeing process, the effect of which is white blooms. This cover the surface of the skin by mycelium which significantly reduces the aesthetic and functional leather value.

It is reported that the species of fungus most commonly seen in leather production are from the genus *Aspergillus* [8]. While Birbir et al.[18]. reported the list of some fungus types encountered in leather processing as follows: *Penicillium, Absidia, Acremomium, Aspergillus, Basipetospora, Byssochlomys, Chrysonilia, Cladosporium, Emericella, Eupenicillium, Euratum, Fusarium, Monoscus, Paecilomyces, Mucor, Moniliella, Neosortorya, Phialophora, Scopulariopsis, Stachobotrys, Trichoderma, Trichosporon* and *Verticillium*.

In the tannery, the growth of fungi normally occurs on pickled skins, since fungi are capable of growing at a lower pH [19]. Growth of fungi also occurred on vegetable tanned, chrome tanned and finished leather. *Aspergillus spp., Penicillium spp.* and *Paecilomyces spp*. were the most common type of fungi isolated from leather and may be responsible for the discoloration on the skins or leathers. Tanning takes place in an acidic medium. The tanned hides (wet blues) are therefore especially sensitive to the growth of mould fungi. Moulds such as; *Aspergillus niger, Mucor spp., Paecilomyces spp., Penicillium funiculosum,*

Trichoderma viride, Chaetomium globosum, Aureobasidium pullulans, Rhizopus spp., Cladosporium spp., Fusarium spp. and yeasts such as; *Candida albicans, Torula rubra, Saccaromyces cerevisiae, Rhodotorula spp*. may attack leather [18].

Leather without preservation or treated with insufficient antimicrobial agent to prevent fungal contamination showed changes in the structure, loss of protein material, a reduction in physical and mechanical properties as well as the presence of stains that may compromise the quality of the final product [20]. Although the majority of the isolated microbial species are non-harmful and do not cause infections to humans, studies show that some species in the genera *Bacillus, Staphylococcus, Pseudomonas, Klebsiella, Aspergillus* and *Candida* are considered to be pathogens or potential pathogens [19].

2.4. Coffee Fruit

The taxonomical classification of coffee is as under:

Kingdom	Plantae
Subkingdom	Tracheobionta
Superdivision	Spermatophyta
Division	Magnoliophyta
Class	Magnoliopsida
Subclass	Asteridae
Order	Rubiales
Family	Rubiaceae

Coffee is one of the most popular drinks nowadays all over the world. The province of Kaffa in Ethiopia is considered to be the original habitat of Arabica coffee and Central Africa is reckoned to be the native of robusta coffee [21]. With extensive and wide spread cultivation of coffee across the globe, at present Brazil is the largest producer and exporter of coffee in the world. In 2014, the estimated amount consumed globally was 149 million bags à 60 kg of coffee [22]. The shrubs start blooming after three to four years and provide a full harvest after another six years. The maximum yield is obtained after 10-15 years and it bears fruit for approx. 40 years. The shrubs can depending on specie grow to a height of 3-12 meter. During cultivation, the shrubs is pruned to a height of 2-2.5 meter to facilitate harvest. The shrubs is an evergreen with leathery short-steam leaves and white flower with Jasmin-like fragrance.

The coffee fruit, which is a stone fruit and also is called coffee berry or coffee cherry, is cherry-like and grows to 1.5 cm in diameter. The fruit has a green unripe skin which turns red-violet or deep red during ripening, which occurs eight to twelve month after flowering [23]. Coffee contains more than 700 compounds which are responsible for its aromatic and unique flavor. Genus *Coffea arabica* and *Coffea canephora* (*robusta*) are the most important species of Coffea, and they constitute 60–40 % of world production. Arabica usually comes from South America (mainly from Brazil) and upland and mountain areas of East Africa while Robusta (mainly from Vietnam) from lowland of Central and West Africa and South Asia [21].

2.4.1. Anatomy of coffee bean

Inside the skin, the epicarp, is a sweet-tasting mesocarp called pulp. Within which is a thin layer of endocarp called parchment. The endosperm, the coffee bean, is also covered with a spermoderm called silver skin. The bean consists of two hemispheres with flattening adjacent sides. Each bean has an inner layer of silverskin while the parchment both covers the spheres and separates them from each other. (Figure 5)

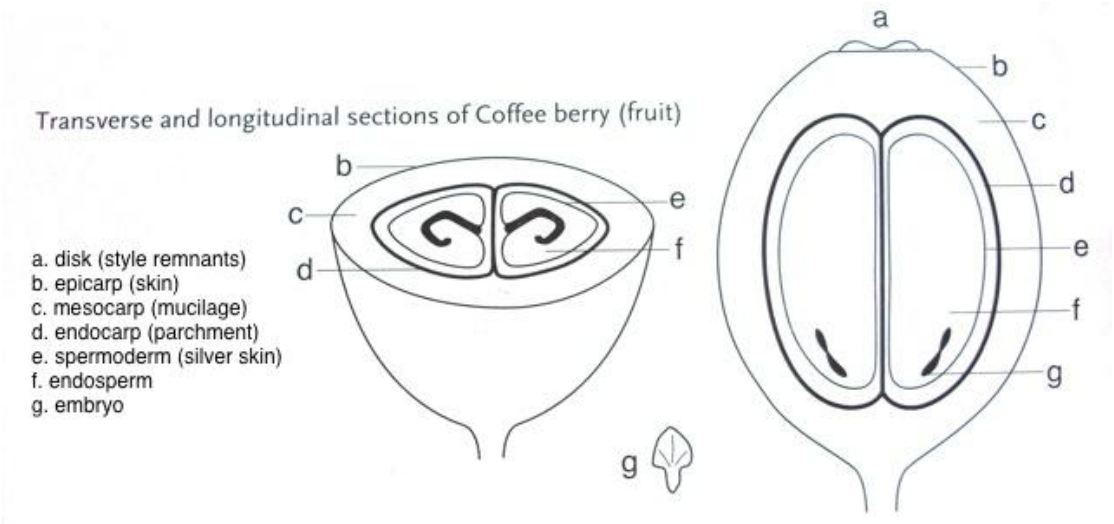


Figure 5: Parts of Coffee Bean

Pericarp

The pericarp is the outer three layers of the fruit: the exocarp (skin), mesocarp (mucilage) and endocarp (parchment).

Exocarp(Skin)

The exocarp, also referred to as the peel, skin, or epicarp, is the outermost layer of the coffee fruit. It is formed by a single layer of compact parenchyma cells (cells with thin primary

walls that contain chloroplasts and are capable of absorbing water). The color of the exocarp at the beginning of fruit development is green due to the presence of chloroplasts which then disappear as the fruit matures. Color upon maturation depends upon coffee variety, but is most commonly red or yellow. Red skin color comes from anthocyanin pigments, while yellow skin color is attributed to luteolin.

Mesocarp(Mucilage)

The mesocarp, also referred to as the mucilage, is the flesh of the coffee fruit. While "pulp" can sometimes refer to solely the mesocarp, the term usually refers to a combination of the exocarp and part of the mesocarp removed during pulping. In unripe coffee fruit, the tissue is rigid. With maturation, pectolytic enzymes break down pectic chains, resulting in an insoluble hydrogel that is rich in sugars and pectins. Studies have shown that the mucilage/water ratio of the mesocarp increases as growing altitude increases. In the wet processing method, this mucilage layer is removed through controlled fermentation. In the dry method, the mucilage, along with the exocarp and endocarp, is left intact during drying.

Endocarp(Parchment)

The endocarp, or parchment, is the innermost layer of the pericarp and is the hull that envelops the coffee bean. It is formed of three to seven layers of sclerenchyma cells (fibrous cells that serve as the principal support cells in plants). The cells of the endocarp harden during coffee fruit maturation, thus limiting the final size of the coffee seed, or bean. In arabica coffee, the average weight of the parchment with 11% moisture content is around 3.8% of total coffee fruit weight.

Seed

The coffee seed, or bean, comprises a silver skin, an endosperm, and an embryo. Coffee seed (bean) sizes vary; however, they average 10mm long and 6mm wide.

SilverSkin

The silver skin, also called the perisperm or spermoderm, is the outermost layer that wraps the seed. It is formed from the nucellus, or central portion, of the ovule. Generally some remnants of the silver skin remain on the bean pre-roast, and come off during coffee roasting as chaff. The silver skin may be polished off of the bean; however, it is generally accepted that this diminishes coffee flavour. It has also been proposed that the presence of a large amount of silver skin on milled coffee is a sign of coffee picked before its ideal ripeness.

Endosperm

The endosperm is the principal reserve tissue of the seed, and is composed of only one tissue, though the cells in the exterior and interior portion of the endosperm vary in oil content and cell wall thickness. The chemical content of the endosperm is of utmost importance since it is the precursor to the flavour and aroma of roasted coffee. The chemical compounds found in the endosperm can be classified as soluble or insoluble in water. The water-soluble compounds are caffeine, trigonelline, nicotinic acid (niacin), at least 18 chlorogenic acids, mono-, di-, and oligosaccharides, some proteins and minerals, and carboxylic acids. Components insoluble in water include cellulose, polysaccharides, lignin, and hemicullulose, as well as some proteins, minerals, and lipids.

Embryo

The embyro is composed of a hypocotyl (embryo axis) and two cotyledons and is 3-4 mm long. Coffee seeds germinate via epigeal germination, in which the hypocotyl elongates and pushes the seed upward above ground. The original cotyledons stay underground; however, new cotyledons will form [23].

In wet processing (Figure 6), the coffee berry is pulped, fermented, washed, and sun or artificially dried. The pulping process removes the exocarp and most of the mesocarp, thereby removing the pulp from the bean. The by-products and waste resulting from wet processing of coffee berries are the pulp, the mucilage, and the waste water. Wet processing in the Arabica coffee industry contributes to a large amount of by products: approximately 41-45 % of the weight of the coffee berry or 6.3 million tonnes worldwide production annually [25].

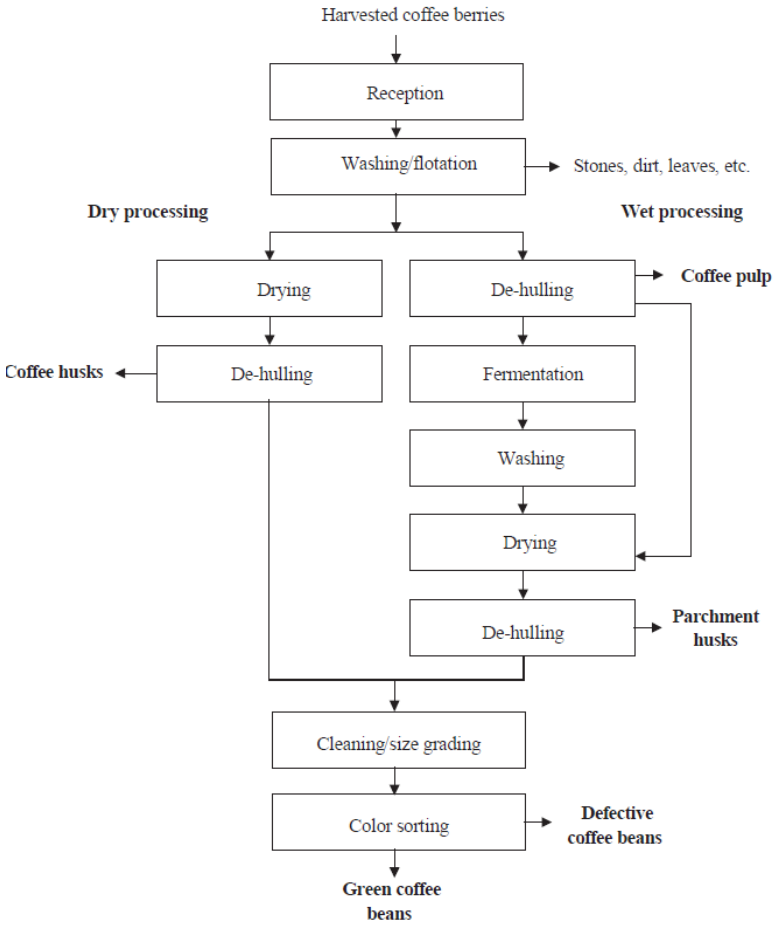


Figure 6: Dry and wet processing methods of coffee berry

2.5. Chemical composition of coffee fruit and husk

Adriana and Oliveira [26] briefly reported on their paper, dry processed Coffee husk have moisture contents ranging from 7% to 18%; such an extensive range is attributed to variations in processing and storage conditions. Wet processed coffee husk (coffee pulp) contain approximately 75% moisture and are usually left to dry to approximately 13% moisture. Average values for the chemical composition of coffee husk (CHs) and coffee pulp (CP) are displayed in Table1. The high contents of carbohydrates are expected, given the origin of such solid residue. The major difference relies on its sugar content (dry basis (db)): 29%total sugars, 24% reducing sugars, and 4% sucrose for sticky coffee husk and 12% total sugars, 14% reducing sugars, and 2% sucrose for coffee pulp. These significant differences have encouraged research studies of sticky CHs in association with specific applications such as animal feed and fermentation studies. CHs and CP are rich in organic matter and nutrients

and contain compounds such as caffeine, tannins, and polyphenols. Because of the presence of the latter compounds, these residues have a toxic nature that not only adds to the problem of environmental pollution but also restricts its use as animal feed. Caffeine, one of nature's most powerful stimulants, is present in CHs at approximately 1.3% concentration (db). Tannins are generally thought to be an antinutritional factor and to prevent CHs from being used in animal feed at percentages >10%. Such compounds are found in CHs at approximate levels of 1% and 2.3% for Arabica and Robusta species, respectively.

Table 1: Chemical Composition of Coffee Husks and Pulp (g/100 g Dry Basis)

	Coffee Husk (Dry Processed)	**Coffee Pulp** (Wet Processed)
Protein	8 – 11	4 - 12
Lipids	0.5 - 3	1 - 2
Minerals	3 – 7	6 - 10
Carbohydrates	58 – 85	45 - 89
caffeine	1	1
Tannins	5	1 - 9

From Table 2, it can be analysed that green coffee beans mainly contain insoluble carbohydrates and cellulose. The amount of caffeine and chlorogenic acid in coffee Arabica is less than that of coffee Robusta [23].

Table 2: The chemical composition of green beans from Arabica and Robusta in % of solids

	Arabica	***Robusta***
Soluble carbohydrates	9.0- 12.5	6.0- 11.5
Monosaccharides	0.2-0.5	
Oligosaccharides	6.0- 9.0	3.0- 7.0
Polysaccharides	3.0-4.0	
Insoluble carbohydrates	46- 53	34- 44
Hemicellulose	5.0- 10	3.0- 4.0
Cellulose	41- 43	32-40

Chlorogenic acid	6.7- 9.2		7.1- 12.1
Lignin		1.0-3.0	
Lipids	15- 18		8.0- 12
Protein		8.5- 12	
Caffeine	0.8- 1.4		1.7- 4.0
Minerals		3.0- 5.4	

From Table 3, it can be concluded that the amount of Chlorogenic acid, caffeic acid and Rutin in coffee husk is significantly higher than that of coffee pulp.

Table 3: Phenolic compounds in fermented Coffee husk and pulp

phenolics	**Chlorogenic acid**	**Caffeic acid**	**Rutin**
Coffee husk (CH)	132.50 ± 0.7	28.27 ± 0.62	8.26 ± 0.32
Coffee pulp (CP)	22.83 ± 0.16	4.29 ± 0.24	1.95 ± 0.27

Each value is the mean (mg/g sample) of three replications ± standard deviation.

According to Ayelign and Sabally [27], the amount Chlorogenic acid extracted from coffee bean and coffee husk collected from different regions of Ethiopia, has different result. Both the coffee bean and the coffee husk from Jimma region has high amount of Chlorogenic acid, 46.144 mg/g and 0.981mg/g respectively.

2.5.1. The Chlorogenic acid and Phenolic composition of coffee pulp and skin

Phenolic compounds are secondary metabolites generally involved in plant adaptation to environmental stress conditions. Phenolics are the most widespread dietary antioxidants and among these chlorogenic acid (CGA) accumulates to high levels in some crop plants. Such plants are; coffee, strawberries, pineapple, apple, sunflower, and blueberries. Coffee is among the highest found in plants, ranging from 4 to 14% of CGA [28]. Chlorogenic acids are a group of phytochemical compounds, the most common being caffeic acid, ferulic acid and coumaric acid which form esters with quinic acid. They belong to the hydroxycinnamic acids, a type of phenolic compounds having a C6-C3 skeleton [29].

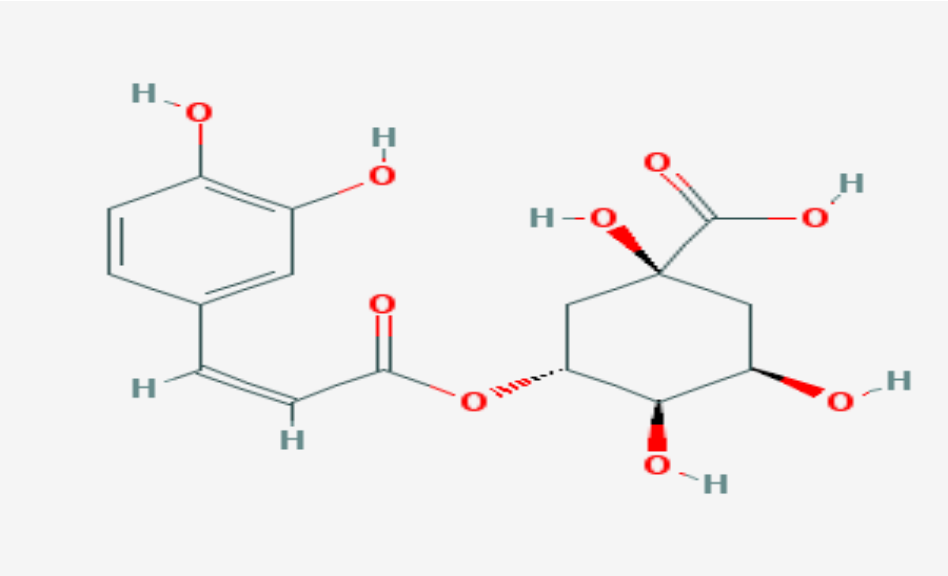


Figure 7: Chlorogenic Acid (1,3,4,5-tetrahydroxycyclohexane carboxylic acid)

The disposal of these by-products, such as coffee pulp, is an environmental concern. Coffee pulp is rich in organic compounds such as sugar, protein and nitrogen although application as livestock feed is very limited. This is due to its high content of anti-nutrient phenolics such as pectin and tannin, and some phenolic acids that are toxic [30].

In recent years, the number of studies on the chemical composition of coffee skin and pulp has been increasing due to their potential use in animal feeding, especially in coffee producing countries, where the disposal of wastes represents an important pollution problem [31].

Farah et al.[32]. illustrated that tannins, which are the main phenolic compounds in these parts of the coffee fruit, have received a special attention because they are considered as anti-nutrients for ruminants.

Coffee as a functional food with antioxidant properties reduces the incidence of cancer, diabetes and liver disease, protects against Parkinson's disease and reduces mortality risk. Green coffee bean extract shows a hypotensive effect in rats and reduces visceral fat and body weight. These properties are connected with bioactive compounds, not only chlorogenic acids and their derivatives, but also caffeine, theophylline and theobromine, cafestol, kahweol, tocopherols and trigonelline [33].

Green coffee beans contain higher level of 5-O-caffeoylquinic acid (5-CQA), even twofold higher than in roasted coffee depending on the time of roasting. Caffeine in coffee reduces oxidative stress and protects antioxidant system: in hypoxia-induced pulmonary epithelial

cells; it is an inhibitor of hydrogen peroxide-induced lipid peroxidation products in human skin fibroblasts, and it reduces tissue lipid peroxidation.

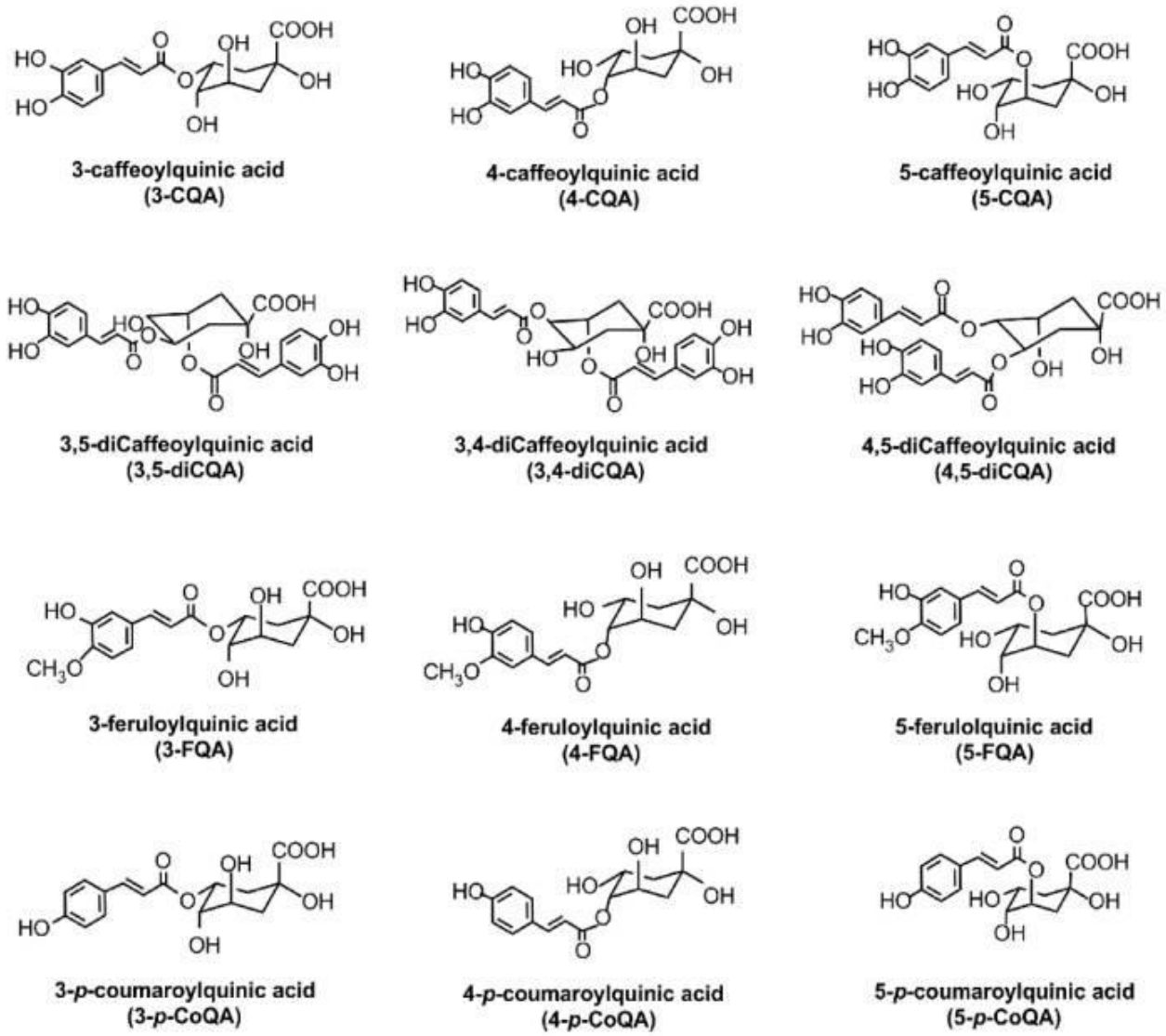


Figure 8: Chemical structures of Phenols present in coffee

CGAs are phenolic acids with vicinal hydroxyl groups on aromatic residues that are derived from esterification of cinnamic acids, including caffeic, ferulic and p-coumaric acids with quinic acid. A number of conjugated structures, such as caffeoylquinic acids (CQA), dicaffeoylquinic acids (di-CQA), feruloylquinic acids (FQA), and *p*-coumaroylquinic acids (*p*-CoQA), exist in several isomeric forms in coffee beans. Coffee arguably is one of the most popular consumed beverages in the world and is also a very rich source of CGAs. The major CGAs in coffee include 3-caffeoylquinic acid (3-CQA), 4-caffeoylquinic acid (4-CQA), 5-caffeoylquinic acid (5-CQA), 3,4-dicaffeoylquinic acid (3,4-diCQA), 3,5-dicaffeoylquinic acid (3,5-diCQA), and 4,5-dicaffeoylquinic acid (4,5-diCQA). Additional, minor CGAs

including 3-feruloylquinic acid (3-FQA), 4-feruloylquinic acid (4-FQA), 5-feruloylquinic acid (5-FQA), 3-p-coumaroylquinic acid (3-p-CoQA), 4-p-coumaroylquinic acid (4-p-CoQA), and 5-p-coumaroylquinic acid (5-p-CoQA) are also present in traceable amounts in coffee beverages [34].

CGA isomer composition in green coffee beans is complex and varies in part according to the specific coffee variety, the geographic location where it is grown, and the processes used in post-harvest, washing/drying procedures, all of which precedes roasting of the coffee beans. The most abundant CGA in green coffee beans is 5-CQA, which accounts for 76%–84% of the total CGAs, or approximately 10 g/100 g coffee beans [32]. Farah et al [35] measured the CGA composition in two major coffee plant species and reported that green *Coffea Robusta* beans contained a higher content of CGAs compared to green *Coffea arabica* beans and reported that total content of CGAs in green *Coffea canephora* beans grown at Conillon was 86 milligram per gram of dry weight, whereas the total CGA contents in green *Coffea arabica* beans grown at Mundo Novo and Catuai Vermelho ranged from 63 to 55 milligram per gram of dry weight, respectively.

2.6. Antimicrobial properties of phenolics from coffee

Shetty et al. [36] reported that coffee (Coffea arabica) showed bactericidal activity against *Vibrio cholerae*, *Salmonella typhimurium* and *S.typhi*. Tannins and caffeine from coffee were found to inhibit *Staphylococcus aureus*, *Vibrio parahaemolyticus, V. cholerae*, and *Salmonella*. Almeida et al. [37] reported that coffee extracts and selected coffee chemical compounds such as caffeine, chlorogenic acid and protocatechuic exhibited antibacterial activity against Enterobacteria. The presence of caffeine in coffee beans also inhibits *Aspergillus parasiticus* and the formation of aflatoxin. According to Mazzafera P. [38] the presence of caffeine at 0.1 % also reversibly inhibited protein synthesis in bacteria and yeast. However, some microorganisms have the ability to grow in the presence of caffeine and survival would be related to their capacity to degrade the alkaloid.

Nirmal et al. [39] reported that the extracted CGA from coffee waste has inhibited the growth of bread spooling pathogens and as a result the shelf life of sliced bread has increase to seven day without being attacked by fungi. The zone of inhibition measured in mill meter for

Bacillus spp., *Pseudomona spp.*, and *E.coli* was found to be 15±2.7, 12±1.8, and 5.5±0.5 respectively.

Mold is a principle spoiling organism in food products such as bread and butter. Low acidic condition can inhibit mold. Molds formation in both bread slices were monitored at regular intervals using magnifying lens. Mold was observed in bread slice 1 without CGA after two days and mold formation in CGA added bread slice 2 was visible only after seven days of incubation.

2.7. Chlorogenic Acid Content in Other Plant Sources

Fresh potatoes contain CGAs that range from 0.10 to 0.19 mg of 5-CGA per 100 g potato, which is equivalent to 90% of the total phenolic compounds present in potato tubers. 5-CQA, 5-FQA, and 3,5-diCQA 3,4-diCQA were also detected in different varieties of the vegetable *Chicorium endivia.* Genetically modified tomatoes with increased CGA content have also been developed to enhance their antioxidant properties. Popular citrus fruits such as pears and apples are additional rich sources of CGAs. The content of 5-CQA in pears ranged from 0.02 to 3.72 mg per gram of fresh fruit depending on the ripeness of the fruit and type of cultivar. Apples are a rich source of CGAs with the core part having the highest level (2.10 mg per gram of dry fruit), followed by the apple seed (1.10 mg per gram of dry fruit) and then apple flesh (0.48 mg per gram of dry fruit) [40].

The CGA profiles in beverages prepared from chrysanthemum, purple sweet potato stem, kuding tea, and honeysuckle flower and reported that 5-CQA and 3,5-diCQA were the dominant isomers with 3-CQA, 4-CQA, 3-FQA, 4-FQA, 5-FQA, 3,4-diCQA, and 4,5-diCQA relatively minor isomers in these beverages. CGAs are the main phenolic compounds in the tea infusions prepared from the herb *Artemisia annua*. In summary, CGAs are widely present in the plant kingdom; many of these plants are important in the human diet. Beverages prepared from coffee beans, fruits, vegetables, and herbs constitute important dietary sources of CGAs. It is interesting to note that the “di-CGA” may contribute different taste qualities, such as producing the bitter/metallic taste found in certain coffees. The significance of this in respect to the taste profile of coffee could be particularly relevant to Robusta coffees, or blends of coffees that contain a proportion of Robusta beans and hence higher amounts of di-CGA [34].

2.8. Mode of antibacterial action of phenolics

2.8.1. Membrane Damage

Various studies have reported the action of phenolics against Gram positive and negative bacteria. Prigent S. [41] proposed several mechanisms for the inhibition such as disintegration of cell membrane due to binding of phenolics to protein membrane bilayers, or outer membrane protein (OMP), either by non-covalent or hydrogen bonding (reversible), and covalent or hydrophobic interaction (irreversible). Cytoplasmic membrane has been used as a target of biocide for antimicrobial test [42]. In a study by Shapiro and Gugenheim [43] the interaction of phenolics to membrane bilayers will change the permeability of the membrane and lead to membrane disruption. The effect of carvacrol on the cell membrane of *B.cereus*, a Gram positive bacterium, also was also reported by Burt S. [44]. In this study the carvacrol apparently dissolved in the phospholipid bilayer and causes distortion, expansion and destabilization of the cell membrane. Sanchez et al. [45] reported the action of phenolic compound, oleuropein, against phospatidylglycerol, a model membrane of Gram-positive bacteria and phosphatidylethanoalamine, a model membrane of Gram negative bacteria. In this study, Gram positive bacteria were more susceptible than Gram negative bacteria to oleuropein. This might be because Gram negative bacteria have three essential layers (cytoplasmic, peptidoglican and an outer membrane layer) while Gram positive bacteria have two layer [46].

2.8.2. Leakage of intracellular constituents

Antimicrobial actions of phenolic compounds are due to the leakage of ion and other intracellular constituents [47]. Phenolic compounds caused membrane leakage in bacteria resulting from the presence of protons, phosphate, and potassium in the growth medium [41,48]. Lambert et al. [49] reported that carvacrol and thymol caused membrane leakage as indicated by the presence of protons, phosphate, and potassium in the growth media of *S. aureus* ATCC 6538. Loss of potassium from cells of *E.coli* and *S. aureus* which were treated with thymol and eugenol also was also observed [47]. Phenolic extracts from berries exhibited disintegration of the outer membrane which led to the leakage of lipopolisaccharide into the growth medium of Gram negative bacteria *S. typhimurium*.

2.8.3. Inhibition of enzyme activities

Phenolic compounds can exhibit antimicrobial activities through enzyme inhibition. According to Pimia et al. [50], phenolics extracted from berries exhibited antibacterial activity through inhibition of extracellular microbial enzymes. In *E. coli*, chlorogenic acid inhibited β-ketoacyl-ACP reductase, an enzyme responsible for fatty acid synthesis in bacteria [41]. Chlorogenic acid competitively inhibited the binding of NADPH in fatty acid synthesis which could lead to suppression of growth of bacteria. Flavonoids such as quercetin inhibited DNA gyrase which functions as catalyst of DNA super coiling in prokaryotes [36].

2.8.4. Inhibition of nucleic acid formation

Inhibition of nucleic acid formation was proposed as another mechanism of antibacterial action of phenolics [36]. Caffeine inhibited protein synthesis by causing damage to bacterial DNA, inhibiting DNA repair and synthesis of protein in bacteria [26]. According to Kaur and Singh [48], phenolics from the *Livistona chinensis* fruit extract inhibited the growth of *S. aureus* through DNA, enzyme, and protein denaturation. A group of phenolics that consisted of biphenol, catechol, 1,2,4-benzenetriol, 2-methoxyestradiol, 2-hydroxyestradiol, diethylstilbestrol, butylated hydroxytoluene, butylated hydroxyanisole, fert-butylhydroquinone, and some phenolic acids such as ferulic acid, caffeic acid, chlorogenic acid, and eugenol caused strand breaks of model DNA [51].

2.9. Extraction of phenolic compounds

The extraction of a phenolic compound depends on its solubility in water. Generally, water soluble antimicrobial compounds can be released or extracted from plant tissue by rupturing the tissue during sample preparation. Other methods such as juicing, steam distillation, or solvent extraction have also been employed [52]. Steam distillation is used to extract phenolic compounds in essential oils. According to Cowan [53] and Zhong et al. [54] solvent extraction, either single or combination, has been used frequently for extraction of active compounds. Methanol, Ethanol, Propanol, Acetone, Diethyl ether, Ethyl acetate, Chloroform, or their combinations with water in different proportions have been used to extract phenolic compounds [25]. In addition, extraction of phenolic compounds can be modified using hot extraction or superheated extraction [55].

The extraction of phenolics is influenced by factors such as solvent polarity, ratio of solvent to sample and extraction time [56]. Different polarity of solvent might influence the type of chemical or phenolic compounds recovered. Polyphenol, phenolic acids such as chlorogenic, gallic, protocatechuic, and caffeic and essential oil [25] have been extracted with water by distillation or hydro-distillation. Methanol was used to extract essential oil, phenol, proantocyanidins and flavonoids [57], while ethanol was used to extract some phenolic acids, and flavonoids [58]. A non-polar solvent such as diethyl ether combined with ethyl acetate was used to extract phenolic compounds of grape seeds [59]. Chloroform also was used to extract essential oil, phenol, and alkaloid.

Many extraction methods have been tested by modifying the ratio of solvents. Horax et al. [60] used 80% methanol to extract the phenolic compound from bitter melons. Baydar et al. [61] extracted phenolic compounds of defatted grape material in a soxhlet apparatus using acetone : water : acetic acid (90:9.5:0.5 ratio) at 60^{o}C. In coffee, methanol is the preferred solvent to extract phenolic compounds [62,63]. Hydroxynnamic acids of coffee were extracted with 40% methanol and some phenolics such as caffeine and Chlorogenic acid from raw monsooned *Arabica coffee* extracted using 80 % methanol [62].

Recovery of phenolics was influenced also by the extraction period [56]. Variyar et al. [62] reported that caffeine and Chlorogenic acid were extracted by soaking the sample in 80% methanol for 2 hours. Extraction of litchi fruit pericarp with 60 % ethanol for 3 hours resulted in the highest recovery of phenolics [54]. Extraction method also influenced the extraction time. Markom et al. [64] reported that 3 hours extraction using supercritical fluid extraction in 10% methanol and 10% ethanol yielded the highest hydrolyzable tannins, although, combination of 50% ethanol and water resulted in the same amount of tannins after 2 hours extraction.

2.10. Characterization of phenolic compounds

Several methods have been widely used to separate and identify specific chemical composition of phenolic compounds such as Thin Layer Chromatography (TLC), High Performance Liquid Chromatography (HPLC), and Gas Chromatography. Although TLC is the simplest, least expensive and fastest method for the separation of phenolic compounds, HPLC and GC were the most common methods used [65]. A HPLC method was employed

commonly to identify phenolic acids in berries, fruit and beverages and herbal medicines. It also was used to identify simple phenolics present in coffee [65,66].

Gas chromatography-mass spectrophotometry was able to separate eight phenolic acids and four flavonoids in methanolic and aqueous plant extract [67]. The GC-MS also was used commonly for determination of phenolic compounds of essential oil in cortex cinnamomi [68]

Chapter Three

Materials and Methods

3.1. Materials

3.1.1. Chemicals and Reagents

Coffee husk was obtained from local coffee pulping industry located at Addis Ababa, Ethiopia. The coffee husk was then kept in zipped plastic bags to prevent moisture entrapment. Goat pickled pelt was obtained from Tannery division of CSIR-CLRI, India.

HPLC grade Methanol and analytical grade Ethanol was obtained from Sigma Alidrich India. Acetonitrile was obtained from Sigma Alidrich India. Standard Chlorogenic acid was purchased from Alfa Aesar-Great Britain.

All chemicals used to process pickled pelt of goat skin to wet blue leather were commercial grades.

Staphylococcus aureus ATCC 33592, *Pseudomonas aeruginosa* ATCC 27853, *Bacillus cereus* ATCC 10875, *Escherichia coli* ATCC 25922 and *Aspergillus niger* MTCC 281 were obtained from Intech, Chadiagarh-India by Central Leather Research Institute of India. Agar powder (GRM 026), nutrient broth (GM002), Mueller Hinton Agar (M173), Potato dextrose agar (M403) were purchased from Himedia laboratories pvt. Ltd, India.

3.1.2. Instruments and Equipments

Small testing drum of rpm 4-8 is used for processing goat delimed pelt to wetblue. Soxhlet (Buchi Switzerland E-812) extractor was used for extraction purpose. FT-IR Jasco-4700 used for analyzing functional groups present in the extract. HPLC-DAD was used to quantify the amount of Chlorogenic acid in the coffee husk extract. LCMS (column- AC QUITY UPLC BEA C18, 1.7µm and 2.1*50mm at 20^{o}C temperature) was used to identify the $[M-H]^-$ peak.

Hi antibiotic zone scale was used to measure zone of inhibition in mill meter (mm) and obtained from Himedia laboratories pvt. Ltd, India. Humidity chamber (90% HR and 30^{o}C temperature) is used to put leather samples after application of the test to see if any growth of fungi. Autoclave at 120^{o}C and 1 and half hours was used to sterilization of cultures. Analytical weighing balance, Graduated cylinders, Micro pipette, Sampling tube, Filter paper(0.2µm) are used on the experiment.

3.2. Methods

3.2.1. Optimization of CGA yield

Extraction of the active component-Chlorogenic Acid was done using soxhlet extractor by varying solvent, extraction time and solvent to sample ratio. The temperature for extraction was kept constant at 120 ^{o}C to get better yield [61]. The dilution of the solvents preferred for this study was 80% solvent and 20% water [60] and 10g of coffee husk was Soxhleted.

Table 4: Complete design matrix for optimized yield of CGA

Variable	Factor code	Levels	
		-1	+1
Solvent	A	Methanol (80%)	Ethanol (80%)
Extraction time	B	4 h	6 h
Solvent to sample ratio	C	5:1	10:1

Table 5: Experimental design matrix

Run code	Coded factor			Actual factures			CGA yield (mg/kg)
	A	B	C	solvent	Extraction time	Solvent to sample ratio	
E1	+1	+1	-1	Ethanol	6 h	5:1	
E2	-1	-1	+1	Methanol	4 h	10:1	
E3	-1	+1	-1	Methanol	6 h	5:1	
E4	+1	+1	+1	Ethanol	6 h	10:1	
E5	-1	+1	-1	Methanol	6 h	5:1	
E6	-1	-1	-1	Methanol	4 h	5:1	
E7	+1	-1	+1	Ethanol	4 h	10:1	
E8	+1	-1	-1	Ethanol	4 h	5:1	
E9	-1	+1	+1	Methanol	6 h	10:1	
E10	+1	-1	-1	Ethanol	4 h	5:1	
E11	+1	+1	-1	Ethanol	6 h	5:1	

E12	-1	-1	+1	Methanol	4 h	10:1	
E13	+1	-1	+1	Ethanol	4 h	10:1	
E14	+1	+1	+1	Ethanol	6 h	10:1	
E15	-1	+1	+1	Methanol	6 h	10:1	
E16	-1	-1	-1	Methanol	4 h	5:1	
E17	-1	-1	-1	Methanol	4 h	5:1	
E18	+1	+1	+1	Ethanol	6 h	10:1	
E19	+1	-1	-1	Ethanol	4 h	5:1	
E20	+1	-1	+1	Ethanol	4 h	10:1	
E21	-1	+1	+1	Methanol	6 h	10:1	
E22	-1	-1	+1	Methanol	4 h	10:1	
E23	+1	+1	-1	Ethanol	6 h	5:1	
E24	-1	+1	-1	Methanol	6 h	5:1	

Twenty four experiments were conducted to get the optimized amount of Chlorogenic acid and the data was analyzed using Design-Expert 6.0.8 portable.

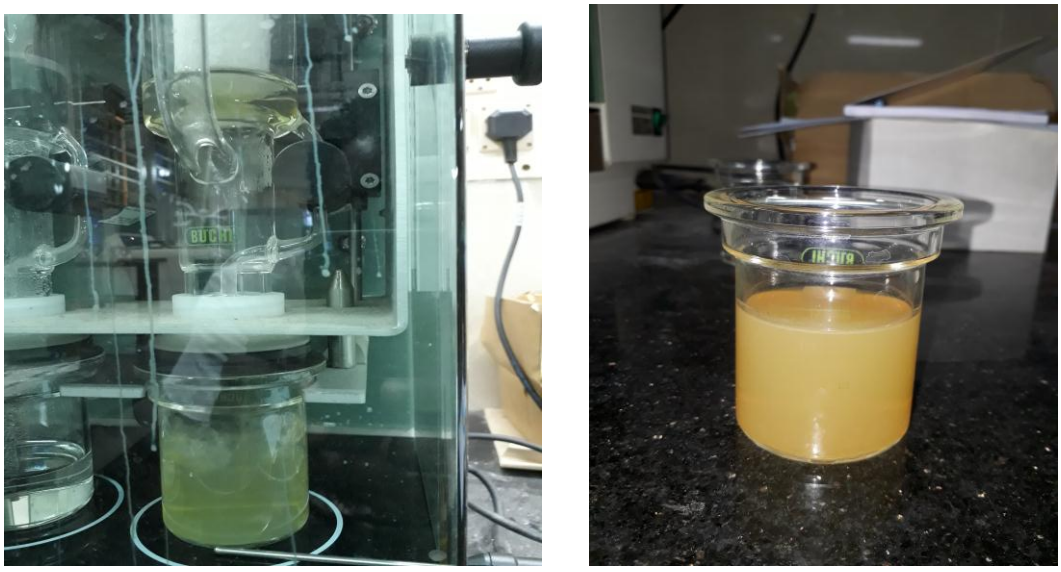


Figure 9: (a) Extraction of CGA using Soxhlet extractor (b) Extracted CGA

The separation of solvent from the coffee husk extract was done using Buchi R-134 rotary evaporator. It works by vacuum distillation where rotating the bottle provides better agitation, better contact of the solvent and extract with warm water and increase surface area of the

solvent which is evaporating, it fundamentally boils under the reduced pressure, so that solvent removed as gas. Then efficient spiral condenser allows to cool the gaseous solvent to returns into liquid phase.

Figure 10: Vacuum drying of crude CGA

3.2.2. Quantification and Characterization of CGA

All extracted samples were analyzed for presence of the active component by high performance liquid chromatography (HPLC). All samples were prepared using HPLC grade Methanol and were filtered through 0.2µm filter prior to analysis by HPLC. The active component-CGA was analyzed using a Reverse Phase- HPLC system with a photodiode array detector.

Separation was performed by a gradient elution system on a phenomenx Gemini 5µm c18 150*4.6mm column at 25^{o}C. The flow rate was adjusted to 1.0 mL/min. For gradient elution mobile phases A and B were employed. Solution A contained HPLC grade methanol and solution B contained 0.6 % of H_3PO_4 in deionized water, these were applied for 65 minutes at 90:10 ratio (v/v). The Photo Diode Array (PDA) was set at 210-600 nm and chromatograms were extracted at 327nm. A standard Chlorogenic acid was prepared and diluted to obtain 10ppm, 15ppm and 20ppm solutions to build calibration curves.

Analysis of data from three replicated experiments was performed using analysis of variance (ANOVA) with Design-Expert 6.0.8 portable. The main and interaction effects were considered significant if $p<0.05$.

Repeated experiment of the optimized yield of CGA was performed and made concentrated for anti-microbial studies and leather tests.

Fourier Transform Infrared (FTIR) spectroscopy analysis was performed to identify the functional groups present in the extract and match them with the required data of the standard Chlorogenic acid for verification. All spectra were performed with 40 scans/second, $4cm^{-1}$ resolution, and recorded at 45˚incident angle using potassium bromide in the region 400 to 4000 cm^{-1}.

Liquid chromatography mass spectroscopy (LCMS) column- AC QUITY UPLC BEA C18, 1.7µm and 2.1*50mm at 20^{o}c temperature was conducted to identify the base peak which is the $[M-H]^{-}$ peak whether it matches with the mass of the CGA. Mobile phase- Acetonitrile (A), 5mm Ammonium Acetete pH 3.5 using Acetic acid (B). flow rate=0.4ml/min (company-Waters and software-Masslynx).

3.2.3. Anti-Microbial Studies

The antimicrobial activity of the coffee extracts against various bacteria was determined by means of the disc diffusion and well diffusion method. Two gram positive bacteria (*Bacillus cereus* and *Staphylococcus aureus)*, two gram negative bacteria (*Pseudomonas aeruginosa* and *Escherichia coli)* and fungi; *Aspergillus niger* were selected to study the effect of extracted Chlorogenic acid. The strains were kept at -70 °C in brain heart infusion broth (BHI) with 20% glycerol, activated by transferring into nutrient agar and incubated at 37˚C for 48 hours. Then 100 µL of the suspensions was inoculated onto the surface of growth media in a 150-mm petri dish and spread homogeneously.

Agar and Potato Dextrose Agar were autoclaved for 1-5 min at 120^{o}C and used for disk diffusion study of susceptibility of bacterial strains and fungi respectively for the extracted CGA from coffee husk.

100µL of a bacterial suspension was swabbed uniformly across the growth media in petri dishes. The plates were left at room temperature for 30 minutes to allow diffusion. Two wells of 6mm in diameter were made in agar media using sterile borer. 10µl of 4.2mg/mL CGA

and 10 µL of methanol as control were inoculated into the well and incubated at 35°C for 48 hours and growth of test microorganisms. For disk diffusion method, paper discs of 6.0 mm diameter were impregnated with CGA extracted from the coffee husk and placed on the surface of the agar containing each bacterium. For each experiment Methanol was used as control. The inhibition zones in mm around wells were measured. The antimicrobial activity was expressed as the diameter of inhibition zones produced by the extracts against test micro-organisms [39].

3.2.4. Determination of Minimum Inhibitory Concentration (MIC)

In microbiology, minimum inhibitory concentration (MIC) is the lowest concentration of an antimicrobial drug that will inhibit the visible growth of a microorganism after 24 hours incubation. MIC is used by diagnostic laboratories mainly to confirm resistance, but most often as a research tool to determine MIC breakpoints. MIC is the minimum concentration of CGA which can inhibit the growth of microorganism. Different concentrations of CGA viz., 500µg/mL, 250µg/mL, 125µg/mL, 62.5µg/mL and 31.2µg/mL were prepared. 10µL of the 24 hours bacterial suspension was added to it and inoculated in a petriplate containing nutrient agar. Standard plate count was done after 24 hours to find MIC. If the organism under study is susceptible to the incorporated antibiotic, no fungi growth is expected in agar plates with higher amounts of the drugs. Fungi growth is observed as the antibiotic concentration in the agar plate diminishes. Inhibition of growth at the minimum or lowest concentration of antibiotic is regarded as the end point and recorded as the Minimum Inhibitory Concentration (MIC).

3.2.5. Leather test

The pickled pelt of goat was processed to wet blue using regular process recipe. Normal production process was followed till tanning. Commercial preservative was used as control and extracted Chlorogenic acid was used as experiment. Since the extracted CGA is not concentrated as commercial preservative, the amount of CGA feed to drum was higher than the commercial one. The extracted CGA was fed at the end of tanning because of the pH.

Table 6: Experimental design for tanning goat pickled pelt

Process		Tanning		Drum no.		experiment-tannery
In put material		pickled goat pelt				
Quantity						
pelt Weight				Date		
All percentage is based on Fleshed weight						
s.no.	Process name	Chemical name	%	Run time	pH	REMARKS
1	Depickling	water	250			
		salt	15	10'		^{o}B=10
		pelt		15'		
		Degreasing agent	0.5	10'		
		Sodium Formate	1			
		Sodium Bicarbonate (1:10@35^{o}C)	1.5	120'	5.5-6.0	1:10@35 ^{o}C
						D/W/D
2	pickling	water	100			
		salt	7	10'		^{o}B =6-7
		pelt		15'		
		Formic acid	0.75	30'		1:10
		Sulfuric acid	0.5	60'	2.6-2.8	1:10
3	tanning	BCS	4			
		Sodium Formate	0.5	30'		
		BCS	4	120'		
	Control	comm. Preservative	0.1-0.2	20'		
		Sodium Bicarbonate (1:10@35 ^{o}C)	1.5	15'	3.8-4.0	3feed, each 15' then 4h
	Experiment	CGA	0.5	30'		check cross section
						and exstution
						Boil test>100 ^{o}C
						pile for 24h

3.2.6. Physical strength characteristics

The physical properties such as tensile strength, tear strength, percentage elongation at break was examined as per standard SATRA procedures for both the experimental and control leather.

Tensile strength is the load per unit area of cross section required to pull apart or break a strip of leather material. The tensile strength of control and experimental leathers were measured as per procedure. A dumb bell shaped leather sample parallel to the direction of backbone of the animal was punched out using a steel press knife of standard dimensions. The samples were of 9cm total length and of 5cm in the experimental region. After condition for 48 hours at temperature 20±2^{o}C and 65±2^{o}C % Relative Humidity, the thickness of the samples at the middle point and at two points midway between the middle and ends were measured as per procedure given in. The samples were tested in an Instron Universal Testing Machine.

Tensile Strength (Kg/cm^2) = (Breaking load (kg)) / (thickness x width (cm^2))

The tear strength of control and experimental samples were measured following a modified procedure based on. A sample of the size 7.5x2.5 cm was slit at right angles to the grain and flesh sides for about half the length. One end of the piece formed by the slit was clamped in the upper jaw of the Instron Universal Testing Machine and the other end was fixed to the lower jaw. When the Instron machine was run, the sample treated. The average load corresponding to the tearing of the tongue was calculated from strip chart recorder. This load is divided by the average thickness of the sample was taken to be the tearing strength of the samples in kg/cm. Control and experimental samples were tested for the determination of tearing strength of the samples in kg/cm. Control and experimental samples were tested for the determination of tearing strength parallel to the direction of the backbone, similar procedure was repeated for equal number of samples taken from direction perpendicular to the direction of backbone.

Tear Strength (Kg/cm) = Load (kg) / Thickness (cm)

3.2.7. Wastewater analysis

Waste water from the tanning bath were collected and filtered through Whatman no.1 filter paper and analyzed for COD and TDS.

Chemical oxygen demand (COD) is defined as the amount of a specified oxidant that reacts with the sample under controlled condition. The quantity of oxidant consumed is expressed in terms of its oxygen equivalence. COD often is used as a measurement of pollutants in waste water and natural water. Reagents used for the determination of the COD include standard potassium dichromate 0.25N, COD acid and mercuric sulphate. Take sample of the spent liquor and made up to 100 mL distilled water and from that take 2.5 mL and transferred to clean COD tube, add 1.5mL potassium dichromate and 3.5mL of COD acid. Add spatula of mercuric sulphate. Also the blank was prepared in the same way with 2.5ml of distilled water, 1.5mL of potassium dichromate and 3.5mL of COD acid and mercuric sulphate. All tubes were kept in the COD incubator at 150^{o}C for 2 hours. After incubation samples were titrated against ferrous ammonium sulphate(FAS) in burette for the COD value using few drops of ferroin indicator and the end point is the persistence of reddish brown colour.

$$\text{COD (mg/l)} = (A-B) \times N \times 8000/\text{volume of sample}$$

A=Volume of FAS used for titration of the blank (ml), B=Volume of FAS used for titration of the sample (mL), 8000=Milliequivalent weight of oxygen x 1000ml/L, N= Normality of FAS

Total Suspended Solid (TSS)

Dry weight of empty dish or crucible is recorded as initial weight. Add 50mL sample and keep it in water bath until dry then keep it in oven(103 to 105^{o}C) for at least 1 hour then take final weight of dish after desiccator.

$$\text{Total solid (mg/L)} = (\text{final weight-initial weight}) \times 1000 \times 1000 / \text{volume of sample}$$

Determination of total dissolved solids

Dry weight of empty dish or crucible recorded as initial weight. Take sample and filter with Whatman No.1 then add 50 ml filtrate sample and keep it in water bath until dry. Then keep it in an oven (103 to 105^{o}C) for at least 1 hour then desiccate before taking final weight of dish.

Total dissolved solid (mg/L) = (final weight-initial weight) ×1000×1000 / volume of sample

Determination of Total Suspended Solid

The difference between the total solids and total dissolved solids is suspended solids.

Total Suspended Solis = Total Solid -Total Dissolved Solid

Figure 11: Sample drum

Chapter Four

Results and Discussions

4.1. Optimized Chlorogenic Acid result

Experimental design was selected for the statistical analysis of the study and the response measured is the percentage yield of Chlorogenic acid extracted from coffee husk. The percentage yield of Chlorogenic acid extracted from coffee husk was investigated by selecting three factors suchas solvent type (A), extraction time (B) and solvent to sample ratio (C). Regression analysis and analysis of variance (ANOVA) was done by using Design Expert 6.0.8 Program. The software program was used to generate surface plots, using the fitted Equation obtained from the regression analysis, keeping one of the independent variables Constant.

Response of the extraction process was used to develop a mathematical model that correlates the percentage yield of Chlorogenic acid under investigating. Design Expert software version 6.0.8 was used for the regression analysis of the experimental data and also for evaluation of the statistical significance of the equation developed. The responses and the statistical analysis of the ANOVA are given in Table 9 and Table 10 respectively.

Table 7: Experimental values for percentage yield of Chlorogenic acid

Run code	Coded factor			Actual factures			CGA yield (mg/kg)
	A	B	C	solvent	Extraction time	Solvent to sample ratio	
E1	+1	+1	-1	Ethanol	6 h	5:1	468
E2	-1	-1	+1	Methanol	4 h	10:1	681
E3	-1	+1	-1	Methanol	6 h	5:1	574
E4	+1	+1	+1	Ethanol	6 h	10:1	502
E5	-1	+1	-1	Methanol	6 h	5:1	569
E6	-1	-1	-1	Methanol	4 h	5:1	557
E7	+1	-1	+1	Ethanol	4 h	10:1	538
E8	+1	-1	-1	Ethanol	4 h	5:1	501
E9	-1	+1	+1	Methanol	6 h	10:1	610

E10	+1	-1	-1	Ethanol	4 h	5:1	523
E11	+1	+1	-1	Ethanol	6 h	5:1	471
E12	-1	-1	+1	Methanol	4 h	10:1	679
E13	+1	-1	+1	Ethanol	4 h	10:1	527
E14	+1	+1	+1	Ethanol	6 h	10:1	510
E15	-1	+1	+1	Methanol	6 h	10:1	625
E16	-1	-1	-1	Methanol	4 h	5:1	549
E17	-1	-1	-1	Methanol	4 h	5:1	553
E18	+1	+1	+1	Ethanol	6 h	10:1	505
E19	+1	-1	-1	Ethanol	4 h	5:1	528
E20	+1	-1	+1	Ethanol	4 h	10:1	522
E21	-1	+1	+1	Methanol	6 h	10:1	640
E22	-1	-1	+1	Methanol	4 h	10:1	675
E23	+1	+1	-1	Ethanol	6 h	5:1	470
E24	-1	+1	-1	Methanol	6 h	5:1	570

As evident from Table 9, the maximum yield of Chlorogenic acid extracted from coffee husk was 681ppm at experiment number 2 or product code E2, while the minimum percentage of lignin degradation was 468ppm at experiment number 1 (E1). Also from the table experiment numbers 9, 12, 15, 21 and 22 were maximum amount of yield gained. Therefore it was concluded that the maximum percentage yield of Chlorogenic acid was gained at Methanol as solvent, extraction time of 4 hours and 10:1 ratio of sample to solvent. The minimum yield was obtained at Ethanol as solvent, extraction time of 6 hours and 5:1 ratio of sample to solvent.

The model equation that correlates the response to process variables in terms of actual and coded value was given below in equation.

Final Equation in Terms of Coded Factors:

%CGA = +533.11 – 29.09*A – 7.73*B + 10.10*C + 0.38*A*B – 2.62*A*C + 0.026*B*C + 1.49*A*B*C

Where A= 80% Methanol, B= 4 hours extraction time and C= 10:1 solvent to sample ratio.

Based on the model, it can be concluded that the most desirabile yield of Chlorogenic acid will be

Solvent	Ex. Time	Ratio	%CGA	Desirability
-1.00	-1.00	1.00	678	0.987

4.1.1. Analysis of variance

The Model F-value of 200.75 implies the model is significant. There is only a 0.01% chance that a "Model F-Value" this large could occur due to noise. Values of "Prob > F" less than 0.0500 indicate model terms are significant. In this case A, B, C, AC, ABC are significant model terms

Table 8: Analysis of Variance

Source	Sum of Squares	DF	Mean Square	F value	Prob>F
Model	96513.96	7	13787.71	200.79	< 0.0001
A	51987.04	1	51987.04	757.09	< 0.0001
B	3675.38	1	3675.38	53.52	< 0.0001
C	6266.20	1	6266.20	91.26	< 0.0001
AB	44.41	1	44.41	0.65	0.4331
AC	2108.41	1	2108.41	30.70	< 0.0001
BC	0.21	1	0.21	3.034E-003	0.9568
ABC	3432.04	1	3432.04	49.98	< 0.0001

Values greater than 0.1000 indicate the model terms are not significant. Adjusted R-Squared of 0.9838 indicates that 98.38% of data reasonable agreement with pridicted R-square of 0.9747.

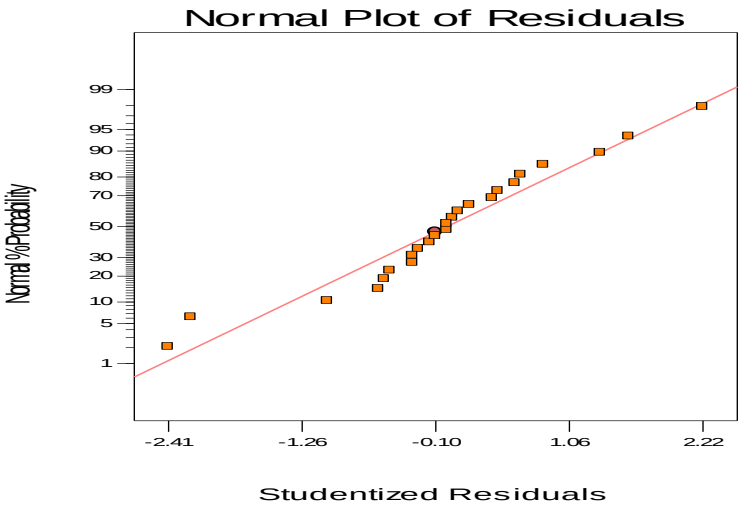


Figure 12: Normal probability plot

4.1.2. Effect of solvent type (A) on CGA yield

The solvents used were Methanol (80%) and Ethanol (80%) and the former is more effective for extraction of CGA from coffee husk which was reported by Horax et al. [46]. The result of the extraction also matches with the literature in that the percentage yield of Chlorogenic acid is significantly affected by the type of solvent used.

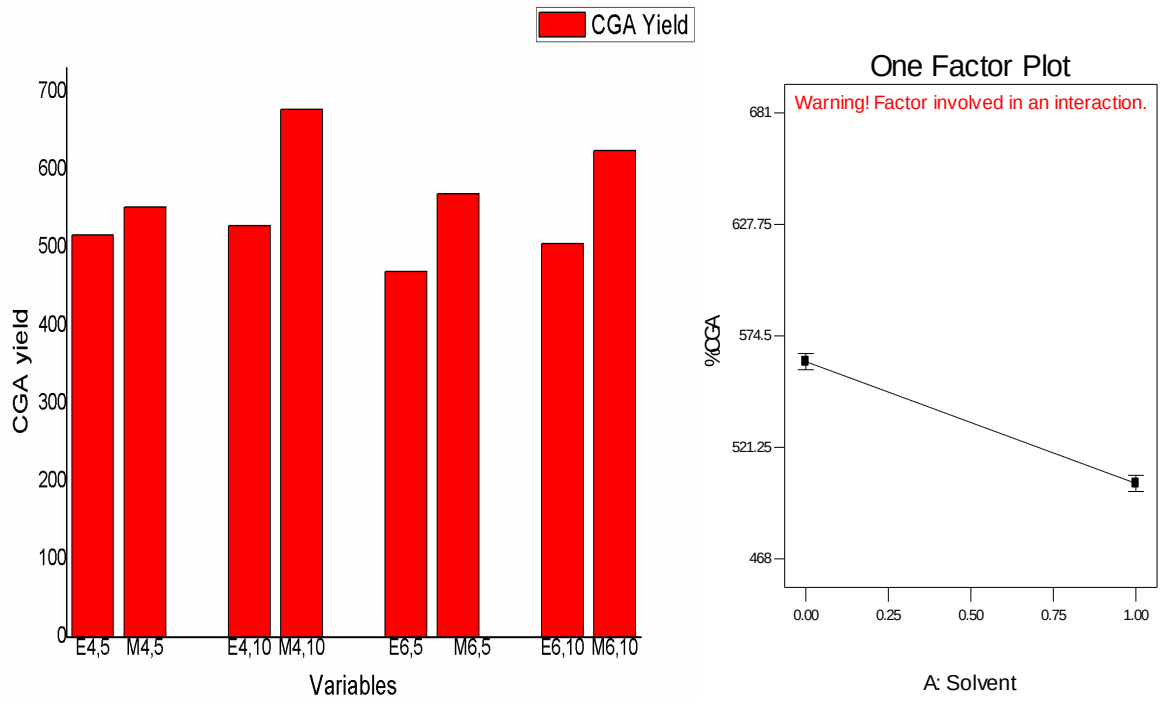


Figure 13: Effect of solvent type on CGA yield, where variables are E=Ethanol, M=Methanol, 4=4 hours, 5=5:1 solvent to sample ratio, 6=6hours and 10=10:1 solvent to sample ratio

4.1.3. Effect of extraction time (B) on CGA yield

The extraction time for the extraction procedure used were 4 hours and 6 hours and which has significant effect on the percentage yield of Chlorogenic acid. As the time increased from 4 to 6 hours, the yield of Chlorogenic acid decreases due to the possible structural decomposition of Chlorogenic acid which occurs when the reaction proceeds for long period of time.

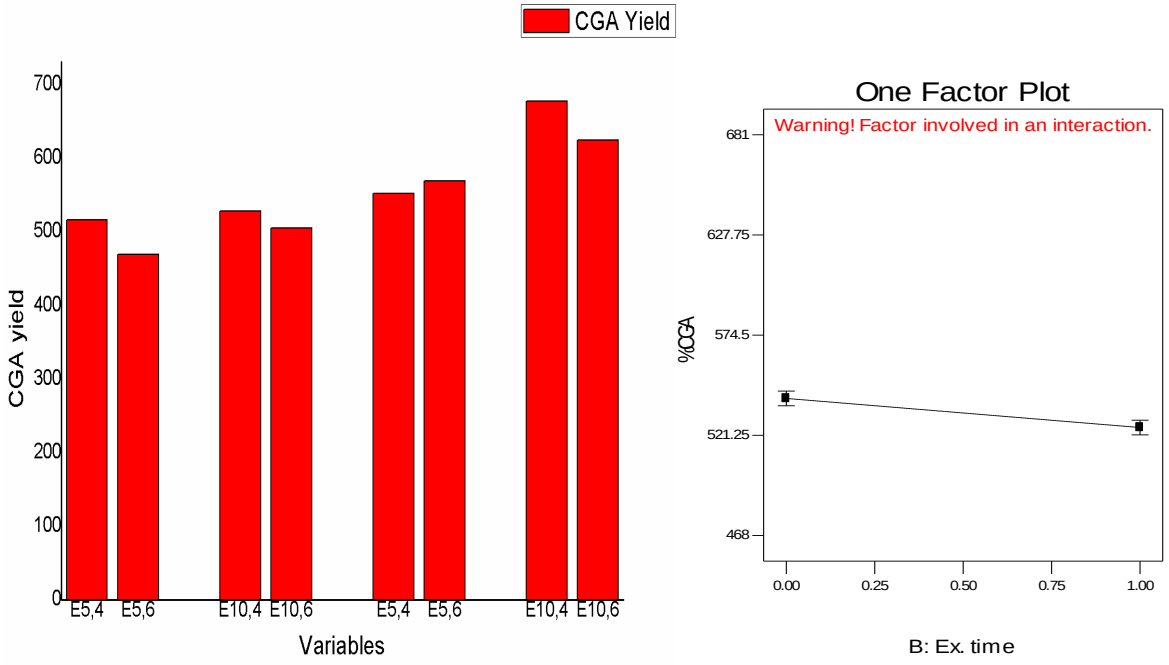


Figure 14: Effect of Extraction time on CGA yield (mg/kg), where Variables are E=Ethanol, M=Methanol, 4=4 hours, 5=5:1 solvent to sample ratio, 6=6hours and 10=10:1 solvent to sample ratio

4.1.4. Effect of solvent to sample ratio (C) on CGA yield

The solvent to sample ratios used in the experiments were 5:1 and 10:1 and the result indicates that as the ratio of solvent to sample increases, the percentage yield of Chlorogenic acid also increases.

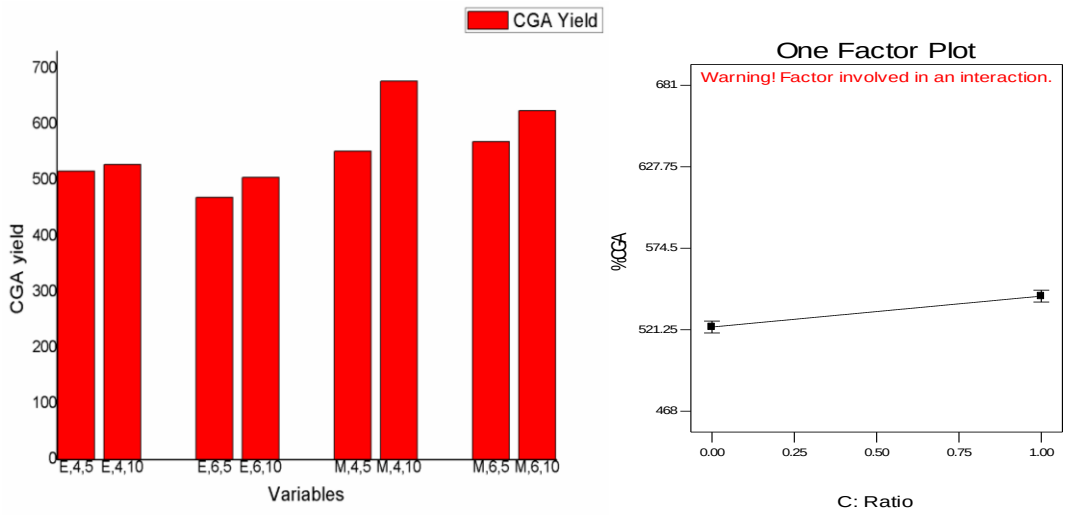


Figure 15: Effect of solvent to sample ratio on CGA yield (mg/kg), where E=Ethanol, M=Methanol, 4=4 hours, 5=5:1 solvent to sample ratio, 6=6hours and 10=10:1 solvent to sample ratio

4.1.5. Interaction Effect

The only interaction effect in the model is AC (Solvent type and ratio of solvent to sample). It can be concluded that, as the reaction shifts from Ethanol to Methanol and as the ratio of solvent to sample increases from 5: 1to 10:1, the yield of Chlorogenic acid increases.

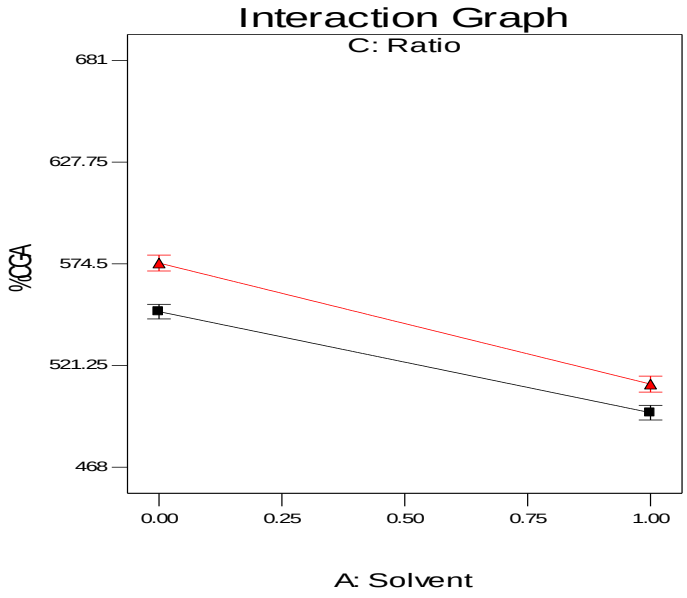


Figure 16: Interaction effect of solvent to sample ratio and solvent type on %CGA

4.2. Characterization and Quantification of CGA

4.2.1. HPLC

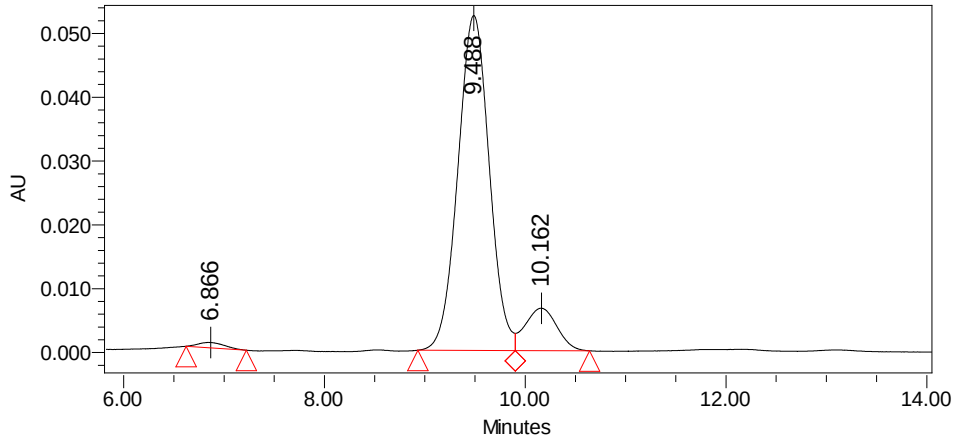


Figure 17: Retention Time

Under the described chromatographic conditions, the retention times of the two phenolic acids were 9.488 min for Chlorogenic acid and 10.162 for Caffeic acid, as seen in Figure 17.

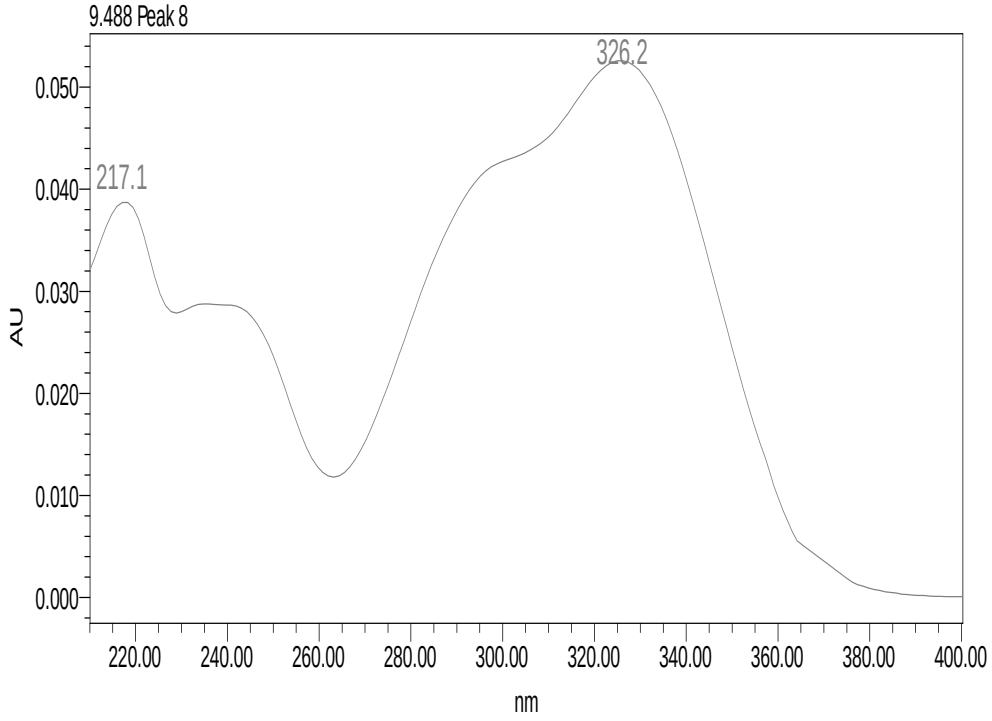


Figure 18: UV peaks of the Extracted CGA

Figure 18 shows the UV-Vis absorption spectra of CGA in the wavelength regions of 200 – 400 nm at room temperature. In these regions CGA has two maximum peaks; the first maximum being at 217nm with shoulder at 240nm and the second peak was at 326 nm with shoulder at 295nm and the minimum point was at 262 nm. The peak point at 326 nm was the highest peak corresponding to the HOMO to LUMO transition presents mainly ππ* character with electron density localization on the benzene ring and carbon chain and this large absorbance seem to be promising to improve the sensitivity for CGA determination in coffee beans. The other peaks and shoulders are also present the same ππ * transitions [39].

4.2.2. FT-IR

FT-IR analysis result shown in Figure 19 indicates that there is high intensity band in the region 3409 cm^{-1} which is the stretching band for aromatic OH. 2927cm^{-1} band corresponds to C-H aliphatic stretching. Benzene C=C stretching and aliphatic C=C stretching bands occur at 1651cm^{-1} and 1601 cm^{-1} respectively. From the FT-IR spectrum it can also be analysed that Carbonyl (C=O) stretching occurred at 1698 $cm^{-}1$. According to literatures [69,70] on coffee analysis, bands that appear at lower intensity in the range of 1600–1000 cm^{-1} corresponds to Chlorogenic acids. The region with bands 1510, 1458, 1383, 1267, 1124 and 1033 cm^{-1} can be attributed to Chlorogenic acids. Some of the bands can be interpreted as, 1033 cm^{-1} C-H in-plane bending, 764 cm^{-1} C-H out-plane bending, 1124 cm^{-1} C-O-C stretching, 1458 cm^{-1} C-O-H in-plane bending and 1267 cm^{-1} C-O stretching.

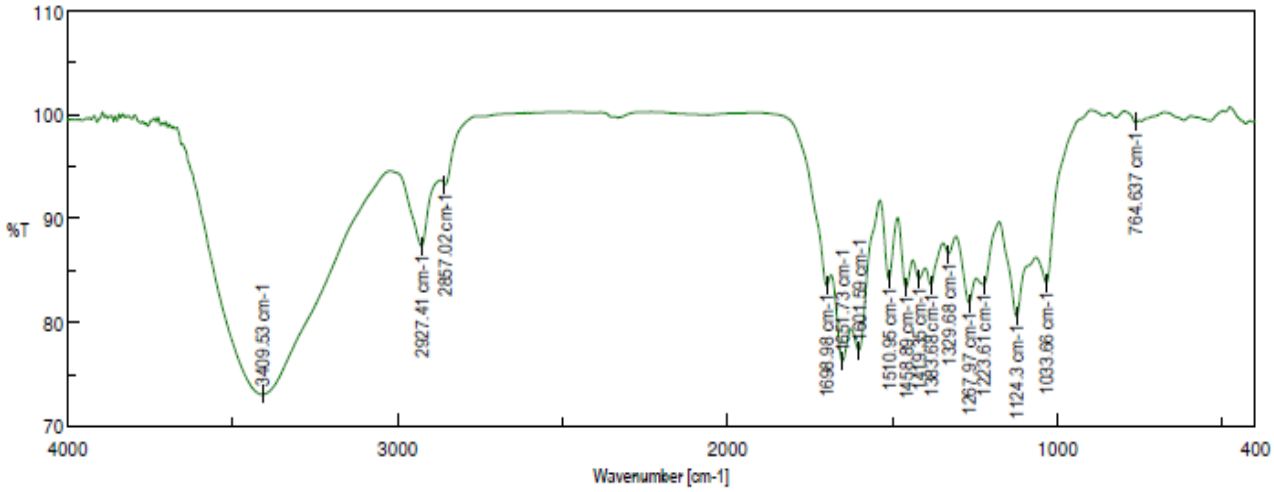


Figure 19: FT-IR spectra of extracted CGA

4.2.3. LCMS

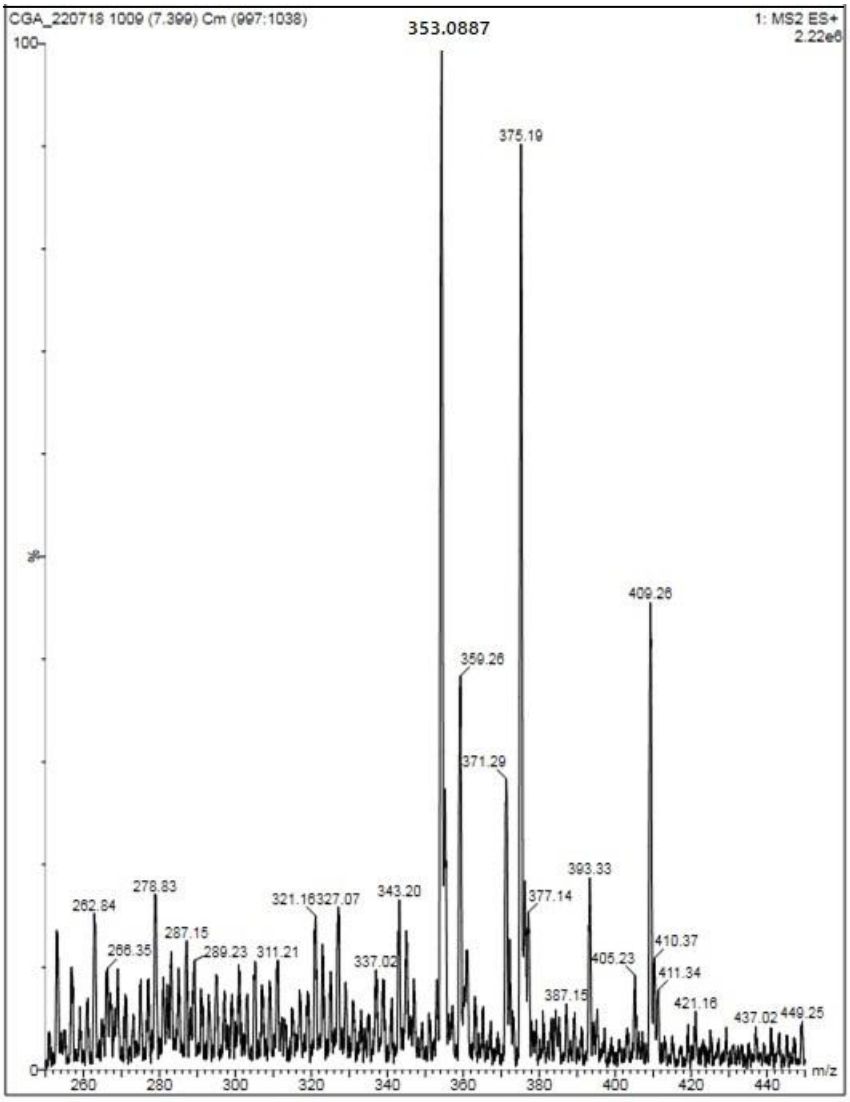
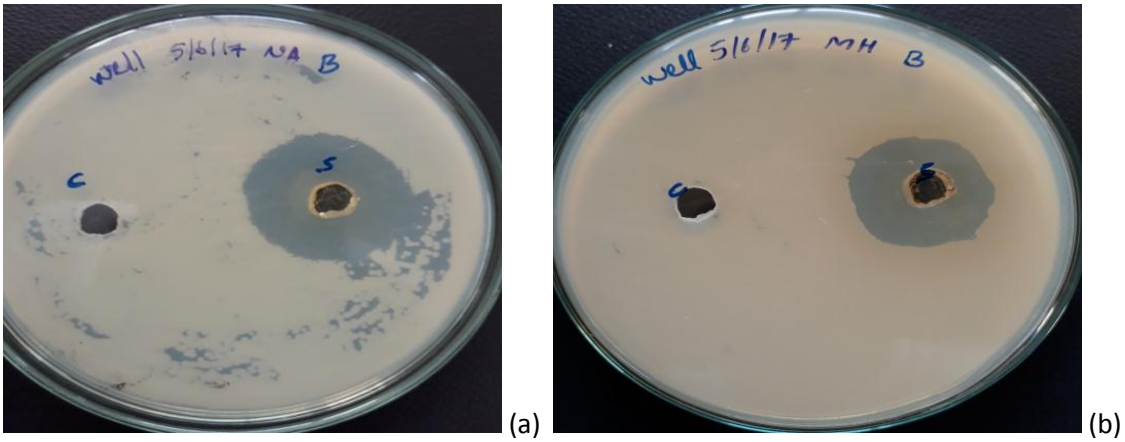


Figure 20: LC-MS spectra of extracted CGA

The chemical formulas corresponding to the peaks were first established after automatic calculation with the exact mass of the molecular ions ($[M-H]^-$) obtained from the LC-MS analysis. In this study, excellent agreement was obtained between the theoretical and the actual experimental mass data of the peaks. Besides exact mass measurements, the abundances of the isotope peaks and fragment ions of the molecular ions ($[M-H]^-$) were also monitored to confirm the identities of chemical formulas. With this exact mass data, the

molecular formula of base peak was calculated as $C_{16}H_{18}O_9$. The experimental mass data of m/z 353.0887.

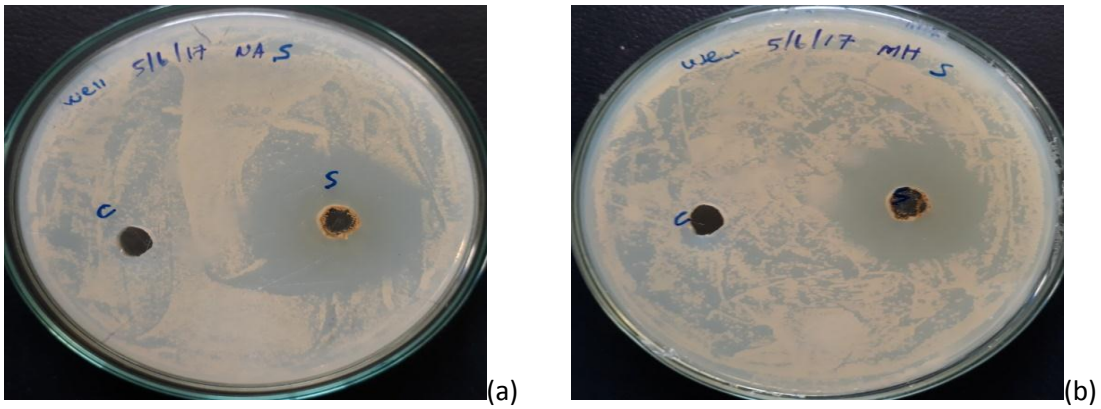
4.3. Anti-Microbial study



(a) (b)

Figure 21: Well diffusion method for *Bacillus cereus* using (a) Nutrient agar and (b) Mueller Hinton Agar

The zone of inhibition for the extracted Chlorogenic acid on *Bacillus cereus* is measured to be 24 mm for (a) and 21 mm for (b) Figure 21. The result indicates *Bacillus cereus* is susceptible or sensitive to Chlorogenic acid extracted from coffee husk and from the Figure 21, it can be concluded that both Nutrient agar and Mueller Hinton agar are favourable for the growth of *Bacillus cereus.*

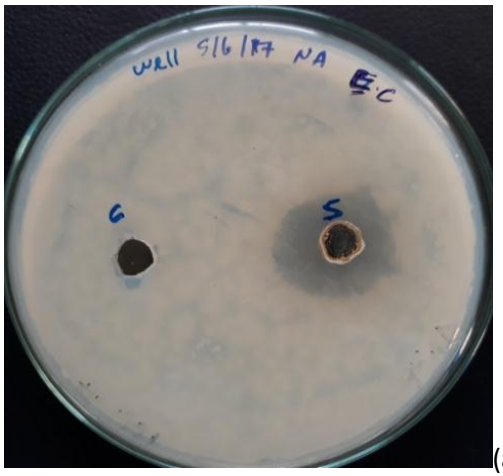


(a) (b)

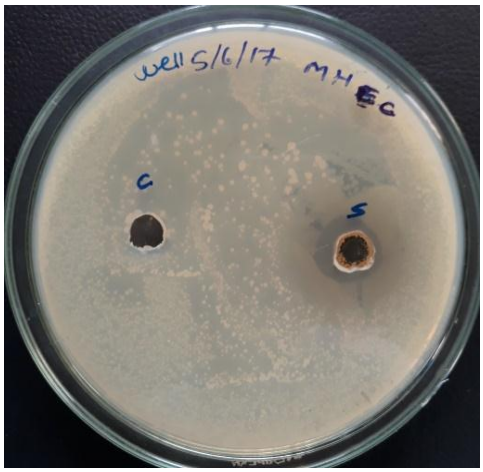
Figure 22: Well diffusion method for *Staphylococcus aureus* using (a) Nutrient agar and (b) Mueller Hinton Agar

The zone of inhibition for the extracted Chlorogenic acid on *Staphylococcus aureus* is measured to be 28 mm for (a) and 20 mm for (b) Figure 22. The result indicates

Staphylococcus aureus is susceptible or sensitive to Chlorogenic acid extracted from coffee husk and from the Figure 22, it can be noticed that the zone of inhibition in Nutrient agar is significantly greater than that in Mueller Hinton.



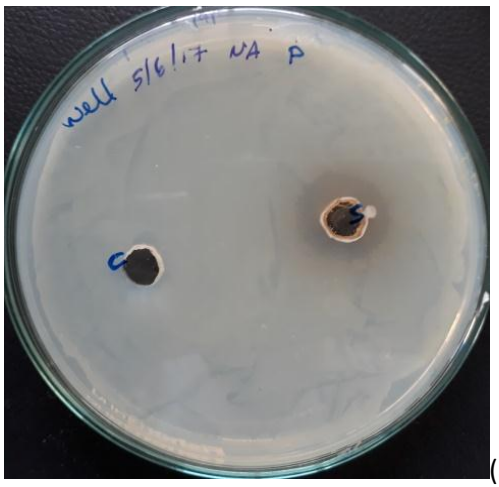
(a)



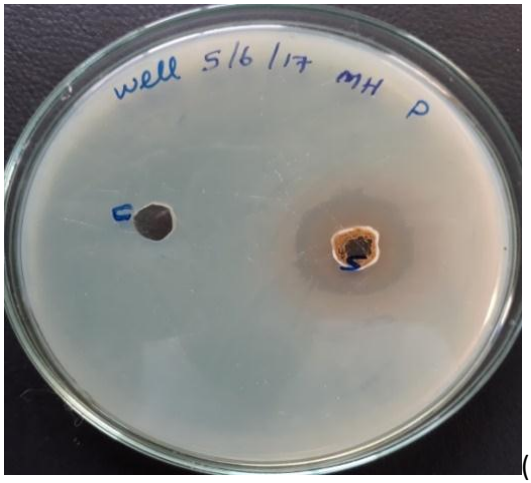
(b)

Figure 23: Well diffusion method for *Escherichia coli* using (a) Nutrient agar and (b) Mueller Hinton Agar

The zone of inhibition for the extracted Chlorogenic acid on *Escherichia coli* is measured to be 15 mm for (a) and 5 mm for (b) Figure 23. The result indicates *Escherichia coli* is susceptible or sensitive to Chlorogenic acid extracted from coffee husk and from the Figure 23, it can be noticed that Nutrient agar is a favourable growth media for *Escherichia coli* than Mueller Hinton Agar.



(a)



(b)

Figure 24: Well diffusion method for *Pseudomonas aeruginosa* using (a) Nutrient agar and (b) Mueller Hinton Agar

The zone of inhibition for the extracted Chlorogenic acid on *Pseudomonas aeruginosa* is measured to be 10 mm for (a) and 15 mm for (b) Figure 24. The result indicates *Pseudomonas aeruginosa* is susceptible or sensitive to Chlorogenic acid extracted from coffee husk and from the figure, it can be noticed that Mueller Hinton agar is a favourable growth media for *Pseudomonas aeruginosa* than Nutrient agar.

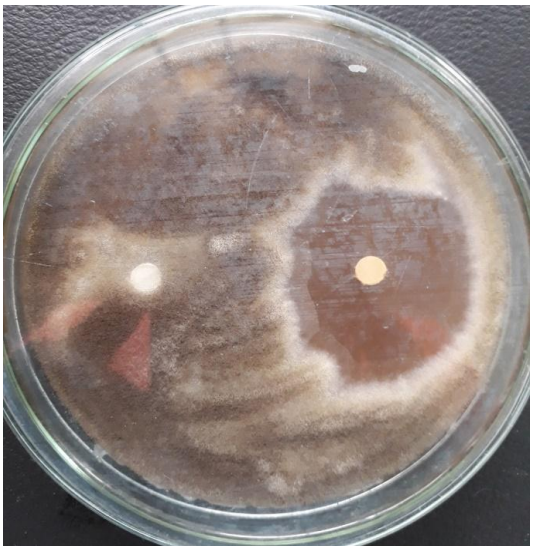


Figure 25: Disk diffusion method for Aspergillus niger using Potato Dextrose agar

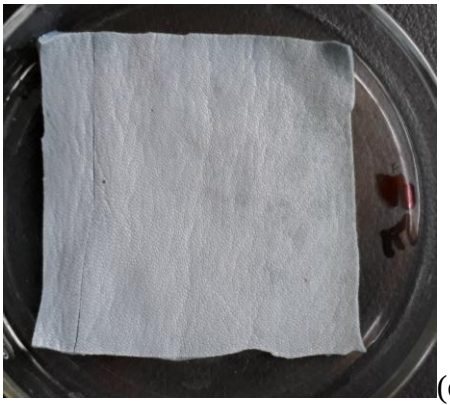
The zone of inhibition of the extracted Chlorogenic acid on *Aspergillus niger* is measured to be 34 mm Figure 25. The result indicates *Aspergillus niger* is susceptible or sensitive to Chlorogenic acid extracted from coffee husk.

From the microbial study, it can be concluded that, the two gram positive bacteria; *Bacillus cereus* and *Staphylococcus aureus* are more susceptible or sensitive to the extracted Chlorogenic acid from coffee husk than that of the other two gram negative bacteria; *Pseudomonas aeruginosa* and *Escherichia coli*. And moreover, the measured zone of inhibition for *Aspergillus nigger* indicates that Chlorogenic acid inhibits the growth of fungi in a more effective way. Minimum Inhibitory Concentration (MIC) calculated for *Aspergillus niger* was found to be 62.5µg/mL resembles with literatures [39].

4.4. Leather study

(a)

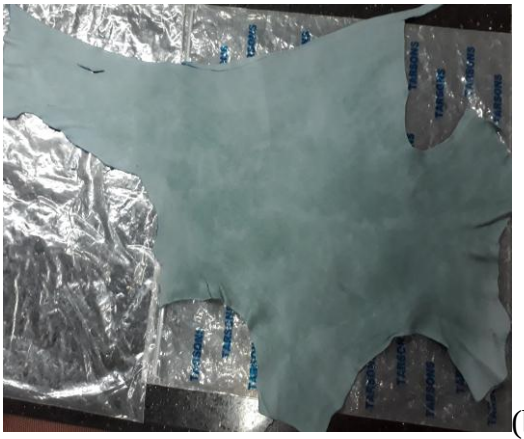

(b)


(c)

(d)

Figure 26: Leather cuts in the humidity chamber; 0 day of experiment (a) and control (b) and 15 days of experiment (c) and control (d)

From the above Figures 26, the leather cuts treated with Chlorogenic acid extracted from coffee husk and kept in humidity chamber showed resistance to microbial growth for 15 days.


(a)

(b)


(c)

(d)

Figure 27: Leather samples in the tannery open air 0 day of experiment (a) and control (b) and 15 days of experiment (c) and control (d)

From the above Figures 27, the wetblue leather treated with Chlorogenic acid extracted from coffee husk and kept in tannery where microorganisms, especially fungi are available everywhere, showed resistance to microbial growth for 15 days.

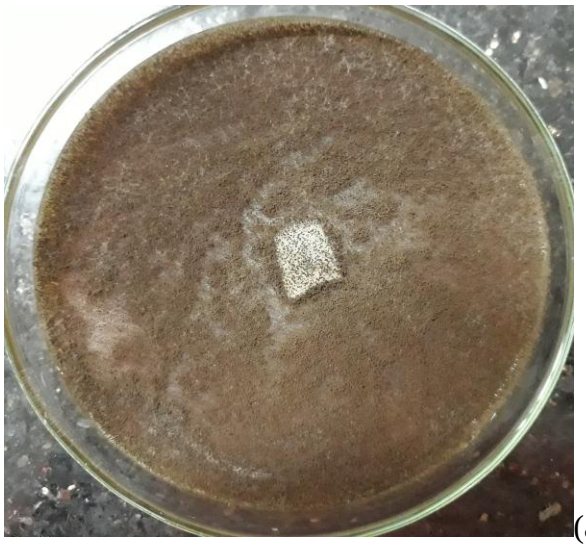
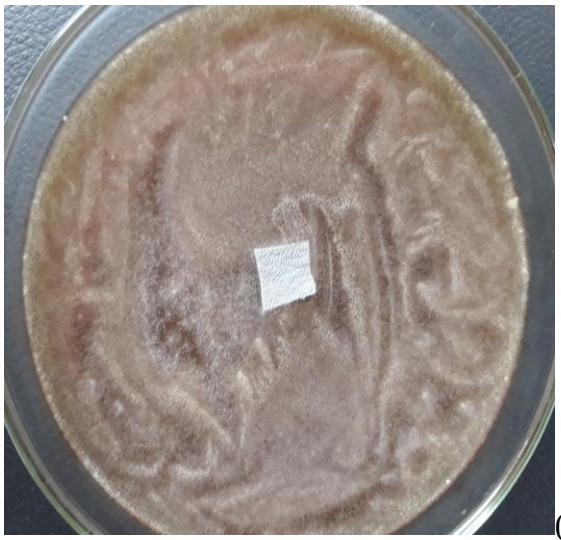

(a)

(b)

Figure 28: Fungal development on leather cuts with no preservative (a) and preserved with CGA (b)

From Figure 28, it can be noticed that, there is a fungal growth on the surface of leather cut from wet blue with no preservative while there is no growth on the surface of leather cut from wet blue preserved with Chlorogenic acid extracted from coffee husk indicating the extracted CGA from coffee husk is preventing the wetblue leather from fungal attack.

4.5. Wastewater Analysis

Characterization result of the wastewater left after completion of tanning process indicates a significant decrement of COD and TDS for preservation of wetblue using Chlorogenic acid. The COD and TDS values of wastewater of leather made using commercial preservative was to be 4468±95mg/L and 948±23 mg/L respectively while of Chlorogenic acid extract from coffee husk was 2908±47mg/L and 890±18mg/L respectively. The result can be corelated with the COD test carried out with commercial preservative and extracted CGA. The COD result obtained was 14,000mg/L and 168000mg/L for extracted CGA and commercial preservative.

Table 9: Physical test result of Crust

S.NO	PROPERTY	TEST RESULTS		CLRI RECOMENDATIONS
		Control	Experiment	
1	Tear Strength, N	25.5±1.1	33.95±0.91	Min. 20
	Avg. Thickness, mm	0.83	0.94	
2	Tensile Strength, N/mm^2	12.55±0.21	15.9±1.9	Min. 10
	Elongation at Break, %	72.8	67.6	40-80
3	Lastometer			
	Load at grain crack, Kg	17.5	28.5	Min. 20
	Distension at grain crack, mm	12.8	12.1	Min. 7
4	Water vapor permeability, $mg/cm^2/h$	8.32	8.3	Upper – Min. 0.8
				Lining – Min. 2.0

From Table 12, it can be noticed that, the effect of using Chlorogenic acid extracted from coffee husk used for wet blue leather preservation has no effect on the strength property of leather. The difference in the figures of the tests was only due to thickness variation.

Chapter Five

Conclusions and Recommendations

5.1. Conclusions

The extraction and optimization result shows the highest yield obtained in extracting Chlorogenic acid from coffee husk using Soxhlet extract is 681mg/kg at operating temperature of 120^{o}C, Methanol solvent, extraction time of 4 hours and solvent to sample ratio of 10:1.

The active component of the extract from coffee husk has potential capacity of blocking the growth of microorganisms especially fungi in different mechanisms. The tested microorganisms for susceptibility test has resulted significant sensitivity to fungal strain; *Aspergillus niger* and resulted about 34mm diameter of clearance and 62.5µg/mL of Minimum Inhibitory Concentration (MIC).

From the characterization of wastewater, it can be concluded that the wastewater examined using Chlorogenic acid for preservation, showed less COD and TSS than that of wet blue processed using commercial preservative indicating the decrement of load of the effluent treatment.

The wet blue preserved using the extracted chlorogenic acid was tested for it resistance to fungal attack and found to resist any attack till 15 day. The wet blues were processed to crusting and tested for it strength properties and found to have no change of those properties up on the use of chlorogenic acid for preservative purpose.

In conclusion, the results in this study showed that the coffee husk extract has a great anti-microbial potential that may be applied as preservative in leather manufacturing.

5.2. Recommendations

Depending on the results obtained in this thesis, available resources and technical feasibility, the following recommendations are suggested

- Further deep investigation to change coffee husk and coffee pulp into leather processing auxiliary is needed to completely utilize these wastes and add value on it. This will at large benefits the country by saving hard currency to import leather chemicals.
- Coffee husk is a major source of antimicrobial bio-preservative for wetblue leather and with its high price in the market, this project can be scaled-up to an industrial scale to consume the coffee husk and pulp produced in the country and in African Horn region by which the only use has been power source for cocking.
- The coffee husk used for this project was dried one. To get a vast knowledge about the yield of active component of coffee husk and the condition of coffee husk, it is recommended to further study the fresh coffee husk and pulp for its chlorogenic acid content.
- The time of extraction selected were 4 and 6 hours. The time below 4 hours has to be investigated to see the effects on CGA yield.
- Further study should be done on the availability of coffee husk and its cost required for the extraction of the active component for leather application.

Reference

1. Ethiopian leather industry development institute(LIDI), 2015, GTP2
2. J. Kanagaraj, V. John Sundar, C. Muralidharan), S. Sadulla, 2005, Alternatives to sodium chloride in prevention of skin protein degradation-a case study, Journal of Cleaner Production 13, pp. 825-831
3. Etinosa O. Igbinosa, Emmanuel E. Odjadjare, Vincent N. Chigor, Isoken H. Igbinosa, Alexander O. Emoghene, Fredrick O. Ekhaise, Nicholas O. Igiehon,1 and Omoruyi G. Idemudia, 2013, Toxicological Profile of Chlorophenols and Their Derivatives in the Environment: The Public Health Perspective, The Scientific World Journal Volume 2013, Article ID 460215, 11 pages
4. United States Environmental Protection Agency, 2006, Reregistration Eligibility Decision for 2-(Thiocyanomethylthio)- benzothiazole (TCMTB), EPA739-R-05-003
5. Woo Sang Sung and Dong Gun Lee, 2010, Antifungal action of Chlorogenic acid against pathogenic fungi, mediated by membrane disruption, Pure Appl. Chem., Volume 82, pp. 219–226
6. Gaurav Saxena, Ram Chandra, and Ram Naresh Bharagava, 2016, Environmental Pollution, Toxicity Profile and Treatment Approaches for Tannery Wastewater and Its Chemical Pollutants, Springer International Publishing Volume 240, pp. 31-69
7. Mohamed N. Abdallh, Walid S. Abdelhalim, Hisham S. Abdelhalim, 2016, Biological Treatment of Leather Tanneries Wastewater Effluent- Bench Scale Modeling, IJESC volume 6, pp. 2271-2286
8. Alois Orlita, 2004, Microbial biodeterioration of leather and its control: a review, International Biodeterioration & Biodegradation, pp. 157 – 163
9. M. K. Kaygusuz, N. O. Isık, 2013, Microorganisms isolated and antimicrobial treatments applied at different stages of leather processing, Antimicrobial Research, pp. 420-426
10. Dalton DL., 2008, New generation fungicide for the leather industry, http://www.leathermag.com
11. Tony Covington, 2009, Tanning Chemistry The Science of Leather, The Royal Society of Chemistry, Cambridge
12. K.T. Sarkar, 2012, Theory and practice of leather manufacturing 8th edition, N.A. Bishaka, Kolkata

13. Abubeker Yimer, 2014, Study on value addition of leathers from Ethiopian cow hides: strategy toward quality improvement, MSc Thesis- AAiT, AAU
14. Luis Z, Elton H, and David O, 2012, Fungal Growth on Wet blue: Methods to Measure Impact on Leather Quality, Buckman Laboratories International, Memphis, USA
15. Anderson H, 2000, The Adaptation of Various Bacteria to Growth in the Presence of Sodium Chloride, Journal of the Society of Leather Technologists and Chemists, Volume 24, pp. 215-217
16. Aslan E, Birbir M., 2011, Examination of Gram-positive Bacteria on Salt-pack Cured Hides, Journal of American Leather Chemists Association, Volume 106(12), pp. 372-380
17. Aslan E, Birbir M., 2012, Examination of Gram-negative Bacteria on Salt-pack Cured Hides, Journal of American Leather Chemists Association, Volume 107(4), pp.106-115
18. Birbir M, Ozyaral O, Johansson C, Ilgaz A., 1994, Mold Strains Isolated From Unfinished and Finished Leather Goods and Shoes, Journal of American Leather Chemists Association, Volume 89(1), pp.1-34
19. Maier RM, Pepper IL, Gerba CP, 2000, Environmental Microbiology, Academic press, London
20. Fontoura JT, Gutterres M., 2015, Damage of pickled hides, wet-blue leather and vegetable tanned leather due to biodeterioration, Journal of American Leather Chemists Association, Volume 110(5), pp. 138-144.
21. P. S. Murthy, M. M. Naidu, 2012, Sustainable management of coffee industry by-products and value addition-A review, Resources, Conservation and Recycling, Volume 66, pp. 45-58
22. International coffee organization, March 2015, Monthly Coffee Market Report, pp.1-5
23. Ebba Bondesson, 2015, A nutritional analysis on the by-product coffee husk and its potential utilization in food production-A literature study, Swedish University of Agricultural Science
24. International Coffee Organization, 2011-2012, Annual Review, pp. 1-12
25. Baiq Rien Handayani, 2009, Study and characterization of antibacterial compounds of Arabica coffee berry pulp, PHD Dissertation-Oklahoma State University, USA

26. Adriana S Franca and Leandro Soares Oliveira, 2015, An Overview of the Potential Uses for Coffee Husks, Research Gate
27. Abebe Ayelign and Kebba Sabally, 2013, Determination of Chlorogenic Acids (CGA) in Coffee Beans using HPLC, American Journal of Research Communication, Volume 1(2), pp. 78-91
28. Rohit Upadhyay & L. Jagan Mohan Rao, 2012, An Outlook on Chlorogenic Acids-Occurrence, Chemistry, Technology, and Biological Activities, Food Science and Nutrition, Volume 53:9, pp.968-984
29. Mullen W, Nemzer B, Stalmach A, Sidrah A, Combet E., 2013, The polyphenolic and hydroxycinnamate contents of whole coffee fruits from China, India and Mexico, Journal of Agricultural and Food Chemistry, Volume ISSN 0021, pp. 61-69
30. http://www.casabrasilcoffees.com/learn/anatomy-of-the-coffee-fruit-and-bean/
31. Habtamu L Didanna, 2014, A critical review on feed value of coffee waste for livestock feeding, World Journal of Biology and Biological Sciences Volume 2, pp. 072-086
32. A Farah and Carmen M Donangelo, 2006, Phenolic compounds in coffee, Brazil J. Plant Physiology, Volume 18, pp. 23-36
33. Magdalena J Skowron, Aleksandra Sentkowska, Krystyna Pyrzynsk, Maria Paz De Pena, 2015, Chlorogenic acids, caffeine content and antioxidant properties of green coffee extracts: influence of green coffee bean preparation, Euro Food Res. Techn., Volume 242, pp. 1403–1409
34. Ningjian Liang and David D. Kitts, 2016, Role of Chlorogenic Acids in Controlling Oxidative and Inflammatory Stress Conditions, Nutrients , Volume 8, pp. 16-20
35. A Farah, Mariana Monteiro, Carmen M. Donangelo, and Sophie Lafay, 2008, Chlorogenic Acids from Green Coffee Extract are Highly Bioavailable in Humans, The Journal of Nutrition, Volume 138, pp. 2309–2315
36. Shetty M, Subbannayya K, and Shivananda, 1994, Antibacterial activity of tea (*Camellia sinensis*) and coffee (*Coffee arabica*) with special reference to *Salmonella typhimurium*, J Common disease, Volume 26(3), pp. 147-50.
37. Ana A. Aalmeida, Adriana Farah, Daniela A. M. Silva, Elziaria A.Nnunan, and Beatriz A. Gloaria, 2006, Antibacterial Activity of Coffee Extracts and Selected Coffee Chemical Compounds against Enterobacteria, Jornal of Agriculture and Food Chemistry

38. Mazzafera P, 2002, Degradation of caffeine by microorganisms and potential use of decaffeinated coffee husk and pulp in animal breeding, Sci. Agric., Volume 59(4), pp. 815-21.
39. Nirmal kumar G, Yogesh S, Saravanakumar P, Dhayananth N1and Rameshbabu N G, 2014, Chlorogenic acid from coffee waste as a preservative against bread spoilage, International journal of engineering research and science and technology, Volume 3, No. 2, pp. 374-378
40. Horax R, Hettiarachchy N, Islam Shahidul , 2005, Total phenolic contents and phenolic acid constituents in 4 varieties of bitter melons (*Momordica charantia*) and antioxidant activities of their extracts, Journal of Food Science, Volume 70:2, pp. 75-80.
41. Prigent S, 2005, Interaction of phenolic compounds with globular proteins and their effect on food related functional properties, Disertation: Wageningen University, The Netherlands
42. Maillard JY, 2002, Bacterial target sites for biocide action, Journal of Applied Microbiology, Volume 90, pp. 16-27.
43. Shapiro S and Gugenheim B., 1995, The action of Thymol on oral bacteria, Oral Microbial and Immunology, Volume 10:24, pp. 1-6.
44. Burt S, 2007, Antibacterial activity of essential oils: potential applications in food, Doctoral Thesis, Universiteit Utrecht, The Netherland.
45. Sanchez JC, Alsina MA, Herrlein MK, Mestres C., 2007, Interaction between the antibacterial compound, oleoropein, and model membranes,. Colloid Polymer Sci., Volume 285: 13, pp. 51-60.
46. Lugtenberg B, Alphen LV, 1983, Molecular architecture and functioning of the outer membrane of Escherichia coli and other Gram-negative bacteria, Biochemical et Biophysica Acta. Volume 737, pp. 51-115.
47. Walsh SE, Maillard JY, Russell AD, Catrenich CE, Charbonneau DL, Bartolo RG., 2003, Activity and mechanism of action of selected biocidal agents on Gram positive and negative bacteria,. J App Microbiology, Volume 94:24, pp. 4-7.
48. Kaur G, and Singh RP, 2008, Antibacterial and membrane damaging activity of *Livistona chinensis* fruit extract, Food Chem. Toxicol., Volume 46, pp. 29-34.

49. Lambert RJW, Skandamis PN, Coote PJ, Nychas GJE., 2001, A study of the minimum inhibitory concentration and mode of action of oregano essential oil, thymol and carvacrol, J Applied Microbiology, Volume 91:4, pp. 53-62.
50. Li Y, Trush MA, 1994, Reactive oxygen-dependent DNA damage resulting from the oxidation of phenolic compounds by a copper-redox cycle mechanism, Canc Research, Volume 54:189, pp.5-8.
51. Pimia RP, Nohynek L, Alakomi HL, Cadentey KMO, 2005, Bioactive berry compounds-novel tool against human pathogens-Mini Review, Applied Microbiology Biotechnology, Volume 67, pp. 8-18.
52. James PN, 2007, Origin and detection of bacterial species associated with lettuce and salad vegetables, Dissertation-School of chemical sciences and engineering, The University of New South Wales
53. Cowan MM, 1999, Plant product as antimicrobial agents, Clinical Microbiology Rev., Volume 12(4), pp. 64-82.
54. Zhong J, Duan XW, Qu HX, Yang B, Chen YL, Ruenroengklin N, Jiang YM., 2008, Effects of various extraction conditions on phenolic contents and their antioxidant activities of litchi fruit pericarp, Acta. Hort., Volume 804:32, pp. 7-9.
55. Palma M, Pineiro Z, Barroso CG., 2001, Stability of phenolic compounds during extraction with superheated solvents, J Chrom A., Volume 921(2), pp. 69-74.
56. Shahidi F, Naczk M., 2004, Phenolics in food and neutraceuticals, CRC Press, Volume 9, pp. 2-4
57. Skerget M, Kotnik P, Hadolin M, Hras AR, Simonic M, Knez Z, 2005, Phenols, proanthocyanidins, flavones and flavonols in some plant materials and their antioxidant activities. Food Chemistry, Volume 89, pp.1-8.
58. Bros I, Soran ML, Briciu RD, Cobzac SC., 2009, HPTLC quantification of some flavonoids in extracts of *Satureja hortensis L*. obtained by use of different techniques, J Plan Chrom., Volume 22(1), pp.89-93
59. Kallithraka S, Viguera CG, Bridle P, Bakker J., 2009, Original paper survey of solvents for the extraction of grape seed phenolics, Phytochemical Analysis, Volume 6(5), pp.5-7.
60. Horax R, Hettiarachchy N, Islam Shahidul, 2005, Total phenolic contents and phenolic acid constituents in 4 varieties of bitter melons (*Momordica charantia*) and

antioxidant activities of their extracs, Journal of Food Science, Volume 70:2, pp. 75-80.

61. Baydar NG, Osman S, Ozkan G, Sema C., 2004, Determination of antibacterial effects and total phenolic contents of grape (*Vitis vinifera* L.) seed extracts. Int J Food Sci Technol., Volume 41(7), pp. 799-804.
62. Variyar PS, Ahmad R, Bhat R, Niyas Z, Sharma A., 2003, Flavoring components of raw monsooned Arabica coffee and their change during radiation processing, Journal of Agriculture and Food Chemistry, Volume 51(27), pp. 45-50.
63. Coronell MAR, Marnet N, Kolli VSK, Roussos S, Guyot S, Augur C., 2004, Characterization and estimation of proanthocyanidins and other phenolics in coffee pulp (*Coffea Arabica*) by Thyolisis High Performance Liquid Chromatography, Journal of Agriculture and Food Chemistry, Volume 52:134, pp. 4-9.
64. Markom M, Hasan M, Daud WRW, Singh H, Jahim JM., 2007, Extraction of hydrolysable tannins from *Phyllanthus niruri* Linn. Effects of solvents and extraction methods, Separat Purification Technology, Volume 52 (3):4, pp. 87-96.
65. Vermerris W, Nicholson R., 2006, Isolation and identification of phenolic compounds (a practical guide), In Phenolic compound biochemistry, Springer, The Netherlands, pp. 151-196.
66. Urakova IN, Pozharitskaya ON, Shikov AN, Kosman VM and Makarov VG., 2008, Comparison of high performance TLC and HPLC for separation and quantification of Chlorogenic acid in green coffee bean extracts, J. Science, Volume 31:2, pp. 37-41
67. Fiamegos YC, Nanos CG, Vervoort J, Stalikas CD., 2004, Analytical procedure for the in-vial derivatization-extraction of phenolic acids and flavonoids in methanolic and aqueous plant extracts followed by gas chromatography with mass-selective detection,. J Chrom A., Volume 1041(1-2), pp. 11-18.
68. Gong F, Liang YZ, Xu QS, Chau FT., 2004, .Gas chromatography–mass spectrometry and chemometric resolution applied to the determination of essential oils in *Cortex Cinnamomi*, J Chrom A., Volume 1041(1-2), pp. 11-18.
69. Amélia E. Terrile, Gustavo G. Marcheafave, Guilherme S. Oliveira, Miroslava Rakocevic, Roy E. Bruns and Ieda S. Scarminio, 2016, Chemometric Analysis of UV Characteristic Profile and Infrared Fingerprint Variations of Coffea arabica Green Beans under Different Space Management Treatments, J. Braz. Chem. Soc., Volume 27, No. 7, pp. 1254-1263.

70. Ana Paula Craig, Adriana S. Franca, Leandro S. Oliveira, 2011, Evaluation of the potential of FTIR and chemometrics for separation between defective and non-defective coffees, Food Chemistry, Volume 132 (2012), pp. 1368–1374.

Appendices

Appendix A: Gradient Elution time set for LCMS

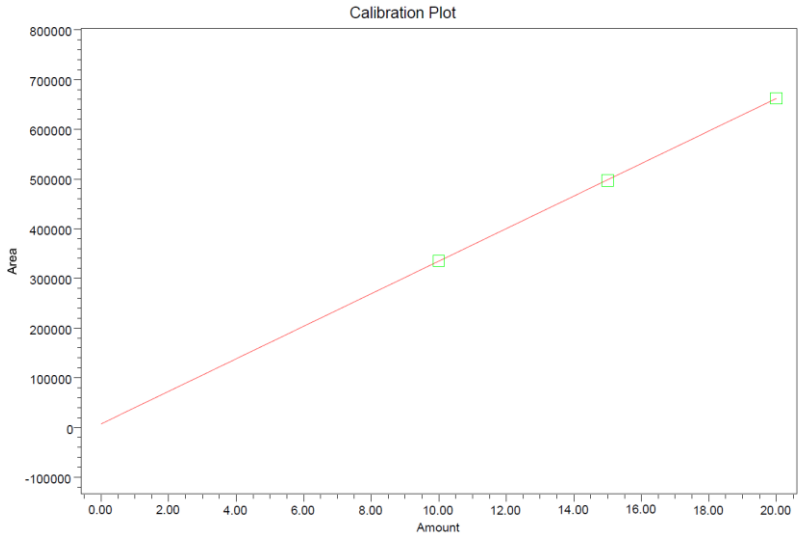
Time(min)	0	1.5	10	14	14.10	16
A	10	10	90	10	10	10
B	90	90	10	90	90	90

Appendix B*:* Calibration curve of HPLC

Standard solution of 10ppm, 15ppm and 20ppm was prepared to draw the calibration curve.

All calibration curves showed an r^2 of 0.999.

Name	**Level**	**X value**	**Response**	**Calc. Value**	**% Deviation**	**Manual**	**Ignore**
1 Chlorogenic Acid		10.000000	335148.575946	10.018504	0.185	No	No
2 Chlorogenic Acid		15.000000	496967.740709	14.962993	-0.247	No	No
3 Chlorogenic Acid		20.000000	662420.352158	20.018504	0.093	No	No



Declaration

I, the under signed, declare that this thesis is my own work and that all sources of material used for the thesis have been accordingly acknowledged.

_____ _____

Daniel Tesfaye Date

This is to certify that the above declaration made by the candidate is correct to the best of my Knowledge.

_____ _____

Advisor, Dr. Eng. Shegawu Ahmed (Ass. Professor)