ADDIS ABABA UNIVERSITY

ADDIS ABABA INSTITUTE OF TECHNOLOGY (AAiT)

SCHOOL OF CHEMICAL AND BIO ENGINEERING

IMPROVING EFFICIENCY OF VEGETABLE TANNIN FROM WATTLE BARK AND ITS COMPARISON WITH DIFFERENT CONVENTIONAL EXTRACTION METHODS

FANUEL TADESSE

ADVISORS: Dr. SHEGAW AHMED
Dr. KJ SREERAM

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This is to certify that the thesis prepared by Fanuel Tadesse, entitled: improving extraction efficiency of vegetable tannin from wattle bark and its comparison with different conventional extraction methods and submitted in partial fulfillment of the requirement for the Degree of Master of Science (chemical and Bio Engineering) complies with the regulation of the university and meets the accepted standards concerning to originality and quality.

Signed by the examining committee:

	Signatures	Date
Advisors:		
Dr. Shegaw Ahmed	_____	_____
Dr. KJ Sreeram	_____	_____
Internal examiner:		
Dr. Anwadh J.	_____	_____
External examiner:		
Dr. Kumsai D.	_____	_____

DECLARATION

I declare that this thesis, titled "improving extraction efficiency of vegetable tannin from wattle bark and its comparison with different conventional extraction methods," has not been submitted in any form to just about any University or other tertiary education institution for another degree, diploma, or award. When other people's contributions are involved, every attempt is taken to make this explicit, including appropriate references to literature and discussions. Information from others' published and unpublished work has been mentioned in the text, and a list of references is included. The work was under the guidance of Dr.KJ Sreeram senior researcher in Central Leather Research Institute (India) and Dr.Shegaw Ahmed, Assistant Professor of Chemical Engineering School of Chemical and Bio Engineering, Addis Ababa University.

Signature: - …… Date: - ……

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ABSTRACT

Conventional extraction processes affect our environment and consume a huge amount of solvent and these problems have stimulated considerable interest in the development of environmentally friendly extraction methods, which are prepared by enzyme and microwave pre-treatment methods. The objective of this research is to improve the extraction efficiency of vegetable tannin and compare it to other extraction methods. Extraction pre-treated with Microwave pre-treated was found to be easier and time effective than other extraction. But the percent yield relative to enzyme pre-treated extraction methods is small. The extraction of wattle bark was also performed by enzyme pre-treatment at 48°C for 60 minutes using 2% of concentration. Enzyme pre-treatment extraction method is best method among all the extraction methods which been 64.585 % yield obtained which is (30.52%) much higher than the conventional extraction methods. The enzyme pre-treated wattle powder was characterized using dynamic light scattering (DLS) and a spectrophotometer. DLS determined the average particle size to be 167.0 nm, with a poly disparity index of 0.0369. Color the extract was measured using a Minolta CR400 Chroma meter. The spectrophotometer result shows that a higher breakdown of the desired product and increased extraction efficiency that the total phenolic contents in 2% of the enzyme were the highest (78.1669) mg GAE/g dry extract. The total phenolic content of the control was 55.6286, which was much lower than the enzymatic pre-treatment method. The tannins were applied to leather after characterizing the tannin extract. it is observed that the strength characteristics like tensile strength, % of elongation at break, tear load (double edge tear), ball burst (distention at a crack and burst, and load at burst) of the crust leather produced by wattle powder as re-tanning material is found to be good when compared to the conventional re-tanning process. Finally, study organoleptic properties in comparison to locally used commercial tannins were studied and a good result was achieved.

***Keywords**: Microwave, conventional, extraction, TPC, Enzyme, DLS, Tear, organoleptic, chroma*

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List of abbreviations

ANOVA- Analysis of variance

DLS- Dynamic Light Scattering

TPC- total phenolic content

TS- Total solid

Uv-vis spec- Ultraviolet-visible spectrophotometer

ml- milliliter

mm-millimeter

L-liter

g- Gram

GAE- gallic acid equivalent

V- Volume of the filtrate

C*- chroma

L*- lightness

a* -redness

b* -yellowness

E-Weight of empty crucible

F-Weight of fuel

S-Solid content

1. INTRODUCTION

1.1 Background

Leather manufacturing is a much older manufacturing sector that produces items such as leather boots, purses, and clothing, among other things. The raw material utilized in the leather industry comes from food manufacturing waste, specifically from meat processing. Natural tannins found in different parts of trees are known as vegetable tannins (e.g. bark, wood, pods, etc.). Leathers made from natural vegetable extracts, like all-naturals, have their own. Vegetable-tanned leathers do not remain the same throughout their lives, but they do make things better. When the leather is brand new, it has a vivid hue, a warm touch, and a pleasant feel. Leathers tanned with these extracts alter slightly in appearance with wear and aging, becoming less bright but maintaining a warm and velvety feel. In today's tanning process, liquid and powdered vegetable tannins are employed (Li, B. (2006).

Solvent-based tannin extraction produces low yields, takes too long to extract, and the end product sometimes contains traces of organic solvents, decreasing product quality (Yang, B. (2011). As a result, it's critical to create a method for extracting bioactive compounds that is both effective and selective. Cold pressing, supercritical fluid extraction, and solvent extraction are all methods used to extract tannin from plants. However, applying organic solvents to recover natural goods has a variety of drawbacks, including safety concerns, high energy consumption, poor product quality, pollution to the environment, and toxicological consequences (Yang, C. and Yu, L. (2010).

It is necessary to create effective and comprehensive approaches for enhanced tannin recovery, especially from plants where the cell wall can limit extraction efficiency. Enzymes could be a viable alternative to existing solvent extraction processes for extracting tannin components from plants. Enzymes are excellent catalysts for extracting, modifying, or synthesizing complex tannin compounds from natural sources. The natural capacity of enzymes to catalyze reactions with high specificity, region selectivity, and the ability to act in an aqueous solution under mild processing conditions is used in enzyme-based extraction (Gardossi, L. (2009). Enzyme-assisted extraction methods are gaining importance as a result of the need for ecologically friendly extraction technologies. A quantitative aspect of enzymatic processing in the industry is that just a few

enzyme applications are represented in the literature. Enzymes can improve the effect of solvent pre-treatment, lowering the amount of solvent needed for the extraction or increasing the yield of extractable compounds. Enzymes such as pectinases, cellulases, and hemicellulases are often employed in juice processing and beer clarity to dissolve cell walls and enhance juice extractability. Breaking the cell wall matrix releases components into the juice, such as phenolic compounds, which improve duct quality (Xueqin, X. and Xiuzhen, Q. (2005).

Using enzyme-assisted extraction methods, high extraction yields for polysaccharides, oils, natural colors, tastes, and medicinal compounds have been reported (Wu, Y. (2005). Recent investigations on enzyme-assisted extraction have shown faster extraction, higher recovery, reduced solvent usage, and lower energy consumption when compared to non-enzymatic techniques. In this work, we compare the overall energy consumption of systems that use enzymatic processing to systems that use standard chemical processing, as well as a brief discussion of quantitative enzyme application screening. We give a brief overview of enzyme-assisted extraction of tannin components from plants and describe recent developments in this field, with a focus on stevioside (Chitra, V. (2010).

Because it requires more pressure and temperature than other methods, autoclaving has been demonstrated to extract tannic chemicals more efficiently than other procedures, resulting in a larger extraction yield. To put it another way, using an autoclave to apply pressure at a high temperature can increase the contact between the solvent and the raw material, resulting in a greater extraction yield. The extraction efficiency of wattle tannin from plant material can be improved by microwave pretreatment. The water present within the cells of the vegetable material evaporates during the microwave pretreatment, creating a strong pressure against the cell walls. The cell walls are broken by the high pressure, making it easier for chemicals to migrate from plant cells. Wattle tannins have an average molecular mass of 1250 units and have strong tanning action (Antonio, P. (2008)

Improving Extraction Efficiency of Vegetable Tannin from Wattle Bark and Its Comparison With Different Conventional Extraction Methods

1.2 Problem statement

Conventional extraction processes are the most popular and classic extraction methods. They usually use organic solvents and require a large volume of solvents and a long extraction time. In addition to this, when disposed of in the environment, they are not environmentally friendly and are not easily biodegradable. Today's challenges, particularly in the leather sector, include reducing the amount of solvent utilized and rising solvent costs. Finding efficient, cost-effective, and environmentally friendly extraction processes is a concern.

Additionally, the major component of commercial hexane, n-hexane, is ranked No. 1 on the US Environmental Protection Agency's list of 189 harmful air pollutants, prompting widespread condemnation of traditional soxhlet extraction procedures. Lweller, Wang (2006). Conventional extraction methods have the following drawbacks a huge amount of heat energy to complete the process. Because the technique takes so long, it needs more labor-intensive steps and minimizes the number of samples that can be handled. Along with the above disadvantages, the evaporation and concentration of the extract consume a large number of organic solvents, causing environmental risks. A viable solution to overcome these problems we will design pre-treatment procedures using enzymes, and microwaves. The application of the Microwave pretreatment method provides many advantages, such as increasing the extract yield, decreasing the thermal degradation, and selective heating of vegetal material. The microwave pretreatment method is also regarded as a green technology because it reduces the usage of organic solvents.

1.2. Objective of the study

1.2.1 General objective

The general objective of this study was to improve the extraction efficiency of vegetable tannin from wattle bark and its comparison with different conventional extraction methods.

1.2.2 Specific objectives

- ✓ To prepare tannin from wattle bark
- ✓ To extract tannin from wattle bark by enzyme pretreatment and microwave
- ✓ To characterize the extracted tannin materials
- ✓ To study the optimum conditions of enzyme and microwave pretreatment parameters

- ✓ To develop a post tanning process using extracted tannin
- ✓ To characterize the physical properties of experimental leathers and compare them with conventional leathers

1.3. Significance of the Study

This research, once completed, can be used in a variety of ways to ultimately benefit the environment. Because this design extraction process was so successful, it will help to solve the socio-economic issues that come with it. Additionally, traditional extraction methods take a long time to accomplish, need a large amount of solvent, and occur at the boiling point of the solvent for an extended length of time but, by using this project save every significant amount of money and time, achieve moderately high recoveries, minimize sample manipulation during the extraction process, reduce consumption and energy consumption.

2. LITERATURE REVIEW

Tannins are phenolic compounds with molecular weights ranging from 500 to over 3000 Da and up to 20,000 Da that is often found in plants. Tannin has an extremely complex and unique chemical structure. Approximately 8000 different tannins have been found. Tannins have been found in both free and bound forms in plant cells. The four primary tannin families found in terrestrial plants are gallotannins, ellagitannins, proanthocyanidins (condensed tannins), and complex tannins. Sea plants have been shown to contain phlorotannins, which are oligomers or polymers of phloroglucinol. Tannin content varies between 0.2 and 25% DW, depending on plant species, harvest period, plant habitat, and extraction process **(**Margarete D, Valerie M, 2018).

Tannins are amorphous, astringent compounds found in abundance in plant bark, wood, leaves, and resinous exudations. They are phenolic chemicals that are water-soluble and found in a wide range of vascular plants (J. S. Martin 1983). Sequim used the word in 1796 to describe the chemicals found in a variety of vegetable extracts that can turn animal hides into leather. The majority of authors prefer to use the term "tannin extracts" instead of "tannin." (1965, T. Swain). Tannins are polymeric phenolic compounds with a variety of chemical structures and many hydroxyl groups. Some tannins hydrolyze to produce the simple seven-carbon Gallic acid, whereas others create ellagic acid or other phenolic acids. Tannins are split into two types: hydrolyzable and condensed tannins (T. Okuda and H. Ito, 2011).

Condensed tannins have been reported to have a molecular weight of up to 20,000. Hydrolyzable tannins have molecular weights ranging from 500 to 5,000. Tannins are phenolic chemicals found in plants that have molecular weights ranging from 500 to over 3000 Da and as high as 20,000 Da (J. S. Martin and M. M. Martin, "1982). Condensed tannins are flavan-3-ol oligomers or polymers linked by carbon-carbon bonds, which are commonly generated by linking the C–4 of one catechin to the C–8 of the next, while C–4 to C–6 linkages are also conceivable but less common. Tannins polymerize through catechin condensation; when the number of condensing units is between two and ten, they are called flavolans. The complexity of the starting molecule is increased by additional processes such as hydroxylation, methylation, glycosylation, or galloylation. They can be divided into subgroups based on the chemical structure and processes that occurred in the

substance. Procyanidins, for example, are molecules with hydroxyl groups at positions 30, 30, 40, 50, and 70, and are differentiated from prodelphinidins, which have an extra hydroxyl at position 5'.(Khanbabaee 2001)

Medicinal plants are typically made by decoction the plant material, extracting the dried plant in boiling water. Because plant tannins can retain their original structure throughout the drying process, hydrolysis occurs during decoction, as in the particular instance of geraniin, which produces corilagin, ellagic acid, and brevifolin carboxylic acid. (Okuda and Ito, 2011). Traditionally, solvents such as ethanol, methanol, acetone, or aqueous mixes of these solvents are used to extract tannins from plants. To remove lipophilic contaminants, the plant material is treated with petroleum ether or dichloromethane before starting the extraction. Because they are low-molecular-weight molecules, proanthocyanidins with a low degree of polymerization or simple Gallic acid esters can be extracted easily using ethyl acetate. (Serrano, 2009). The traditional method of extracting plant secondary metabolites has a long history; however, microwave pretreatment and enzyme pretreatment are modern innovations to the area. The use of enzymes and microwave pretreatment for the extraction of polyphenols and tannins from vegetables has generated a lot of research interest. Both enzyme and microwave pretreatment proved to be more advantageous than traditional methods since they require less time for extraction, use less solvent, and produce a higher yield. The efficiency of microwave pretreatment is attributed to the mechanical action of microwave treatment, which heats the solvent mixture directly (Al-Harahsheh, (2004).

Tannins are currently used in a variety of industries, including medicine, food, beverage, ink and glue manufacturing, the dye and tanning business, plastic resins, water purification, and surface coatings. Their applicability as a complexing agent or a precipitating agent is dependent on their concentration (Antonio P.2008). Because of the aforementioned issues, it is necessary to extract and utilize tannins effectively. As a result, this study focuses on tannin extraction methods such as microwave, enzyme, and autoclave pretreatment. The project also discusses the benefits and drawbacks of tannin extraction.

2.1 Enzyme Pre-treatment extraction method

Enzyme pretreatment extraction is an environmentally friendly process that involves disrupting the material cell with an enzyme or a complex enzyme to boost bioactive extraction yield. This method combines several techniques, but it does not employ high temperatures to extract tannins since protein precipitates at high temperatures. Extraction time or enzyme treatment time should be sufficient, not excessive because excessive time causes tannins to be released and protein-tannin complexes to form. Naturally, not all tannins precipitate all proteins; this is selective precipitation. Only proteins and tannins with a large molecular weight, an open flexible structure, and hydrophobic proteins with a high proline content form tannin-protein complexes (Hagerman AE, Buttler LG, 1981).

Before tannin extraction, the material cell should be treated with an enzyme complex to improve extraction efficacy. Enzymatic pretreatment shortens extraction time and lowers energy usage (Puri M, Sharma, 2012). In comparison to other enzymes, cellulose enzyme has high efficiency in extracting tannin from seaweed at various temperatures. Enzyme-assisted extraction allows for the extraction of tannins that are both stable and active. Because catalytic reactions and region selectivity of enzymes can take place in an aqueous medium under mild conditions, this is completely feasible (Meyer AS, 2010). The efficiency of tannin extraction is mostly determined by ionic strength, ions in solution, and protein and tannin characteristics. Before large-scale production, some technological issues must be considered, such as enzyme price, hydrolyze capacity, and enzyme working conditions (Puri M, Sharma, 2012).

2.1.1 Factors affecting enzyme pre-treatment extraction method

2.1.1.2 Enzyme concentration

In general, as the enzyme concentration rises, it interacts more with the substrate, causing the peptide bonds to break down. Increased enzyme concentration until substrate active sites are saturated results in increased degradation of the desired product and improved recovery. Additionally, increase beyond saturation levels the material extraction process (Rhee KC, Cater CM, Mattil KF (1972). According to James et al. (2005), increasing enzyme concentration has a stronger effect on reducing hydrolysis time than increasing temperature. Thus, the effect of cellulase on wattle bark extract was first investigated using enzyme doses of 1, 1.5, 2.5, and 3%.

The amount of tannin extracted rose as the cellulases concentration increased. When comparing 1.5 percent and 2 percent cellulases concentrations, tannin yield was significantly higher at 2 percent. Because some of the peptides released were more hydrolyzed as the cellulases concentration increased, the result obtained could be due to an increase in amino acids and smaller peptides contained in the hydrolysate. After a fast initial phase of extraction, the rate of extraction will tend to slow down, resulting in a stationary phase. Increasing the enzyme concentration may not result in a higher degree of extraction at this point because the concentration of peptide bonds exposed for hydrolysis may become the limiting factor. Bhaskar et al. (2008) discovered that when enzyme levels exceed 2%, tannin yield enters a stationary phase.

2.1.1.3 pH factor

The pH factor has a significant impact on the efficiency of enzyme tannin extraction. Extraction efficiency can be maximized at optimum pH because each enzyme has its optimum value. The extraction must take place at a distance from the isoelectric point. The extraction process may be hampered if the protein is insoluble at a specific isoelectric point of an enzyme. According to Yu and Ahmad (1998), in many enzyme-catalyzed reactions, normal pH can cause changes in the reactants, including possible denaturation of the enzyme's protein structure or disturbance of the ionic character of the substrates, which can affect the substrate's ability to bind to the enzyme. When treated with acidic cellulase at pH 4.5–5.0, extraction efficiency was generally higher (Cater CM, Rhee KC, Hagenrnaier RO, Mattil KF (1974).

2.1.1.4 Temperature

Temperature is another key aspect to consider when it comes to optimizing enzyme activity. When the temperature was raised from 30°C to 50°C, the extraction efficiency significantly improved. However, when the temperature was raised to 60°C, the tannin yield significantly dropped. According to Novikov (2000), rate extraction increased as temperature increased for most reactions. Nielsen (1995) further said that the activity of an enzyme can be reduced by thermal denaturation at higher temperatures, resulting in a decline in extraction efficiency.

2.1.1.5 Particle size

In general, smaller particles produce more, but when exposed to solvents, they lose their micro porosity, which can lead to lower extraction due to non-uniform distribution. Different particle

sizes, for example, improved extraction performance, whereas the same substrate demonstrated insufficient wattle recovery because of enzyme access issues. (Zhang, 2007). Different authors reported the particle sizes between 0.3 nm to 1000 nm ranges. (Zhou, 2012).

2.1.1.6 Effect of liquid to solid ratio

Diffusion, enzyme mobility, and hydrolytic reactions are all dependent on moisture concentration. The creation of a thick suspension occurs when a material has a low moisture content. As a result, the enzyme's function may be inhibited. If the substance has a high moisture content, however, it may dilute the enzyme and substrate concentrations, delaying the process. As a result, moisture content optimization is expected to achieve an intense enzymatic reaction on the target (Teixeira CB, 2013).

2.3 Microwave pre-treatment method

Microwaves are electromagnetic fields with wavelengths between 1 cm and 1 meter and frequencies between 300 MHz and 300 GHz. Microwave radiation is used to heat the solvent in contact with the sample during the microwave pre-treatment extraction technique. Microwave-induced dipole rotation of molecules happens when a hydrogen bond is broken and ions migrate, allowing the solvent to penetrate deeper into the matrix and the extraction of the components to be completed. The efficiency of tannin extraction is affected by both the depth of microwave penetration into plants and the power of microwaves. The dielectric constant, plant moisture content and temperature, and electrical field frequency, on the other hand, determine microwave penetration depth. Plant water absorbs microwave energy and becomes superheated, causing cell structural damage.

2.3.1 Factors affecting microwave pre-treatment extraction method

The choice of solvent, microwave application time, microwave power, uniqueness of the matrix, effect of contact sample surface area, and influence of temperature are all aspects to consider during the microwave pre-treatment extraction procedure (Chen et al., 2008).

2.3.1.1 Volume of solvent to material ratio

The solvent utilized has a big impact on the microwave pre-treatment extraction procedure. When choosing a solvent for an experimental environment, think about how solubility affects the targeted

analyte, the solvent-matrix interaction, and the solvent's potential to absorb microwaves. The solvent you select should have a high selectivity for the analyte and value you're looking for, as well as good compatibility with other chromatographic analytical steps. A solvent with a high microwave absorption capacity warms up much faster, leading to a faster extraction. A solvent with a high microwave absorption capacity warms up more quickly, allowing for quicker extraction. A solvent with a high microwave absorption capacity warms up faster, resulting in faster extraction. Researchers continue to use a combination of high and low microwave absorption solvents to achieve optimal extraction yields. (Chen et al., 2008). According to certain studies, tannin yields increased with increasing solvent-to-material ratios in the 25–35 ml/g range. However, there was no significant difference or enhancement in the range of 35 - 50 ml/g, which was probably due to the larger volume of acetone solution causing excessive swelling of the materials by water and absorbing the effective constituent (Guo et al., 2001) or that a higher concentration could cause proteins to coagulate and some lipid compounds to be extracted, resulting in higher diffusion resistance and, thus, limiting the extraction content.

2.3.1.2 Effect of Microwave application time

Another key factor that affects the extraction process of microwave pre-treatment is the heating time. In addition to increasing the amount of analyte removed, increasing the extraction time increases the risk of thermal labile components degrading. Different extraction times are necessary for different matrices, however, simply a few seconds of exposure has been shown to provide great efficiency. (Al-Harahsheh and Kingman, 2004). Zhe-xiong (2010) report shows that tannin extraction yields increased fast as irradiation periods increased from 30 to 250 seconds, maximum at 250 seconds. The extraction yields then declined when the irradiation time was increased. This could be caused to tannin breakdown over a long period of irradiation

2.3.1.3 Effect of Microwave-power

Irradiation times and microwave power have a substantial impact on each other. A combination of low or moderate power and a prolonged exposure technique is commonly utilized to maximize the microwave pre-treatment extraction process. Even if there is a risk of thermal degradation connected with the usage of a higher power. The matrix properties, such as particle size and material nature, will have a significant impact on the compound recoveries. (Raner et al., 1993).

3. MATERIAL AND METHOD

3.1. Materials, chemicals, and equipment

3.1.1 Materials

The raw material used for the experimental work was wattle bark species. The samples were collected from a central leather research institution. Wattle bark was crushed to achieve a uniform powder for extraction using a crusher. The crushed powder was sieved using a 40-60 mesh sieve before being selected for extraction.

3.1.2. Chemicals and reagents

The major chemicals and reagents used in the experiment are Folin–Ciocâlteu reagent (1:1) ($C_{10}H_5NaO_5S$), Sodium carbonate (Na_2CO_3), Gallic acid ($C_7H_6O_5$), and Acidic cellulase enzyme ($C_{18}H_{32}O_{16}$). (All chemicals used in the experiment were analytical grade).

Source of enzyme

Microorganisms from fungi create the enzyme cellulases (Aspergillus). Cellulase generated by Aspergillus niger cultivated was isolated, purified, and characterized. As a carbon source, Aspergillus niger produced crude cellulase enzyme using a submerged fermentation process. Purification of cellulase was also conducted using ammonium sulfate precipitation and column chromatography. It was used to produce a high-yield biosynthesis of a high-activity preparation.

3.1.3. Equipment and instruments

This study's experiments were carried out with a variety of equipment and devices: beaker, stirrer, filter paper, crucible, crusher, hot air oven, hot plate magnetic stirrer, rotary evaporator, testing drum, FT-IR HPLC chromatogram, and meter, Oven, Centrifuge, Refrigerator, and Particle Size Analyzer.

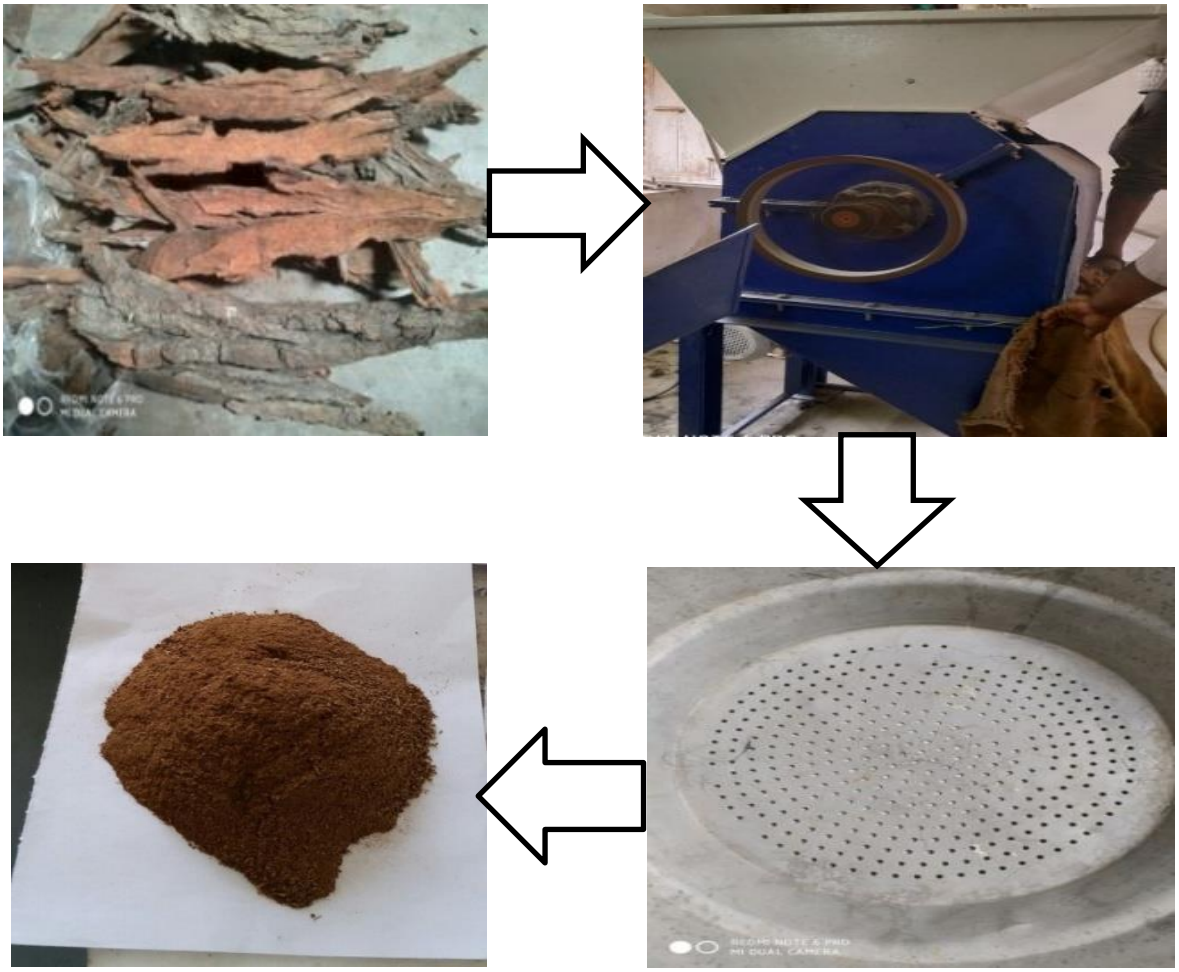

Figure 3. 1 Raw material preparation

3.2 Enzyme pre-treatment extraction methodology

A modern and environmentally acceptable technology was used to extract tannin from wattle bark. Nine separate samples of 10g crushed wattle bark powder and 150ml water was made with varying amounts of enzyme: 1%, 2%, 2.5 %3 %, and 150ml without enzyme. The powder was pre-treated with an enzyme in the beaker for five minutes at room temperature to improve the extraction yield, and it was continuously stirred to achieve a homogenized mixture. The mixture was placed in a mechanical shaker for four hours at a speed of 65 RPM to break down and all contents in the reaction mixture. Finally, the filter paper was used to filter the liquid, which was then placed in a hot air oven to evaporate the liquid. The resulting powder was then used to assess extraction efficiency as well as total phenolic content and particle size.

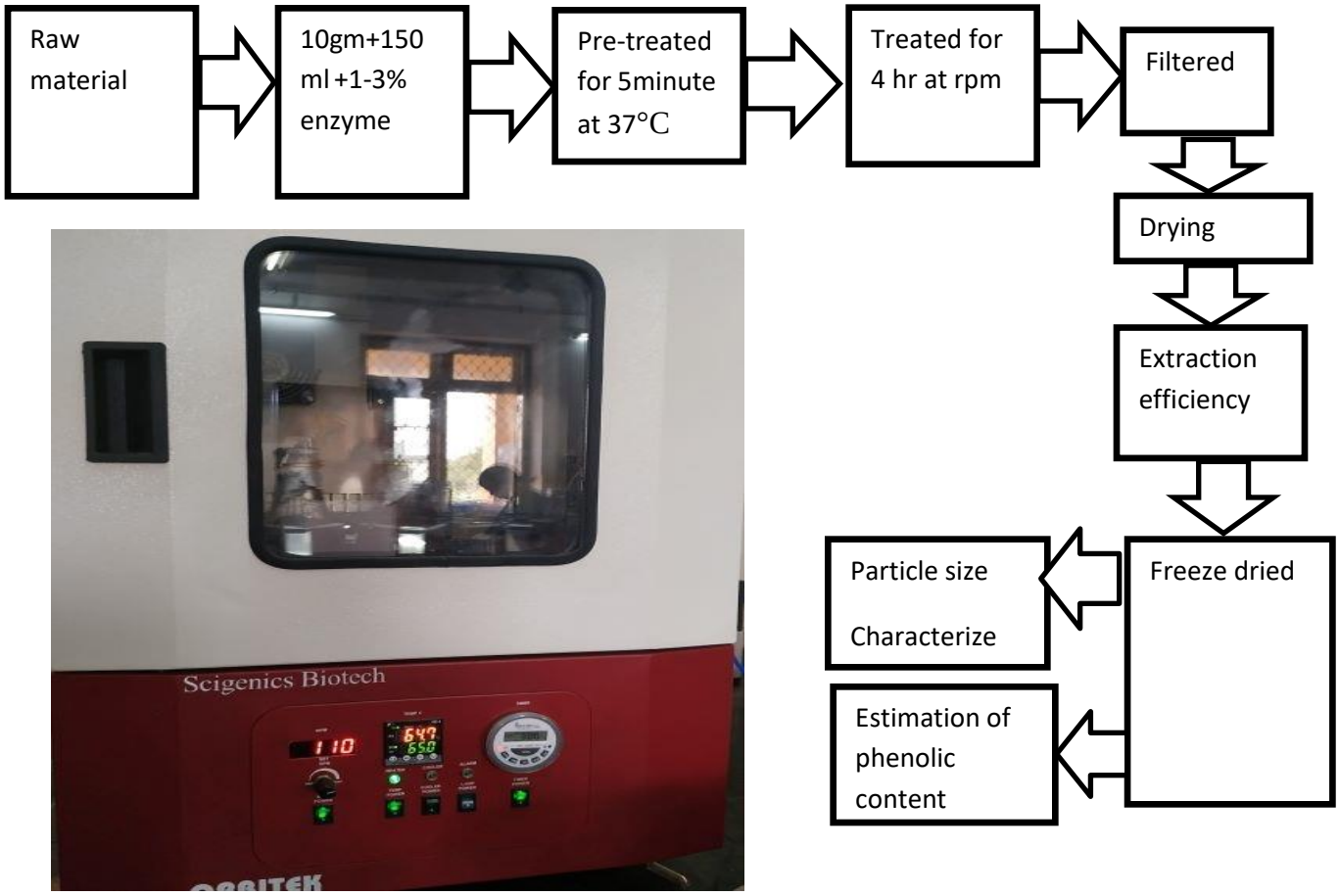

Figure 3. 2 General flow diagram of enzyme pre-treated extraction method

3.3 Microwave pre-treatment extraction methodology

Wattle bark powder was also extracted using the microwave pre-treatment extraction method. Eighteen separate samples of each 10gm crushed wattle bark powder and 150ml of water were made using the microwave pre-treatment procedure. The powder was pre-treated with three different microwave powers (110W, 330W, 550W, and 700W) for 0, 60, 120, 240, 480, and 560 seconds of microwave application time. The combination was then placed in a mechanical shaker at 65 RPM for 4 hours to extract the mixture. Finally, the filter paper was used to filter the liquid, which was then placed in a hot air oven to evaporate the liquid. The resulting powder was then used to assess extraction efficiency as well as total phenolic content and particle size.

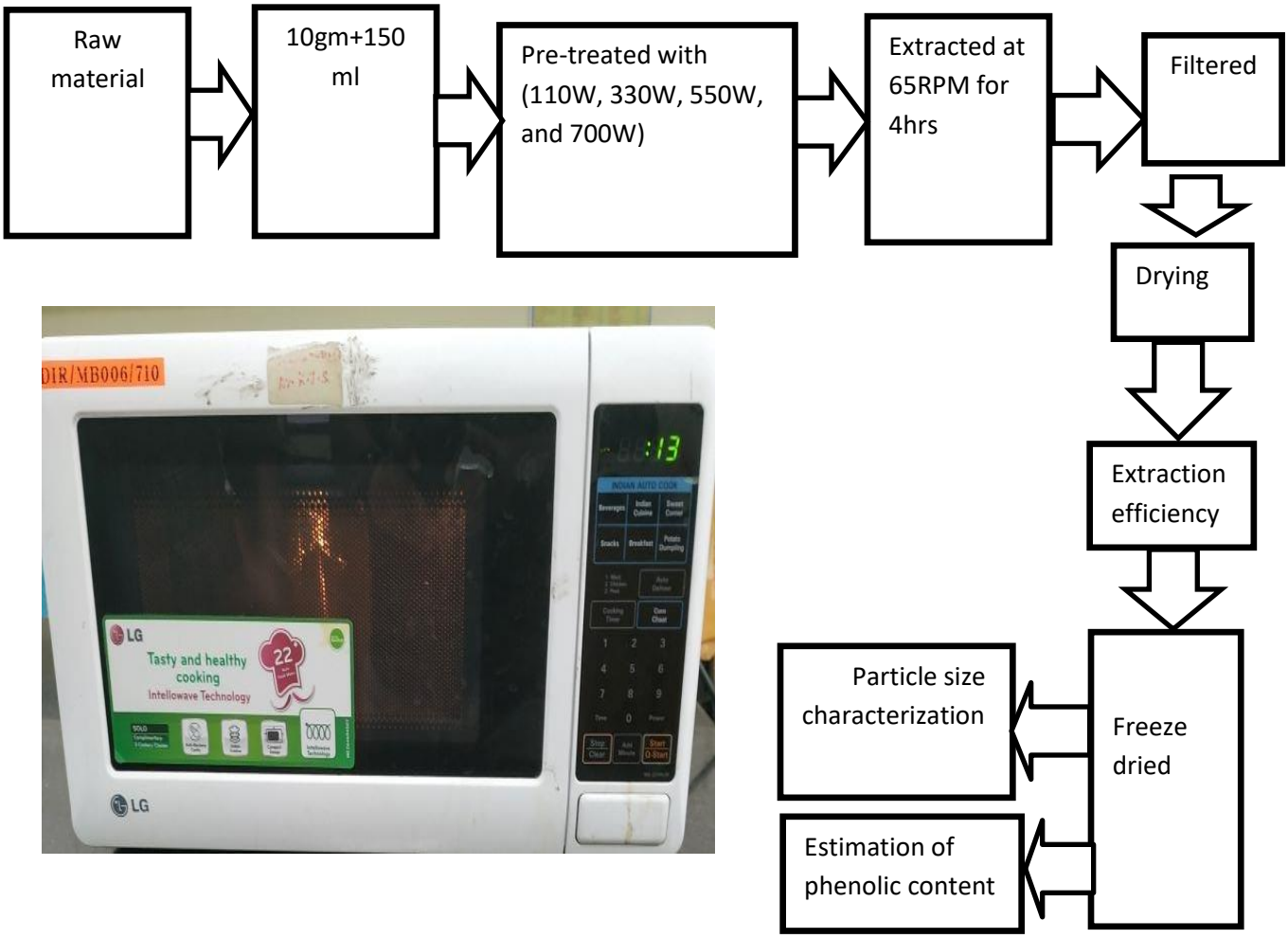

Figure 3. 3 General flow diagram of microwave pre-treatment extraction method

3.3.1Conventional extraction methodology (using soxhlet apparatus)

The extraction of tannin from wattle bark was also carried out by soxhlet extraction methods. In the case of Soxhlet, wattle bark powder of 10gms was taken and packed into a priorly weighed thimble. This thimble was then inserted into the soxhlet apparatus. In a 250mL round bottom flask, 120ml of methylene chloride will be taken and the system was set up to a condenser along with soxhlet and placed onto a heating mantel. The temperature was maintained constant throughout the reaction at the distillable range of solvents. The same steps are repeatedly maintained for other solvents. The solvent slowly gets evaporated and certainly fills the soxhlet where the thimble was

packed. The solvent-soluble part in the thimble is contained by the solvent and returns to the round bottom flask. Sequentially, the extraction process was prolonged to give colorless solvent in the soxhlet. This was identified as the endpoint of extraction. The system was removed and liquor was collected and the solvent was distilled off from the liquor, powdered tannin was then collected.

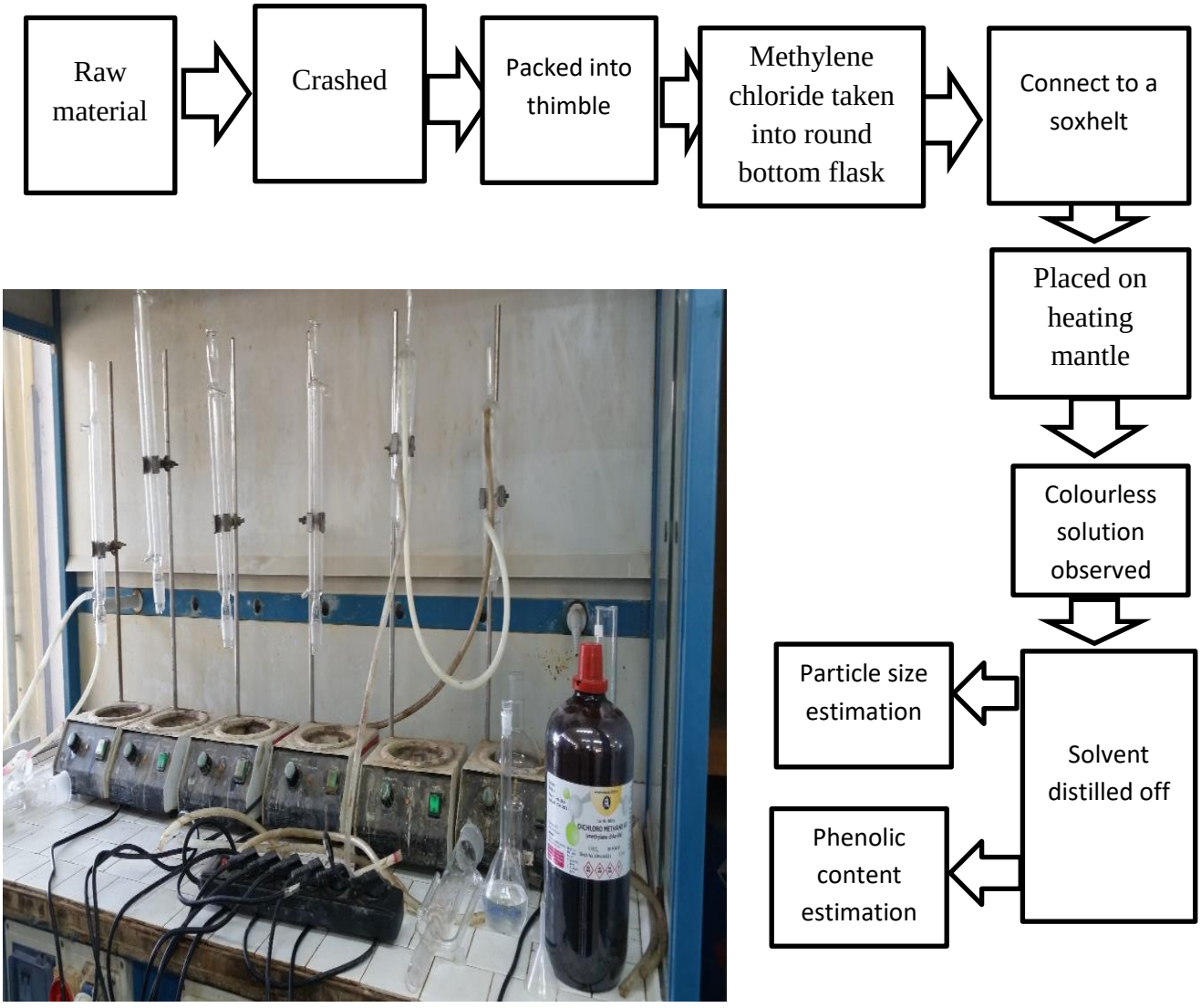

Figure 3.2 Conventional extraction methodology (using soxhlet apparatus

3.4 Characterization of tannin extract

3.4.1 Estimation of particle size

Temperature, time, solvent type, and particle size are some of the extraction characteristics that influence extraction efficiency. The extraction effectiveness of the method is most affected by the size of the sample particles and the temperature. Mass transfer kinetics, as well as solvent (water) access to soluble components, can be affected and controlled by particle size. It's also one of the most thoroughly researched criteria, as it influences the extraction rate, diffusion rate, and extractability.

3.4.2 Procedure for calculating extraction efficiency

After weighing the empty crucible and adding 10gm of sample powder, the crucible was dried in a water bath. Measure the specimen weight and then the solid content after it has dried and cooled.

The volume of the filtrate (V)

Weight of empty crucible (E)

Specimen weight (SW)

Weight of sample = 10gm

The solid content in % (s)

$$\%\ solid = \frac{\text{specimen weight} - \text{weight of empty crucible}}{10\text{gm}} = \frac{Sw - E}{10} * 100 \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots (1)$$

$$\frac{\text{The volume of filtrate (ml)} * \%\text{solid(gm)}}{100\text{ml}} = \text{X gm}$$

$$\text{Extraction efficiency (\%)} = \frac{\text{Xgm}}{10\text{gm of powder}} * 100 \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots (2)$$

3.4.3 Mechanical shakers

Mechanical barriers (cell walls) are broken down and all contents in the reaction mixture are uniformly mixed by shaking or agitation. Tannin extraction from wattle was done at 65rpm with agitation at 65 temperature.

3.4.4 Estimation of total phenolic content

Folin–Ciocalteu technique was used to calculate the total phenolic content. Extracts diluted in distilled water (0.6 mL) were combined with 0.2 mL Folin–Ciocalteu reagent and 1 mL Na2CO3 (0.8N) and kept for 30 minutes in the dark. A spectrophotometer was used to measure the absorbance at 760 nm. The total phenolic content was measured in milligrams per gram of GAE.

3.4.5 Conditions that affect pretreatment extraction method

Temperature, enzyme concentration, and extraction duration are all parameters that can alter the extraction process enzyme pretreatment (Rahimi, 2011). The process parameters were chosen and tuned to assure their effect on the work's reaction. Design experiment software (central composite Design) was used to investigate each factor at three levels (CCD). As a result, the tests were prepared and carried out utilizing three levels for each process parameter and a central composite type response surface design (Ranganath, 2015). The selected ranges of the parameters were duration time between 30 minutes and 120 minutes, reaction temperature between 35°C and 65°C, and enzyme concentration between 1% and 3% based on the literature. The analysis of variance (ANOVA) was used to determine whether the model was appropriate for the experiment's results. It's a statistical decision-making tool for identifying any disparities in the parameters' average performances. It calculates the relative relevance of the researched processing parameters, as well as measurement errors and uncontrolled parameters, using the sum of squares and F facts.

Table 3. 1 Experimental design matrix for central composite Designs

		Factors		
Std	Run	A: concentration (%)	B :time(minute)	Temperature(°C)
14	1	2	60	40
8	2	2	120	40
2	3	3	30	65
9	4	2	60	30

12	5	2	60	40
6	6	3	60	40
11	7	2	60	40
15	8	2	60	40
4	9	1	30	30
3	10	1	120	65
1	11	3	120	30
13	12	2	60	40
5	13	1	60	40
7	14	2	30	40
10	15	2	60	50

3.4.6 Conditions that affect microwave pretreatment extraction method

Temperature, solvent volume, and extraction duration are all parameters that can alter the extraction process microwave. The process parameters were chosen and tuned to assure their effect on the work's reaction. Design experiment software (Box-Behnken Design (BBD) was used to investigate each factor at three levels (*BBD*. As a result, the tests were prepared and carried out utilizing three levels for each process parameter and a Box-Behnken type response surface design (Ranganath, 2015). The selected ranges of the parameters were duration time between 60 seconds and 560 seconds, reaction temperature between 25°C and 35°C, and solvent of volume between 30ml and 40ml based on the literature. The analysis of variance (ANOVA) was used to determine whether the model was appropriate for the experiment's results. It's a statistical decision-making tool for identifying any disparities in the parameters' average performances. It calculates the relative relevance of the researched processing parameters, as well as measurement errors and uncontrolled parameters, using the sum of squares and F facts.

Table 3.2 *Experimental design matrix for Box-Behnken Designs*

Std	Run	A:solvent volume(ml)	B:extraction time(s)	C:extraction temperature(°C)
12	1	40	310	35
9	2	30	310	25
16	3	40	60	30
16	4	35	560	30
4	5	40	310	30
6	6	35	560	25
25	7	35	310	30
13	8	35	60	30
10	9	40	310	25
27	10	35	310	30
24	11	35	310	35
15	12	35	310	30
20	13	40	560	30
21	14	35	310	25
8	15	35	560	35
17	16	30	60	30
11	17	35	310	30

3.5 Tannin extracts for leather processing

Vegetable tanning is another often utilized method for commercial leather production. Tannins are astringent organic compounds originating from plants that can change raw hides/ skins into leather. Tanning extracts are extracted from wattle, quebracho, chestnut, gambier, myrobalan, and other plants and utilized in pits or drums for tanning.

The specific gravity of the tan liquor, which is determined with a hydrometer called a Barkometer, is the most critical control during vegetable tanning. Vegetable tanning normally begins with a

specific gravity of 1.005 (50 Bk) and gradually increases the intensity of the tan liquor as tanning progresses.

Post tanning or wet finishing operations are those that take place after tanning but before finishing, such as neutralization, dying, retaining, and fatliquoring. The main goals of this series of unit operations are to dye the leather to the desired color (dyeing), add softness (fatliquoring), and add fullness and uniformity of substance (fatliquoring) (retanning). These activities are critical because, in most cases, one may start with a wet blue and make a variety of completed leathers without knowing anything about the beam house or tanning operations that the leather has undergone.

3.5.1 Wet back

After adjusting the thickness of the wet blue leathers by splitting (bovine hides) and shaving, the first procedure in the drum is an acid wash. This is done to remove any loosely held or unfixed chrome and to correct the pH for re-chroming. An acid (usually acetic acid) and a fat and chrome dispersion agent are among the substances employed.

3.5.2 Neutralization

To achieve the desired final product, neutralization is a key process in the wet finishing process. This is the process of adjusting the charge characteristics of leathers by neutralizing the free acid present/liberated from chrome complexes due to olation during aging in chrome tanned leathers, and the extent of neutralization will depend on the neutralization is the softness of the final leather, and the extent of neutralization increases with the indentation.

3.5.3 Dyeing

Leathers are dyed to give them the color that the fashion industry demands. Dyeing is the process of completing various completed leathers, such as suede and nubuck leathers. Some leathers or buyers only want surface coloration, while others (glove leather, high-quality garment, and glaze kid) require not only through and through dyeing but also tone-in-tone (grain, cut, and flesh all in nearly the same shade), as well as tone-in-tone with finishing in the case of finished leathers.

3.5.4 Fatliquoring

Leathers are fatliquored to add softness, flexibility, feel, drape, and run, among other qualities. Fatliquoring additionally improves the strength qualities. In effect, fatliquoring aims to coat individual fibers or fiber bundles with a thin layer of oil to reduce internal friction and allow the fibers to slide over one another to achieve the desired softness and flexibility (depending on the extent of fiber opening achieved in beam house operations for different types of leathers).

The raw material was chosen to be wet blue sheepskins. Skins have a tightighter the current investigation, a sheep crust was prepared using the standard post-tanning procedure for upper leather up to the crust. From soaking back through post-tanning, everything was done according to the recipe below. All of the chemicals employed in the leather-processing procedure were of commercial quality. Wet blue was used for the post-tanning studies.

3.5.5 Fixing

Fixing leathers at the end of post tanning is vital. Fixation is obtained by lowering the pH, typically with formic acid.

- To fix the dyes and fatliquors permanently with the leather
- To avoid bleeding even in dry condition and rubbing

Then the leather is Piled and Overnight aged to drain out the water gradually, for proper fixing and distribution of chemicals offered, to avoid folds after unloading and conditioning for next dseting out. Foltructurelowing the recipe in the table below, post tanning was completed.

3.5.5.1 Procedures for leather processing

The raw material was chosen to be wet blue sheepskins. Skins have a tighter structure. For the current investigation, a sheep crust was prepared using the standard post-tanning procedure for upper leather up to the crust. The entire process, from wetting back through post-tanning, followed a standard recipe. All of the chemicals employed in the leather-processing procedure were of commercial grade. Wet blue was used for the post-tanning experiments. Two wet blue tanned sheep of the same breed were collected. One was utilized for experimentation and the other for conventional processing. The only difference between the conventional and experimental recipes

is that the experiment uses extracted wattle powder in the re-tanning stage. For the conventional preparation, Mimosa and Quebracho were utilized.

3.6. Physical Test of Leather

After the leather was made, it must be tested to see if it will fulfill the intended function. Because the qualities of leather are impacted by ambient temperature and humidity, and because they change depending on the season of the year and the hour of the day, it is necessary to condition the leather before testing in a controlled environment. The Indian Standard Specification specifies a temperature of 20°C and relative humidity of 60% R.H.2 (R.H. = relative humidity) during 48 hours. The ISO 2419 test method defines this conditioning procedure for leather. The conditioned samples are put through a series of tests to see what attributes they have. Tensile strength, grip strength, elongation at break, and ball burst were examined for resistance in parallel (//) and perpendicular directions (), with the samples, analyzed parallel and perpendicular to the dorsal line. The following are the principles involved in testing various qualities using Official Standards procedures.

3.6.1Tensile strength

According to the official procedure (IUP/6, 2001), the tensile strength was tested with Instron 1026. A 41 bell-shaped press knife was used to cut the samples parallel and perpendicular to the backbone. Each sample was tested three times. The jaws of the tensile machine (Instron 1026) were set 50 mm apart, and the sample was clamped in the jaws so that the edges of the rod were visible while the surface was monitored for cracking and bursting. The force and distention values recorded jaws along the midline at the place where the grain side of the leather split bursts. The machine was run until the specimen broke, and the breaking load was determined by the maximum load reached.

The tensile strength load is measured in Newtons. $Tensile\ strength(\frac{N}{mm2}) = \frac{\text{Force (N)}}{\text{Area (width}*\text{thickness(mm)}}$ Or $\frac{\text{Breaking Load (Kg)}}{\text{Thickness(cm)}*\text{width(cm)}}$ ..... (3)

3.6.2Tear strength

Tear strength is also an important bulk property test. This test is the most preferable test for leather than tensile strength by many of the customers including BS EN ISO 20345 Safety shoe standards. There are two types of tear strength tests are followed for leather material.

1. Double edge tear strength – Baumann Tear strength
2. Single edge tear strength - Tongue/trouser tear strength

Conduct the test by operating the tensile tester at the rate of 100 ± 10 mm/ minute speed until the test piece is torn apart.

$$Tear\ strength = \frac{\text{Maximum tear force (N)}}{\text{Thickness (mm)}} \quad \text{.......... (4)}$$

Tear strength is also expressed in metric (kgs) and imperial (lbf) units

3.6.3 Elongation at break

In many circumstances, the percentage elongation of leather is also a valuable indicator of Stretching quality. The elongation and tensile strength are both tested at the same time. Before testing, two reference markings are made in the narrow area of the specimen, and the distance between them is measured. The elongation of the sample is also measured at the same time as the tensile test. The percentage elongation at that load can be used to express the extension. (Roig, M., Segarra, 2012).

$$Elongation\ at\ break(\%) = \frac{\text{L2}-\text{LO}}{\text{LO}} * 100 \quad \text{.......... (5)}$$

Where: - L2 = Initial distance between the jaws in mm

Lo= final distance between the jaws in mm

3.6.4 Ball burst test

Tear strength (sometimes called tear resistance) is a measurement of a material's ability to withstand tearing. It is a unit of measurement that expresses a material's resistance to the growth of any cuts when under strain, and is expressed in N/mm (Nalyanya, K.M 2015). According to the official procedure (IUP/9, 2001), the ball burst test was measured with a Lastometer. Between the clamping rings, a disc-shaped piece of leather was tightly held with the grain side up, the

spherical point of the steel rod just brushing the flesh surface. While the surface was being watched for incipient cracking and bursting, the specimen was pressed downward against the rod, distending the grain of the leather directly above the rod. The force and distention values were measured at the spot where the grain side of the leather cracked and burst and the data were recorded.

3. 6 .5 Evaluation of Organoleptic Properties

A touch test was used to evaluate the softness, grain smoothness, fullness, and overall appearance of vegetable tanned leathers. For each functional attribute, experienced tanners scored the leathers on a scale of 0-10 points, with higher pointers suggesting better properties. Professional researchers and tanners from the Central Leather Research Institute's leather processing branch in Chennai, India, conducted this visual and tactile assessment.

Experts from CLRI model tanneries assessed the organoleptic qualities. The crust leather has been inspected by three tanners. For each functional property, the average of the ratings for the leathers used in the experiment was derived.

4. RESULTS AND DISCUSSION

4.1. Particle size distribution (DLS) of enzyme pretreatment wattle bark powder

DLS is a useful method for estimating the particle size distribution of Nanomaterials. A dynamic light scattering methodology was used to measure the powder derived from wattle bark. The particle size distribution of Wattle bark-derived powder is shown in Figures 4.1 and 4.2. The major peak was recorded with an average particle size of 539 (2.1 percent intensity), the second peak was recorded with an average particle size of 187.9nm (89.2 percent intensity), and the minor peak was recorded with an average particle size of 39.19 (8.7 percent intensity) with 0.369 PDI and 0.921 intercepts respectively with z-average 150.2 d. nm (see figure 4.1). This indicates that there is coagulation and non-uniformity of particle size in the suspension. And this variation in particle sizes prevailed due to the orientation of rod-shaped enzyme-treated extracted powder across the scattering light during DLS measurements. The 2% of the enzyme showed as z-average (d. nm) 167.0 nm and PDI of 0.369 with the particle size of 181nm for 100% accounting (fig. 4.1). The obtained result confirmed that the 2% of enzyme was a good concentration to have a maximum extraction yield.

Table 4. 1 Particle size of wattle bark at 2% of enzyme concentration

Z-Average (dnm):167.0		Size (dnm)	% volume	% intensity
PDI: 0.0369	Peak 1	181.0	100.0	100.0
Result quality: good	Peak 2	0.000	0.000	0.000
	Peak 3	0.000	0.000	0.000

Table 4. 2 Particle size of a conventional soxhlet sample

Z-Average (dnm):150.2		Size (dnm)	% intensity	% St Dev(dnm)
PDI: 0.466	Peak 1	539	2.100	311.2
Intercept: 0.921	Peak 2	187.9	89.20	67.20
Result quality: poor	Peak 3	39.19	8.700	8.068

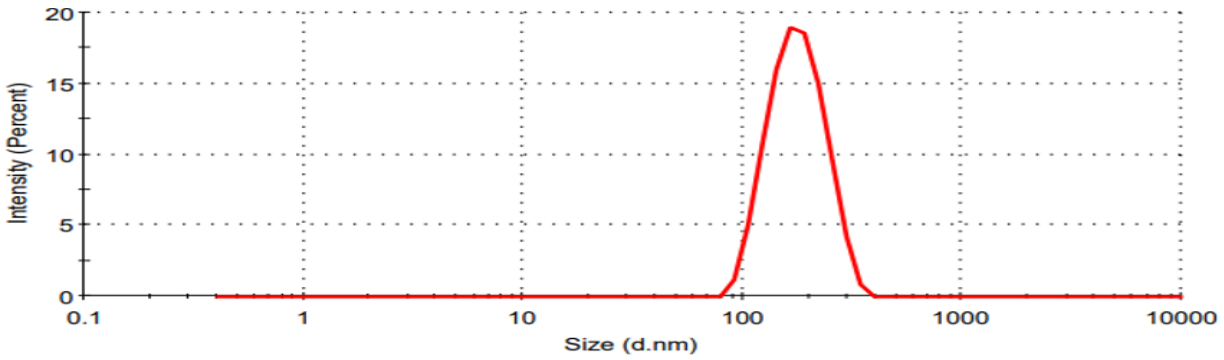

Figure 4. 1 Particle size distribution of 2% enzyme concentration

As a result, the samples with PDI smaller than 0.1 (0.1) belong to monodisperse and give the Unimodal peak, as shown in figure 4.1. Poly scattered samples with a PDI of greater than 0.1 (>0.1) can yield bimodal or trimodal peaks. This product has a PDI of 0.0369, which is less than 0.1, indicating that the enzyme-treated wattle bark powder was uniform.

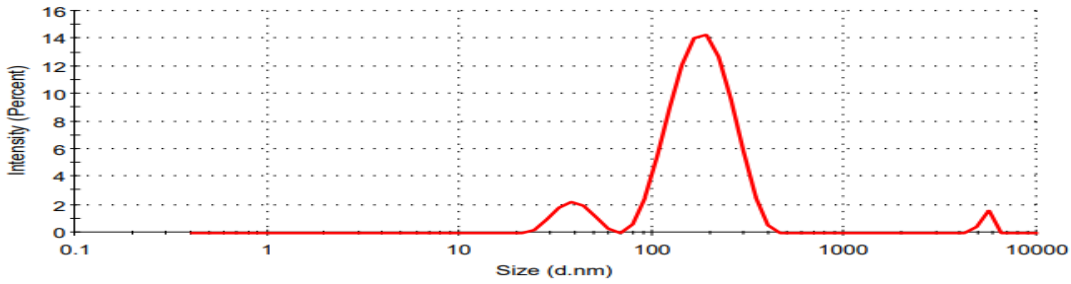

Figure 4. 2 Particle size distribution for the conventional soxhlet sample

As a result of the DLS studies, the reported result was a Nano size dimension. The majority of the particles were between 200 and 700 nm in size, according to the PSD study. The smaller particles (Fig.4.2) were 50 to 300 nm in size. When comparing PSD histograms, it was discovered that a 2 percent enzyme concentration was more effective than the standard one percent. With the same sonication time, a 2 percent enzyme concentration might restrict the particle size distribution Particle sizes range from 0.3 nm to 1000 nm, according to different publications. Wattle tannin size was found to be 120nm and 170nm with a bimodal peak by (Zhou, 2012) and (Fattahi, 2018). The wattle size was estimated to be around 100 nm and 348 nm by (Sofla, 2016) and (Chattopadhyay, 2016), respectively, with a unimodal peak.

A good tanning agent should cover a range of particle sizes to fill the different portions of the leather. The particle size distribution plot for plant bark samples shows that the distribution is good for leather production. However, the bark extract's average particle size (429.9nm) gives excellent results when compared to commercial vegetable tanning products, which have a particle size of around 458nm. As a result, tannin extract would be an excellent choice for filling micro, meso, and macropores during processing.

4.2. Experimental Data of enzyme pretreatment extraction

4.2.1 Experimental Data

The yield of wattle tannin was quantified and examined using the software after the experimental operation. The next section discusses the findings of design expert 13.

Table 4. 3 CCD matrix of independent variables used in RSM with corresponding

Std.	Ru n	A: enzyme concentration (%)	B :time(min)	C:temperature (°C)	Actual yield (%)	Predicted yield (%)
14	1	2	60	40	8.00	7.48
8	2	2	120	40	50.00	46.80
2	3	3	30	65	30.00	31.20
9	4	2	60	30	20.00	19.80
12	5	2	60	40	41.00	42.48
6	6	3	60	40	65.00	64.80
11	7	2	60	40	27.00	28.48
15	8	2	60	40	44.01	44.48
4	9	1	30	30	32.03	35.20
3	10	1	120	65	75.01	76.20
1	11	3	120	30	51.23	52.01
13	12	2	60	40	46.01	44.48

5	13	1	60	40	74.52	76.02
7	14	2	30	40	33.45	34.48
10	15	2	60	50	47.48	48.80

Table.4-3 shows the experimental settings, as well as the experimental and projected responses for various combinations of parameters such as temperature, time, and enzyme concentration. To fit the response function to the equation, regression analysis was used. A quadratic model was found to be acceptable for the prediction of the specified yield from multiple different models of Design expert 13 software, as illustrated by the following equation.

Actual yield = 34.48 3.18 *A +6.01 *B +9.95 *C +4.05 *A* B -9.99 *A *C + 3.32 *B *C +15.91 * A^2, +1.91* B^2, 209* C^2 ... …….(6)

4.2.2. Model Summary Statistics

For this project, the program recommended the Quadratic model. The response function and the independent variables are modeled using RSM.

Table 4. 4 Suggested model for the design

Source	Std. Dev.	R-Squared	**Adjusted R^2**	**Predicted R^2**	
Linear	0.7721	0. 8340	0.5953	0. 7201	
2FI	0.8830	0. 7443	0.6930	0..6301	
Quadratic	**0.7300**	**0.7754**	**0.7711**	**0.6962**	**Suggested**
Cubic	0.5447		0.2913		Aliased

"Summary statistics for the model": Concentrate on improving the model's "Adjusted R2" and "Predicted R 2." The anticipated R-squared reflects how closely the elements in the model are related. As it approaches unity, it indicates that the model is chosen, which is a quadratic model in this work Analysis of Variance, is a good fit. The Model F-value of 35.92 indicates that the model

is significant, according to the design experiment data. An F-value of this magnitude has a 0.01 percent chance of occurring due to noise. Model terms with P values less than 0.0500 are significant. A, B, C, A^2, B^2, C^2, AB are significant model terms in this example, however ,AC and BC do not affect the response. Values greater than 0.1000 indicate the model terms are not significant.

Table 4. 5 Analysis of variance (ANOVA) for Response Surface Quadratic Model

Source	**Sum of Squares**	**Df**	**Mean Square**	**F-value**	**P-value**	**Prob > F**	
Model	3886.08	9	431.79	35.92	0.2450	< 0.0001	**significant**
A-concentration	40.50	1	40.50	0.1799	0.0391	< 0.0001	
B-time	144.50	1	144.50	0.6419	0.0394	< 0.0001	
C-temperature	364.50	1	364.50	1.6200	0.0259	0.0039	
AB	32.74	1	32.74	0.1454	0.0186	< 0.0001	
AC	199.58	1	199.58	0.8865	0.3897	0.0002	
BC	22.02	1	22.02	0.0978	0.7671	0.2855	
A^2	1952.06	1	1952.06	8.6700	0.01321	< 0.0001	
B^2	28.07	1	28.07	0.1247	0.0384	0.0002	
C^2	33.78	1	33.78	0.1501	0.0144	< 0.0001	
Residual	1125.65	5	225.13				
Lack of Fit	110.85	1	110.85	43.69	0.047	0.0049	**significant**

The F-value of 43.69 indicates that the lack of fit is significant. Due to noise, a significant Lack of Fit F-value has a 0.47 percent probability of occurring. It's good if there's a significant lack of fit.

Table 4. 6 Model statics of the design

Std. Dev.	0.73	R-Squared	0.7754
Mean	22.87	Adj R-Squared	0.7711
C.V.	3. 50	Pred R-Squared	0.6962
		Adeq Precision	0.5121

The "Pred R-Squared" of .6962 fits reasonably well with the "Adj R-Squared" of 0.7711. For numerous regression coefficients, the second-degree polynomial equation in Eqn. (4.8) produces an R2 of 0.7754. This suggests that the projected values are closer to the actual data, and thus the quadratic polynomial could be used to represent the system in the specified experimental domain. The stronger the model is and the better it predicts the response, the closer the R2 number is to unity. The signal-to-noise ratio is measured by precision, which is a metric. For this project, a signal of 0.5121 is considered adequate. As a result, you can use this model to navigate the design space. The precision with which the experiments were done is indicated by a lower value of the coefficient of variation, CV = 3.5 percent.

4.2.3. Diagnostic Plot

The normal probability plot (shown below) shows that the residuals follow the normal percent probability distribution. The discrepancy between the value of actual experiment findings and the value of software-predicted results is known as residual. The points in the plots in this experiment are fitted to the straight line in the picture, indicating that the quadratic polynomial model satisfies the analysis of variance assumptions.

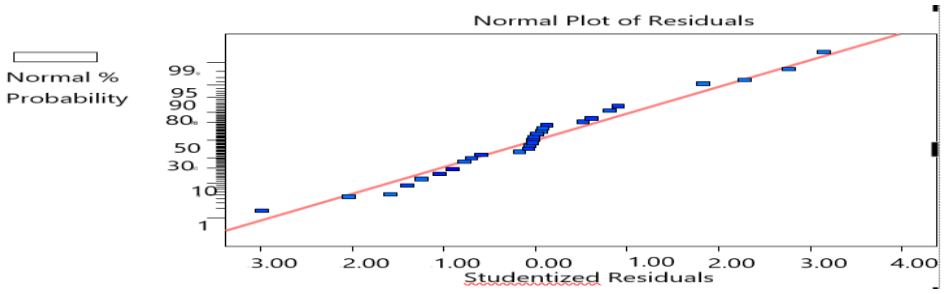

Figure 4. 1 Normal plot of residuals experimental values

If the model is valid and the assumptions are met, the residuals should have no structure and be unrelated to any other variable, including the expected response. Plotting the residuals against the fitted (predicted) values is a straightforward check. The assumption of constant variance is tested by plotting the residuals against the rising expected response values. The plot displays random scatter, indicating that no changes are required to reduce personal error.

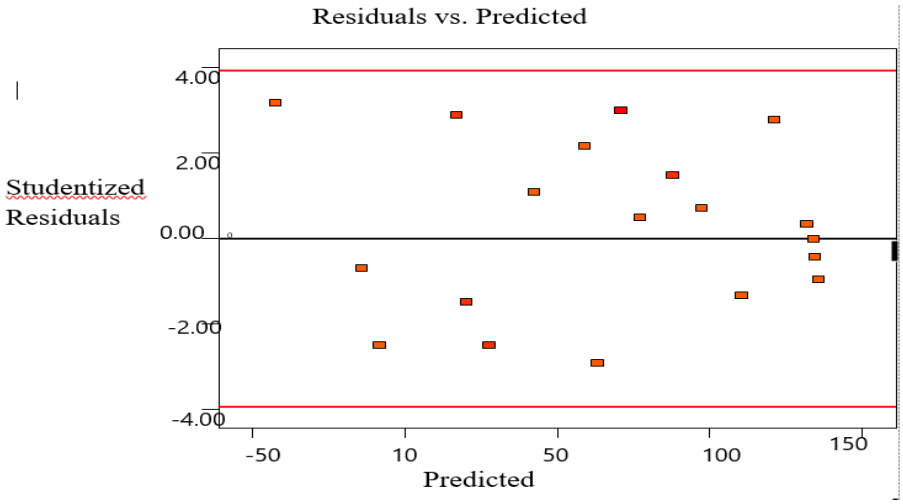

Figure 4. 2 Plot of predicted versus residuals for the yield

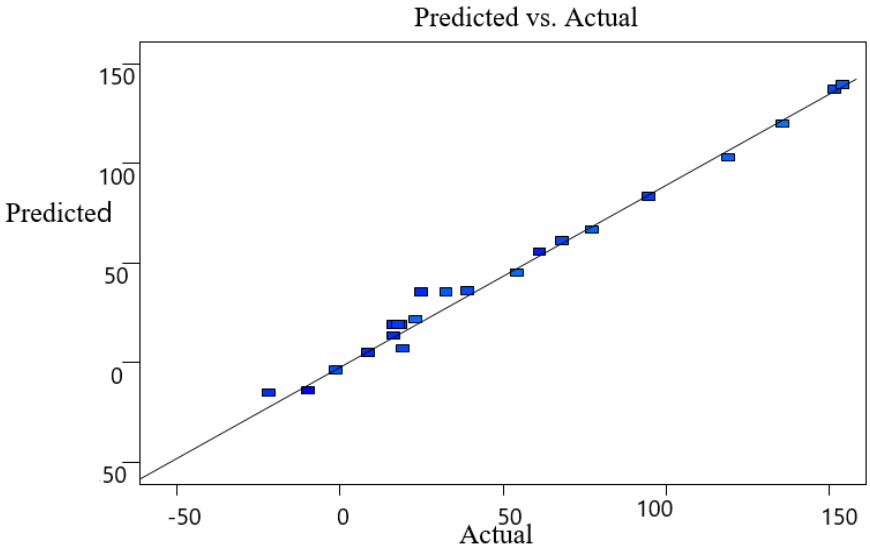

Figure 4. 3 Predicted vs. actual plot of response values

4.2. Effects of Significant Individual Factors on enzyme pretreatment

4.2.1 Effect of Temperature

The yield is lower at lower temperatures and rises as the temperature rises because increasing the temperature speeds up the hydrolysis reaction at the beginning of the reaction. After reaching the optimum temperature of roughly 50°C, increasing the temperature lowers the yield values due to over-degradation of the enzyme as the temperature rises. The temperature has risen. As a result, at low temperatures, the yield was minimal. The negative value of the temperature regression coefficient demonstrates how temperature hurts the reaction after the optimal point, and it coincides with the relationship depicted in Figure 4.6. Therefore, operating at around 50°C of temperature with suitable values of another parameter can give the optimum yield. Similarly, the extraction efficiency improved significantly when the temperature was raised from 30°C to 50°C, according to Novikov (2000). When the temperature was increased to 60°C, however, the tannin extraction declined significantly. For most reactions, according to Novikov (2000), rate extraction increased as temperature increased. The activity of an enzyme can be lowered by thermal denaturation at higher temperatures, according to Nielsen (1995), resulting in a decrease in extraction efficiency.

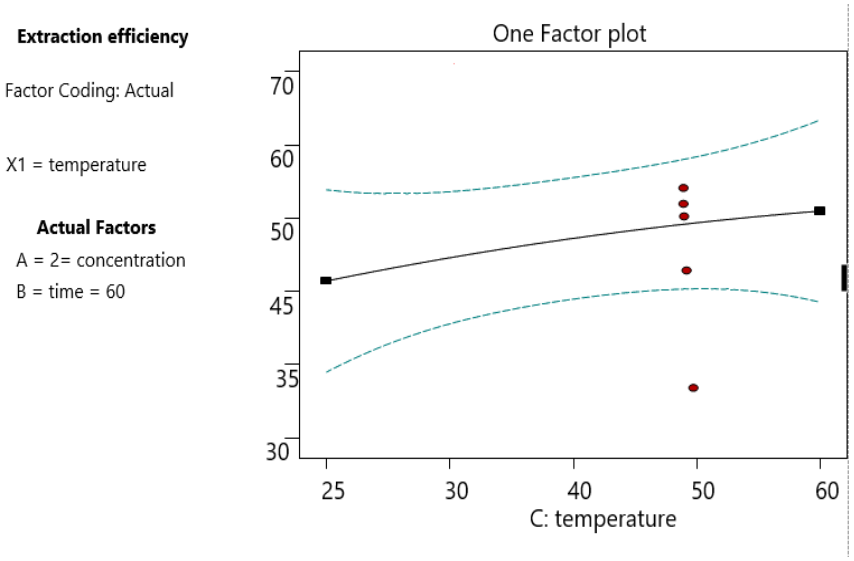

Figure 4. 4 Effect of temperature on the response at constant time and enzyme concentration

4.2.2Effect of extraction time

The graph below shows that the maximum yield was acquired at a lower time level and fell as time grew, whereas other parameters were retained at their fixed values where the maximum yield was obtained. The influence of different times (30, 60, and 120 minutes) on the extraction yield of wattle tannin at other constant conditions of 2 % enzyme concentration and temperature of 50°C was examined in this experiment. The extraction yield was increased up to 60 minutes and then remained unchanged, according to the data. Others have found similar results (Fu et al.) (2008), who found that the best extraction efficiency was reached that during a 60-70 minute duration. As a response, the optimum period for extracting tannin from wattle by enzyme pretreatment was found to be 60 minutes.

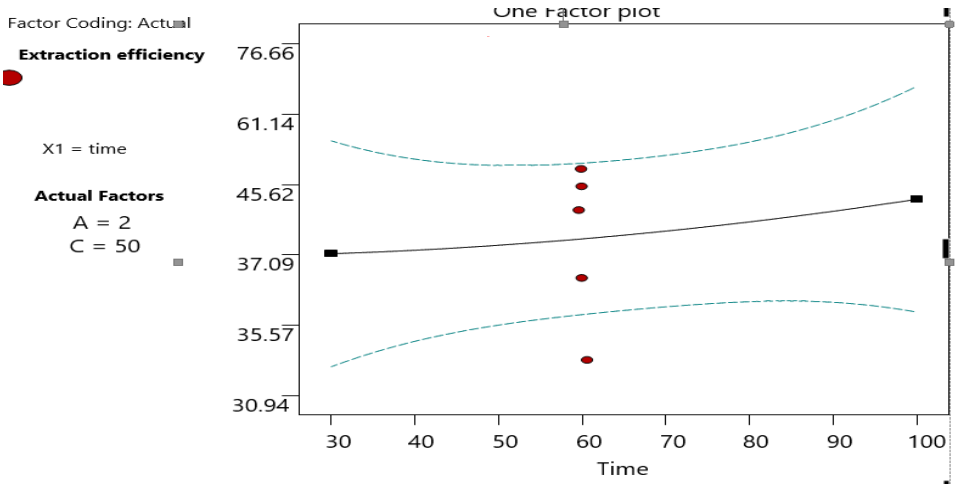

Figure 4. 5 Effect of extraction time on the yield

4.2.3 Effect of enzyme concentration

Because the cell wall is composed up of cellulose, hemicellulose, pectin, and protein, as well as phenolic compounds linked to available polysaccharides by hydrogen and hydrophobic bonds, hydrolyzing enzymes like cellulases, pectinases, and hemicellulases can be used to break down the cell wall structure. These enzymes can also be utilized to improve the penetrating capacity of cell walls, resulting in the release of phenolic compounds and an increase in bioactive component extraction yield (Miron, Herrero, & Ibáez, 2013) The effect of varied cellulase concentrations on extraction yield was similar to that of time. The extraction yield increased by roughly 22% when

2% of the enzyme was added, but increasing the enzyme concentration did not influence the extraction yield. The best concentration of cellulase, according to the results, was 2 %. Fernández et al. reported similar findings (2015)

When indicated in the figure or the RSM table, the influence of enzyme concentration on extraction yield grew for a wide range, remained constant for a limited range, and then began to decrease a

Concentration was increased further. The concentration increases (2%to 3%) and the target product yield decreases when both temperature and time are fixed.

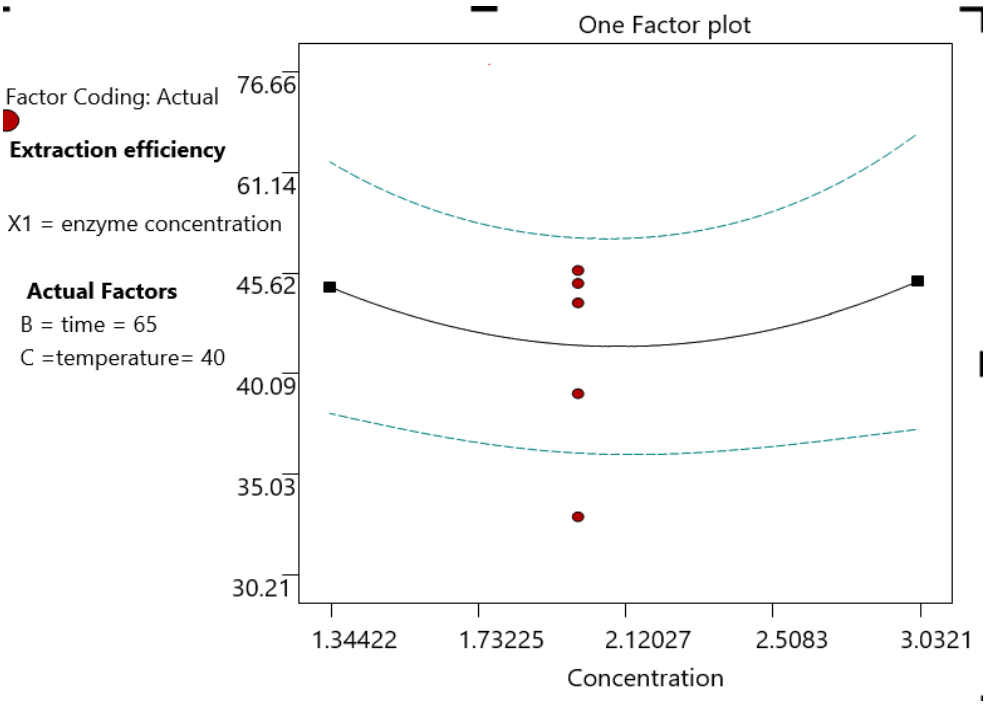

Figure 4. 6 Effect of enzyme concentration on the yield

4.2.4 Interaction effect of temperature and time on enzyme pretreatment

As demonstrated in Figure 4.9, the interplay of temperature and time has a substantial impact on tannin yield. It was discovered that as the temperature and time were reduced, the yield rose. At. When the temperature was lower, time had less of an impact on the yield fluctuation. At a higher level of Extraction, though.

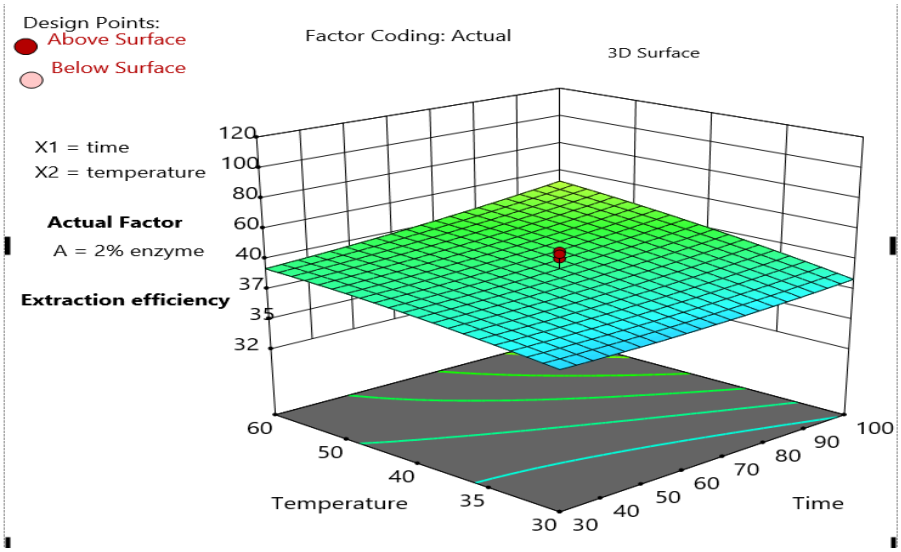

Figure 4. 7 Interaction effects of temperature and time on the yield

4.2.5 Interaction effects of temperature and enzyme concentration

As illustrated in Figure 4.10, the relationship of temperature and enzyme concentration hurts tannin yield after it reaches its optimum point. The regression coefficient of their interaction in the equation indicated the relation between their interaction and yield (4.8). The plot in Figure 4.10 does not have parallel curves. This indicates that the relationship between temperature and enzyme concentration has a greater impact on yield than any other potential interaction.

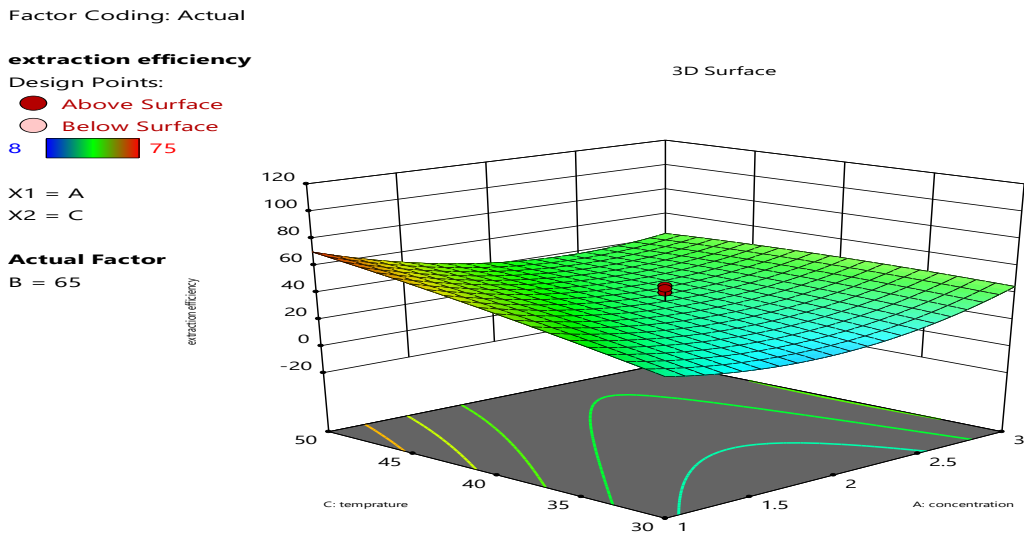

Figure 4. 8 Interaction effect of temperature and enzyme concentration on the yield

4.2.6 Interaction effect of time and enzyme concentration

In comparison to other interactions, the interaction effects of time and enzyme concentration on tannin yield are not very significant, as illustrated in Figure 4.11. It has been discovered that a lower enzyme concentration yields a larger yield at both lower and higher levels of time and that the yield decreases as the hydrolyzing time increases.

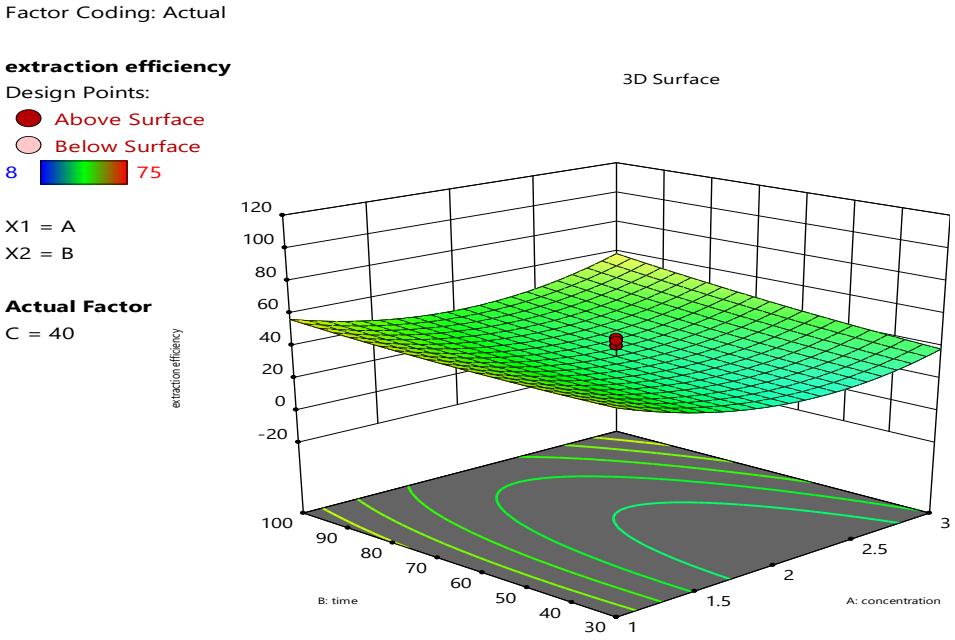

Figure 4. 9 Interaction effect of time and acid concentration on the yield

4.3. Optimization of the parameters

The optimal concentration of the three variables influencing extraction yield was determined using the response surface methodology with CCD. The quantity of enzyme, the temperature, and the length of time it took to extract the enzyme were all crucial factors to consider. The optimal process parameters for temperature, time, and enzyme concentration were found using CCD. For the analysis, the parameters were kept in a range while the yield was set to a maximum. Table 4.7 depicts the optimal process parameter combinations that, when numerically optimized, would yield the best result. Numerical optimization was used to optimize any combination of one or more goals. The optimum values of the process variables were the operating process variables, according to the model capable of predicting the greatest tannin yield. According to the results of the

experiments, the maximum yield of 64.585 percent was reached at 50°C, 60 minutes, and 2 % of enzyme concentration.

This demonstrates that the predicted tannin yield values matched those acquired during experimentation. The tannin yield was adjusted to 64.647 percent using the response optimization technique utilized in this work, with a suitable parameter interaction of 49.705°C, 60 minutes, and 2% enzyme concentration, as shown in Table 4.7 below. In other literatures (Mushtaq, 2016) the extraction yield was obtained 65.89%. As compared to the current study yield was little beat decreasd . Because of its large variety of applications and abundant supply in nature, tannin has attracted a lot of attention. The amount of it in the plant, on the other hand, is determined by geography, biological origin, species, populations, age, and tree position. The amount, structure, and actions of tannin in plants are affected by the plant's growth period, species, and parts. As a result, stabilizing tannin's concentration, structure, and actions is difficult. When materials are stored for a long time, their tannin content decreases.

Table 4. 7 Adjusted process parameters in range to maximize the yield

Name	Goal	Lower Limit	Upper Limit	Importance
A: concentration (%)	minimize	1	3	3
B: time(minute)	minimize	30	120	3
C:temperature(°C)	minimize	35	60	3
Extraction efficiency (%)	maximize	64.494	64.64	3

Solution

No	Enzymes %)	Time(minute)	Temperature° (C)	Actual yield (%)	Desirability	
1	**2.000**	**60.000**	**49.705**	**64.647**	**0.958**	**Selected**
s2	2.000	60.000	47.686	64.617	0.958	
3	2.000	60.000	47.776	64.559	0.58	
4	2.000	60.000	47.609	64.494	0.958	

4.3.1 Effect of pH values

The pH factor has a significant impact on the efficiency of enzyme tannin extraction. Extraction efficiency can be maximized at optimum pH because each enzyme has its optimum value. Extraction should take place far away from the isoelectric point. The extraction process may be hampered if the protein is insoluble at a specific isoelectric point of an enzyme. The number of enzyme increases, the pH values decrease, indicating that the protein is insoluble, which may hinder the extraction process. The acidic cellulose enzyme's optimal pH was 4.8. This value was far from the enzyme's isoelectric point, resulting in the highest extraction efficiency. (Cater CM, Rhee KC, Hagenrnaier RO, Mattil KF (1974) reported shows that when acidic cellulase was treated at pH 4.5– 5, the extraction efficiency of tannin was generally higher and this result was agree with this study.

4.4 Color measurement of enzyme pretreatment

A Minolta CR400 Chromameter was used to determine the color of the extracts. Color value analyses were carried out repeatedly. To investigate color variations, three characteristics were used: L* (lightness), a* (redness), and b* (yellowness). This is how the overall color difference (E*) was computed.

$$\Delta E* = \frac{\sqrt{(L* - Lo*)2 + (a* - ao*)2 + (b* - bo*)2 - 4ac}}{}$$

E* is a single value that accounts for the difference between the sample and standard's L*, a*, and b*. If E* is out of tolerance, it doesn't say which parameter (L*, a*) or b* is out of tolerance. It can also be deceiving in circumstances when L*, a*, and b* are out of tolerance but E* is still acceptable. There are also two other delta values associated with this scale. C* and H* are the letters of the alphabet. C* is the chroma difference between the sample and the standard in the polar coordinate system. H* is the hue angle difference between the sample and the standard in the polar coordinate system.

Table 4. 8 Color measurement of different sample

sample	L*	a*	b*	C	H	color
control	39.132	8.029	12.167	14.577	56.581	
1%	38.448	9.303	12.213	14.568	55.790	
2%	45.652	10.868	15.496	14.553	57.084	
2.5%	46.484	11.889	16.896	14.537	55.807	
3%	37.323	11.935	17.131	15.525	58.060	

The color parameters of wattle tannin powder were measured using a Minolta CR400 Chromameter, which included lightness (L*), chroma (C*), and hue. The chroma values of tannin powder stayed constant (14.5 units) when the enzyme concentration raised from 1 to up to 2.5 %, but the lightness increased. The lightness of the tannin declined as the enzyme concentration increased beyond 2.5%. And the chroma was enhanced the tannin quality derived from wattle bark has a high correlation with its color values.

According to Byamukama [32]. Because a higher enzyme concentration corresponds to higher lightness and chroma values, powders with high L* and C* values are more desirable. As the

amount of enzyme increased from 1% to 3%, the a* (redness) and b* (yellowness) values were increased. As seen in the table, there is no significant color change as the percentage of enzyme grows, implying that the color change of the sample is minor.

4.5 Determination of the Total Phenolic Content

The total phenolic content was calculated using the Folin–Ciocalteu method. Extracts were diluted in distilled water (0.6 mL), combined with 0.2 mL Folin–Ciocalteu reagent and 1 mL Na2CO3 (0.8N), and stored in the dark for 30 minutes. The absorbance at 760 nm was measured using a spectrophotometer.

4.5.1 Preparation of stock solution for enzyme pretreatment

10,000ml distilled water was added to 10gm (10,000mg) of wattle bark material in a 250 ml round bottom flask. The mixture was then cooled to room temperature by being refluxed in a water bath for 30 minutes. Finally, the stock solution was filtrated using filter paper.

4.5.2 Preparation of standard solution for enzyme pretreatment

In 50ml of distilled water, 10mg of each reference standard Gallic acid was dissolved. The standard solution was then transferred to a 250ml volumetric flask at concentrations of 0mg/l, 25mg/l, 50mg/l, 150mg/l, and 200mg/l. Finally, 0.2 mL of this solution, 0.6 mL of folin-ciocalteu(1:1), and 1 mL of distilled water were added to 1 mL of Na2co3 solution. The absorbance of the reference solution was measured at 760nm after 30 minutes using pure water as a zero adjustment.

Table 4. 9 Preparation of standard solution for enzyme pre-treatment

Stock solution	Sample weight (ml)	Distilled Water(ml)	Folin(1:1) reagent(ml)	Na2Co3 (ml)	Distilled Water(ml)
0mg/l	0.2l	0.6ml	0.2ml	1ml	1ml
25mg/l	0.2ml	0.6ml	0.2ml	1ml	1ml
100mg	0.2ml	0.6ml	0.2ml	1ml	1ml
150mg/l	0.2ml	0.6ml	0.2ml	1ml	1ml

200mg/l	0.2ml	0.6ml	0.2ml	1ml	1ml
0%	0.2ml	0.6ml	0.2ml	1ml	1ml
1%	0.2ml	0.6ml	0.2ml	1ml	1ml
2%	0.2ml	0.6ml	0.2ml	1ml	1ml
2.5%	0.2ml	0.6ml	0.2ml	1ml	1ml
3%	0.2ml	0.6ml	0.2ml	1ml	1ml

Table 4. 10 Total phenolic content of the enzyme-pretreated sample

sample	**Absorbance(nm)**	**µg/0.2ml**	**µg/50ml**	**mg/10mg**	**TPC** mg GAE/g dry extract
0%	0.6242	16.4263	4106.5789	4.1065789	55.6286
1%	0.7338	18.6243	4656.0913	4.6560913	61.7343
2%	1.0378	26.3401	6585.0253	6.5850253	78.1669
2.5%	0.8895	22.5761	5644.0355	5.6440355	61.7115
3%	0.7851	21.251	5321.212	5.321312	51.1669

According to Kumar (2019), phenolic compounds occur as free molecules more frequently than esters, hence pretreatment of the enzyme to break hydrogen and covalent bonds and separate the polyphenols from the complex compounds is essential. As shown in table 4.10 the total phenolic compounds (TPC) highly increased in 1% and 2% enzyme pretreatment, then decreased following enzymatic pretreatment, as indicated in the table. It's possible that enzyme pretreatment hydrolyzed the bonds between phenolic compounds and lipoproteins, resulting in more free phenolic compounds that were solubilized by the extraction solvent.

Due to the higher breakdown of the desired product and increased extraction efficiency, total phenolic contents in 2% of the enzyme were the highest (78.1669 mg GAE/g dry extract, the total phenolic content of the control was 55.6286, which was much lower than the enzymatic pre-treatment procedure. The overall phenolic content was found to be lower in 3% of the enzyme

(51.1669mg GAE/g) due to the protein's insoluble nature, which may have hindered the extraction method. In A similar study by (Soto, 2008) enzymatic pretreatment of wattle tannin greatly increased the extract's total phenolic components by 65 %. The concentration of TPC did not considerably rise following enzymatic processing to its maximum values. Similar findings have been observed in previous research.

4.7 Microwave pre-treatment method

4.7.1 Particle size distribution (DLS) of microwave pretreatment

Table 4. 11 Particle size microwave pre-treated sample

Z-Average(dnm):143.7		Size (dnm)	% volume	% intensity
PDI: 0.198	Peak 1	174.0	100.0	100.0
Result quality: good	Peak 2	0.000	0.000	0.000
Intercept :0.955	Peak 3	0.000	0.000	0.000

The 240s of microwave showed as z-average (d. nm) 143.7.nm and PDI of 0.198 with the particle size of 174.5nm for 100% accounting (figure 4.12). The obtained result confirmed that the 240s microwave time was good to have a maximum extraction yield.

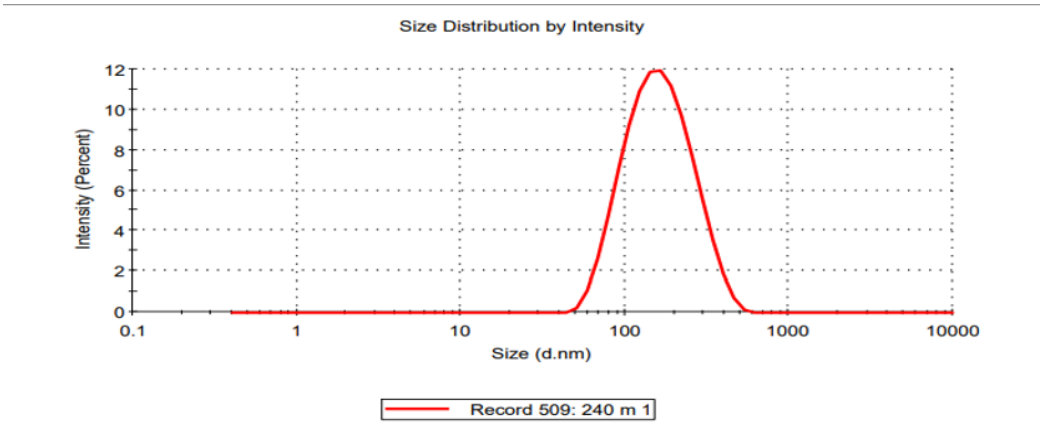

Figure 4. 10 Particle size distribution of 240s sample

There have been differences in the microwave pre-treatment time. 60 and 560-second particle sizes have been raised, whereas 240 and 480-second particle sizes have been reduced. This suggests that

reducing the particle size increased the extraction yield. This experiment is a preliminary look into the impact of Nano-sized particles on extraction yield and pre-treatment time.

4.7.2 Microwave Statistical Analysis of the Experimental Results

4.7.2.1 Experimental Data

After the experimental work, the yield of wattle tannin was calculated and analyzed with the software. The results from design expert 13 were discussed in the following section

Table 4. 12 BBD matrix of independent variables used in RSM with corresponding

Std	Run	A:solvent volume(ml)	B:extraction time(s)	C:extraction temperature(°C)	Actual yield (%)	Predicted yield (%)
12	1	40	310	35	40.25	42.85
9	2	30	310	25	42.10	42.35
16	3	40	60	30	36.52	37.63
16	4	35	560	30	52.01	51.74
4	5	40	310	30	38.41	39.64
6	6	35	560	25	43.05	42.14
25	7	35	310	30	44.72	45.03
13	8	35	60	30	70.21	62.58
10	9	40	310	25	56.34	55.41
27	10	35	310	30	46.23	47.81
24	11	35	310	35	54.01	51.08
15	12	35	310	30	42.14	41.85
20	13	40	560	30	38.54	39.46
21	14	35	310	25	48.16	47.21
8	15	35	560	35	52.46	53.87
17	16	30	60	30	39.23	39.56
211	17	35	310	30	55.65	54.12

The experimental conditions with their experimental and predicted responses with different combinations of parameters such as temperature, time, solvent volume, and microwave power are

shown in Table.4-12. Regression analysis was done to fit the response function as per the equation. From several possible models of Design expert 13 software, a quadratic model was found to be adequate for the prediction of the given yield as shown by the following equation

Actual yield = 8.92+ 16.38*A -9.70*B -7.55*C+ 24.12*AB- 24.71*AC- 0.8275*BC+ 2.13
+23.76 A^2+ 14.47 B^2- 13.34 C^2+ ……..……(7)

4.7.2.2 Model Summary Statistics

The model suggested by software for this work was the Quadratic model. The application of RSM gives an empirical relationship between the response function and the independent variables.

Table 4. 13 Suggested model for the design

Source	Std. Dev.	R^2	Adjusted R^2	Predicted R^2	
Linear	0.6132	0.7321	0.481	0.3848	
2FI	0.1901	0.8371	0.991	0.8188	
Quadratic	0.84	0.6375	0.6750	0.6868	**Suggested**
Cubic	0.0267	0.995	0.7953	-5.2735	**Aliased**

"Model summary statistics": Focus on the model maximizing the "Adjusted R2" and the "Predicted R 2". The predicted R-squared indicates the closeness of the factors for the model. As it approaches unity means a good fit for the model selected which is a quadratic model in this work Analysis of Variance. According to the design experiment data, The Model F-value of 76 implies the model is significant relative to the noise. There is only a 0.02% chance that an F-value this large could occur due to noise. P values less than 0.0500 indicate model terms are significant. In this case, A, B, C, A^2, B^2, C^2, and AB are significant model terms while AC, and BC, are insignificant to the response. Values greater than 0.1000 indicate the model terms are not significant.

Table 4. 14 Analysis of variance (ANOVA) for Response Surface

Source		**Sum of Squares**	**Df**	**Mean Square**	**F-value**	**p-value**	**Prob > F**	
Model		31627.39	14	2259.10	76	0.01513	< 0.0001	**significant**
	A	3219.33	1	3219.33	2.51	0.01357	< 0.0001	
	B	1129.08	1	1129.08	0.8789	0.03644	< 0.0001	
	C	684.79	1	684.79	0.5331	0.04774	0.0039	
	AB	2327.10	1	2327.10	1.81	0.01997	< 0.0001	
	AC	2441.84	1	2441.84	1.90	0.1896	0.0002	
	BC	2.74	1	2.74	0.0021	0.9638	0.2855	
	A^2	3661.38	1	3661.38	2.85	0.0113	< 0.0001	
	B^2	1357.61	1	1357.61	1.06	0.0321	0.0002	
	C^2	1155.01	1	1155.01	0.8991	0.0359	< 0.0001	
Residual		17985.09	14	1284.65				
Lack of Fit		17969.70	10	1796.97	67.14	< 0.0001	0.0002	**significant**

The Lack of Fit F-value of 67.14 implies the Lack of Fit is not significant. There is a 0.35% chance that a Lack of Fit F-value this large could occur due to noise. A significant lack of fit is good.

Table 4. 15 Model statics of the design

Std. Dev.	0.84	**R^2**	0.6375
Mean	40.27	**Adjusted R^2**	0.6750
C.V. %	2.50	**Predicted R^2**	0.6868
PRESS		**Adeq Precision**	25.651

The "Pred R-Squared" of 0.6868 is in reasonable agreement with the "Adj R-Squared" of 0.6375. Multiple regression coefficients R2 is calculated from the second-degree polynomial equation given in Eqn. (4.9) is 0.6375. This indicates that the predicted values are closer to experimental data and the quadratic polynomial was capable of representing the system for the given experimental domain. The closer the R2 value to unity, the stronger the model and the better it predicts the response. Adeq. Precision measures the signal-to-noise ratio. A ratio greater than 4 is desirable. For this work, the ratio of 25.651 indicates an adequate signal. Therefore, this model can be used to navigate the design space. A lower value of the coefficient of variation, CV = 2.5 % indicates the precision with which the experiments were conducted. Similarly, the smaller predicted residual sum of squares (PRESS) statistic shows the better data points fit the model.

4.7.2.3 Diagnostic Plot

From the normal probability plot as shown below, the normal probability plot indicates that the residuals followed by the normal % probability distribution. Residual is the difference between the value of actual results from experiments and the value of predicted results from the software. In the case of this experimental data the points in the plots show fitted to the straight line in the figure, this shows that the quadratic polynomial model satisfies the analysis of the assumptions of variance.

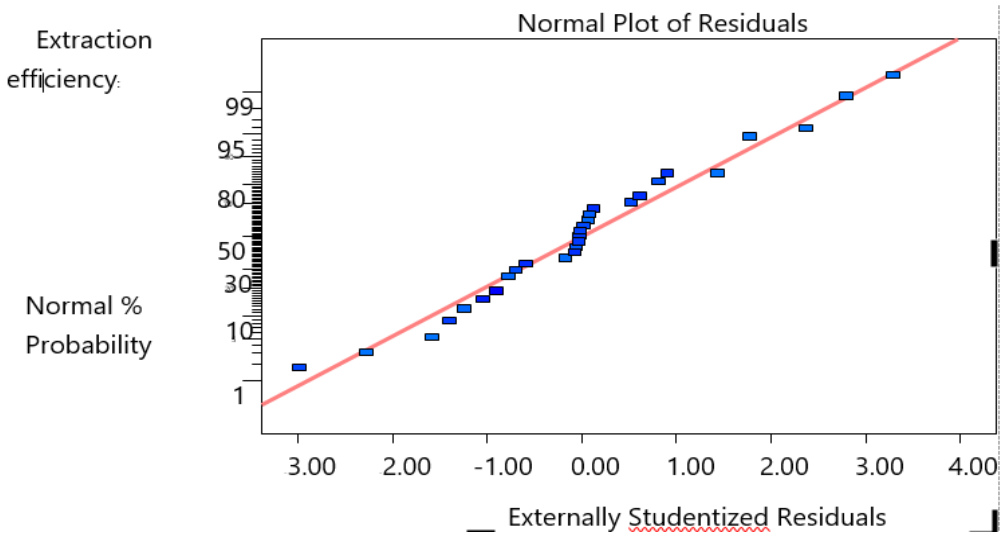

Figure 4. 11 Diagnostic Plot

If the model is correct and the assumptions are satisfied, the residuals should be structureless in particular, they should be unrelated to any other variable including the predicted response. A simple check is to plot the residuals versus the fitted (predicted) values. A plot of the residuals versus the rising predicted response values tests the assumption of constant variance. The plot shows random scatter which justifies no need for any alteration to minimize personal error.

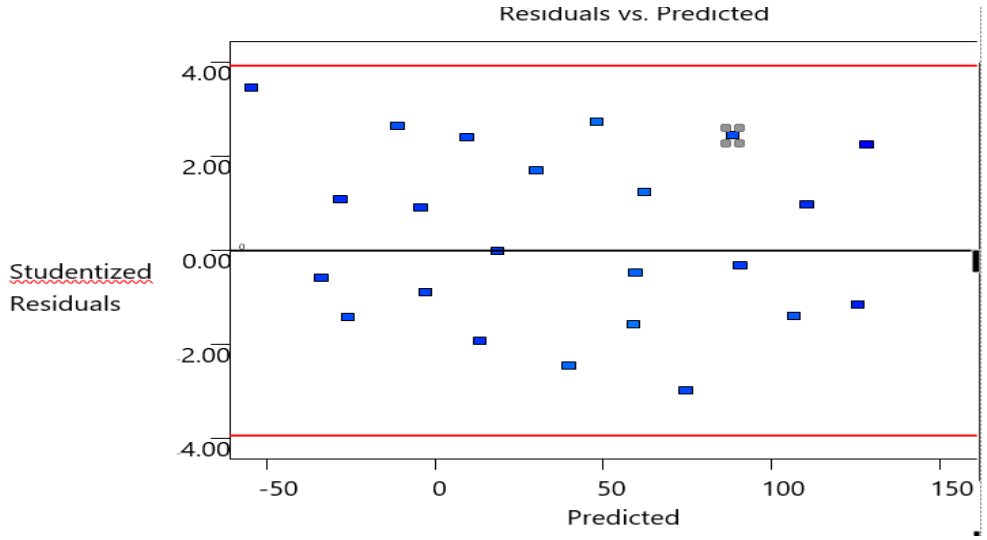

Figure 4. 12 Residual vs predicted

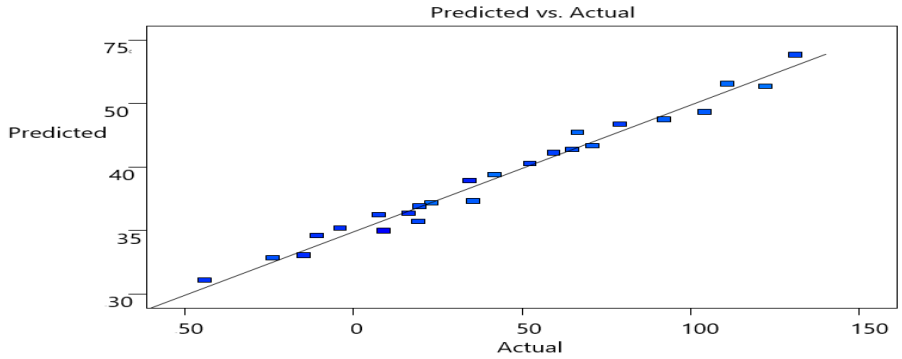

Figure 4. 13 Predicted vs Actual

4.7.3. Effects of Significant Individual Factors

4.7.3.1 Effect of extraction time

To ensure that the extraction is completed, an appropriate irradiation time must be chosen. Studies were conducted at various intervals, including 60, 120, 240, 360, 480, and 560 seconds. As shown in the below graph, the extraction yields of wattle bark increased fast as the irradiation time increased from 60 to 240 seconds, peaking at 240 seconds. The extraction yields then fell when the irradiation time was increased. This could be caused by the disintegration of wattle bark extracted after a long period of irradiation. Other reports like (Al-Harahsheh and Kingman, 2004). Zhe-xiong (2010) report shows that tannin extraction yields increased fast as irradiation periods increased from 30 to 250 seconds, and 250 seconds was its optimum value. As a result, the proper irradiation period was determined to be 240 seconds which is relatively the same to other reports.

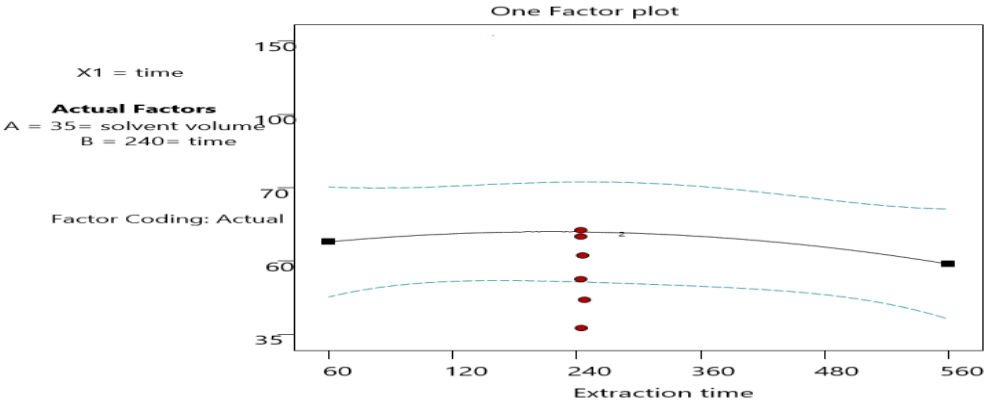

Figure 4. 14 Effect of extraction time on tannin yield

4.7.3.3 Effect of extraction temperature

To increase the efficiency of microwave pretreatment analyte extraction, the temperature must be examined. A higher extraction temperature, in general, produces better results and reduces reaction time. 10.0 g of materials were extracted for 240 seconds with a 35 g/ml solvent-to-material ratio under 110 W microwave irradiation power at three different temperatures to determine how varied extraction temperatures affected the yields of wattle bark extracted during microwave pretreatment. Figure 4.17 demonstrates that as the temperature was raised from 25 to 30°C, the yields of wattle bark removed increased dramatically. The yields of wattle bark extract changed slowly and even declined at 30°C. Increasing moderate temperatures may encourage the cell-matrix to expand, resulting in better wattle bark extraction. However, once the temperature rose above a certain point, the viscosity of the solvent reduced and the diffusivity increased, lowering the extraction efficiency (Camel et al., 2000; Pan et al., 2000). After that, 30°C was chosen as the best temperature for extraction.

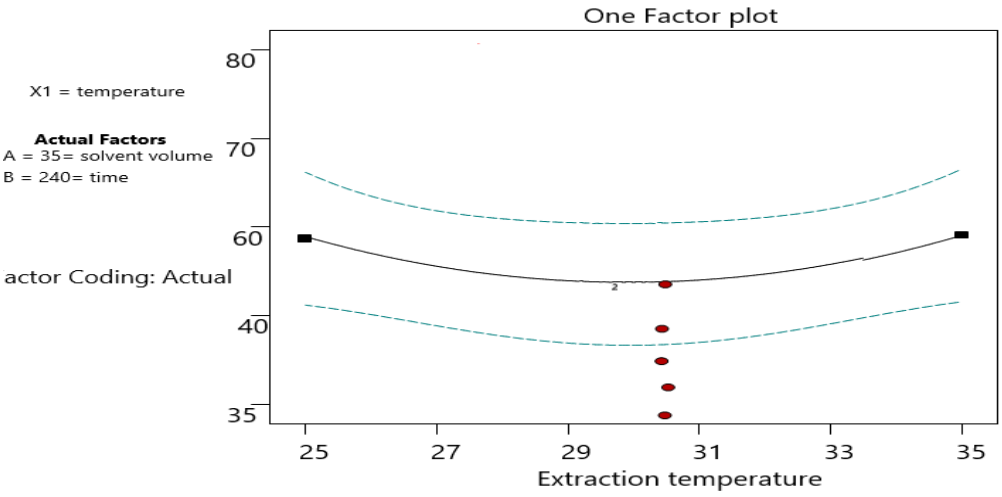

Figure 4. 15 Effect of extraction temperature on the extraction yield of wattle bark Extraction

4.7.3.4 Effect of solvent volume

Effect of solvent volume on wattle bark extraction yield several tests were conducted to determine the effect of the solvent-to-material ratio on tannin yields from wattle bark at various solvent-to-

material ratios. The extraction temperature was 30°C, the extraction time was 240 seconds, and the microwave irradiation power was 330W. The yields of tannins rose with increasing solvent-to-material ratios in the 25–35 ml/g range, as shown in Figure 4.18. However, there was no significant difference or enhancement in the range of 35 - 50 ml/g, which was likely due to the larger volume of water solution causing excessive swelling of the materials by water and absorbing the effective constituent (Guo et al., 2001 may be extracted, which can cause larger diffusion resistance and thus limit the extraction content (Guo et al., 2001 may be extracted, which can cause larger diffusion resistance and thus limit the extraction content (Guo et al., 2001). As a result, 35 ml/g was established to be the optimal solvent-to-material ratio for microwave pretreatment extraction.

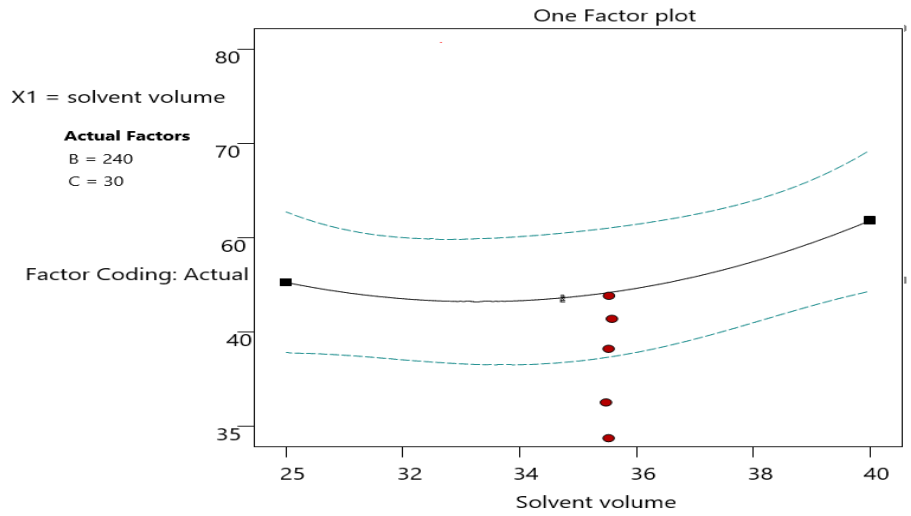

Figure 4. 16 Effect of solvent volume on the extraction yield of wattle bark extracted

4.7.4 Interaction effect of temperature and time

The interaction of temperature and time has a significant effect on the yield of tannin as shown in Figure 4.19. It was observed that the yield was increased with increasing temperature and decreasing time. At. At lower temperature levels, the variation of extraction time had less effect on the variation of the yield. However, at the higher level of Extraction and characterization of tannin temperature, the yield was highly affected by the variation of the time. In such a way that the yield was highly decreased with increasing the time. This is happened due to the over degradation of tannin at a higher level of both temperature and time.

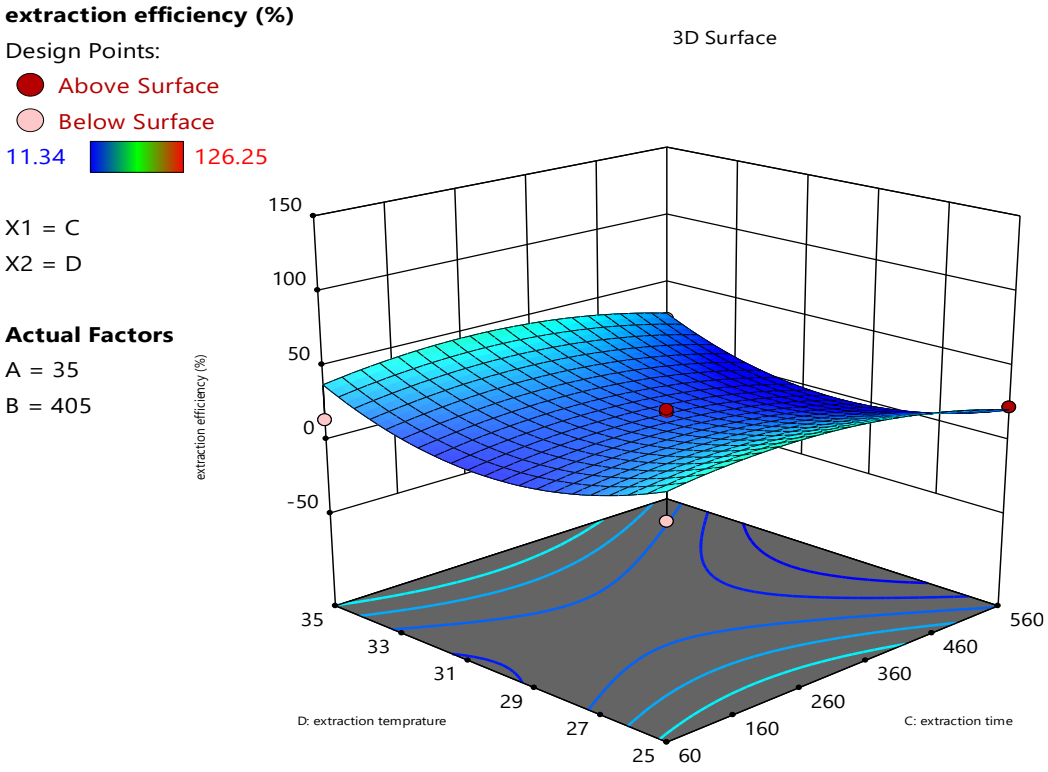

Figure 4. 17 Interaction of temperature and time

4.7.5 Interaction effects of temperature and solvent volume

The interaction of temperature and solvent negatively affect the yield of tannin after its optimum point as shown in Figure 4.20**.** The relationship of their interaction and yield was also shown from the regression coefficient of their interaction in equation (4.9). The curves of the plot shown in Figure 4.20 are not parallel. This shows that the interaction of temperature and acid concentration has a higher effect on the yield than other possible interactions. It was observed that at a lower level of temperature, a lower volume of solvent gives a high yield and decreased with increasing temperature. However, at a higher level of temperature, the variation solvent volume has no significant effect. It was also observed that the effect of volume of solvent variation on the yield at the lower level of temperature was high than that observed at the higher level of temperature.

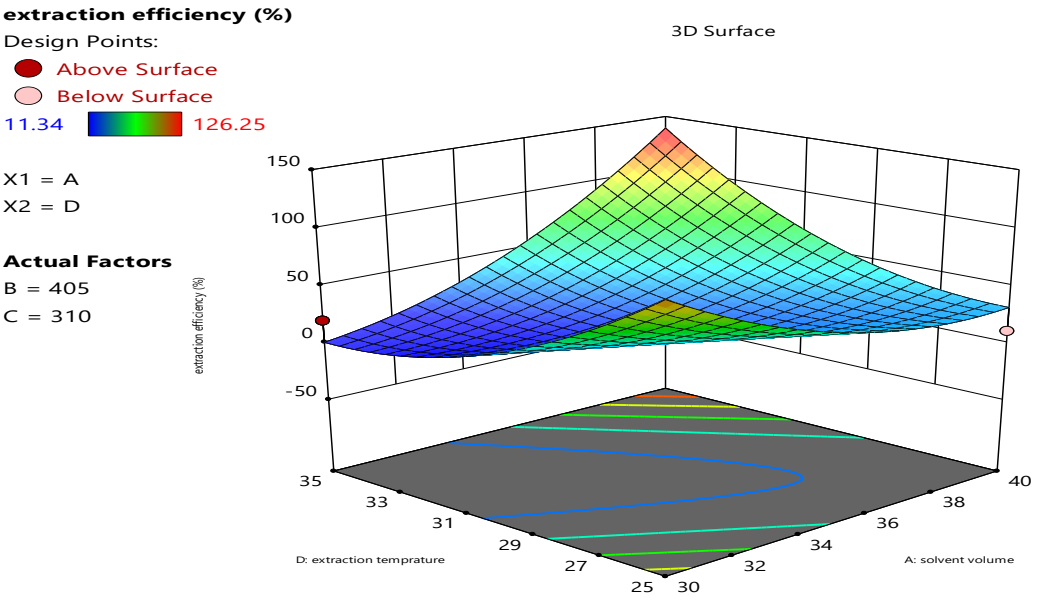

Figure 4. 18 Interaction of temperature and solvent

4.7.6 Interaction effect of time and solvent volume

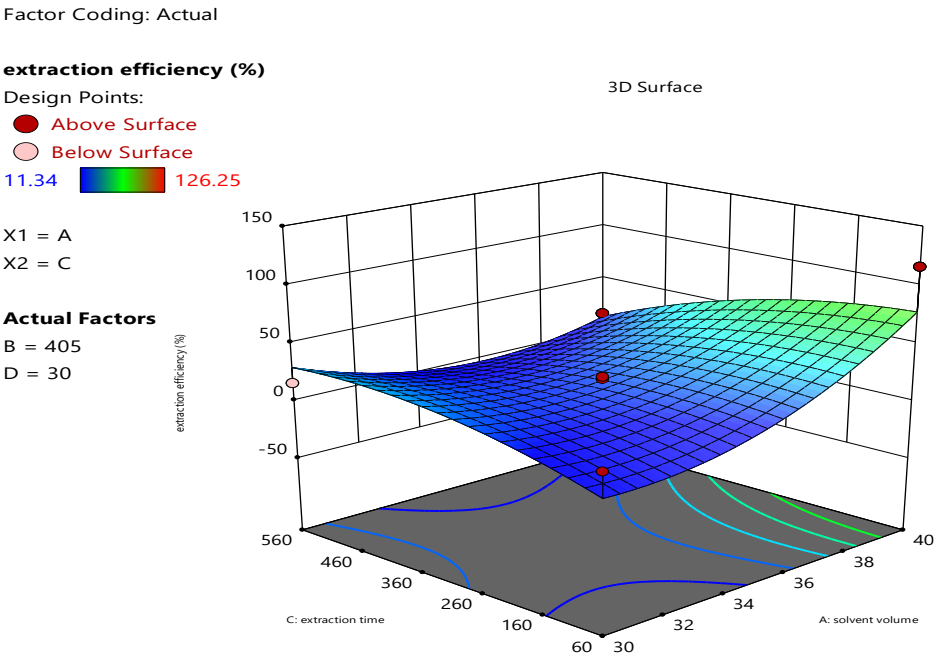

Figure 4.21 Interaction effect of time and solvent volume

Interaction effect of time and solvent volume on yield From Figure 4.20, it is shown that the interaction effects of time and solvent volume on the yield of tannin is not highly significant as compared to other interaction. It is observed that the lower volume of water (35ml) gives higher yield at the both lower level and higher level of time and decreased with increasing hydrolyzing time. This is due to the over degradation of cellulose to undesired products as both time and acid concentration increased

4.7.6 Optimization of the parameters

Methodology of the response surface using Box Behnken Design was used to determine the optimum concentration of the three chosen variables affecting the yield of wattle tannin. The relevant factors were solvent of volume, temperature, and reaction time. The optimum process parameters for temperature, time, and solvent of volume were obtained by BBD. To perform the analysis, the parameters were kept in range while the yield was kept at a maximum. Table 4.16 exhibits the desired combinations of process parameters that would provide the highest response by using numerical optimization. Numerical optimization was used to optimize any combination of one or more goals. The model capable of predicting the maximum tannin extraction of yield showed that the optimum values of the process variables were the operating process variables are putting on the range. According to the experimental work done, the maximum yield of 61.51. % was obtained at the interaction parameter of 25℃, 240 seconds, and 30 ml. Under this condition of parameter interaction, the predicted yield of tannin obtained was 61.38%.

Table 4. 16 Adjusted process parameters in range to maximize the yield

Name	Goal	Lower limit	Upper limit	Importance
Solvent volume(ml)	minimize	25	40	3
Extraction time (s)	minimize	60	560	3
Extraction temperature(℃)	minimize	25	35	3
Actual yield (%)	maximize	60.00	62.38	3

Solution

Numbers	Solvent volume(ml)	Extraction time (s)	Extraction temperature(℃)	Actual yield(%)	Desirability	
1	**30.000**	**60.000**	**25.000**	**61.384**	**0.997**	**Selected**
2	30.000	60.001	25.000	61.380	0.996	
3	30.000	60.020	25.000	61.153	0.958	
4	30.000	60.374	25.006	60.108	0.831	
5	30.024	61.615	25.118	60.002	0.871	

4.8 Effect of microwave power

Microwave irradiation energy breaks hydrogen bonds by inducing dipole rotation of molecules and migration of dissolved ions. Microwave irradiation energy can enhance the penetration of the solvent into the matrix and deliver efficiently to materials, allowing components to be removed through molecular interaction with the electromagnetic field. (Duan , 2001; Hu, 2003; Guo , 2003).

Different microwave irradiation powers, such as 110, 330, 550, and 700 W, were used to investigate the influence of microwave irradiation power on microwave pretreatment. When the microwave irradiation power was less than 110 W, the yields of wattle bark extracted increased**.** When the power was greater than 110 W, however, the yields declined. This could be because different materials require various microwave irradiation powers. Extra energy can be provided to the solvent and matrix by using high irradiation power. It has the capability of disrupting small molecular interactions. Wattle bark extraction yields may be affected by the abnormal molecular interaction structure. This study is in agreement with that of (Uquiche, 2008), who used the best condition for pretreatment microwave power of 112 W for 250 seconds. Microwave pretreatment increased tannin extraction yield, and increasing treatment time had a beneficial influence on tannin extraction yield. (Uquiche, 2008). As a result, a 110W microwave irradiation power was selected.

4.9 Colour measurement of microwave pretreatment

The color of extracts was measured using Minolta CR-400 Chromameter. Analyses of color values were in duplicate. Three parameters, L* (lightness), a* (redness), and b* (yellowness), were used to study changes in the color. The total color difference (ΔE*) was calculated as follows

$$\Delta E* = \sqrt{(L* - Lo*)2 + (a* - ao*)2 + (b* - bo*)2 - 4ac}$$

ΔE* is a single value that takes in account the difference between the L*, a*, and b* of sample and standard. It doesn't indicate which parameter (L*, a*) or b* is out of tolerance if ΔE* is out of tolerance. It may also be misleading in some cases where ΔL*, Δa*Δ b* is out of tolerance, but ΔE* is still within tolerance. In addition, there are two other delta values that are related to this scale ΔC*, ΔH*. ΔC* is the difference in chroma between sample and standard as described in a polar coordinate system. ΔH* is the difference in hue angle between the sample and standard as described in a polar coordinate system.

Variations in hue have been studied because one of the useful applications of this extract is as a colorant. Table 4.17 lists the CIELAB parameters that were used in this study (L*, a*, and b*). It should be noted that the color was prepared using the dried extract; thus, these values can only be used to identify differences, not to characterize the color of the final extract. However, because the dry extract is expected to be the preferred form of the commercial product, this may be considered an important study.

Table 4. 17 Color measurement of different samples

sample	L	a*	b*	C	H	Color
control	46.538	11.463	16.289	19.918	5.863	
60	46.090	12.366	11.753	20.245	55.845	
120	46.391	12.471	11.405	22.232	55.879	

240	46.181	11.557	12.026	21.038	54.028	
480	46.699	14.066	11.089	20.919	54.773	
560	46.114	15.732	11.860	22.756	55.977	

Based on the findings, it can be concluded that microwave pretreatment had only a minor impact on the color of the extract. When microwaves were used instead of traditional extraction, an average total color difference of 5.34 was determined. The most significant difference was in chroma, while hue (L*) was nearly constant. However, for each of the CIELAB parameters, a* and b* darker extracts were obtained, and red hue (a* tended to higher values) and blue hue (b* inclined to negative) were enhanced. According to the results, the blue hue was similar to that of the conventional extraction, but the a* parameter was lower, indicating a green tendency.

4.9.1 Total phenolic content of microwave pretreatment

4.9.1.1. Preparation of stock solution for microwave pre-*treatment*

10gm (10,000mg) of wattle bark material was transferred to 250 ml of the round bottom flask and 10,000ml distilled was added. Then the mixture was refluxed in a water bath for 30 minutes cooled to room temperature. Finally, filer using filter paper, the filtrate was the stock solution.

4.9.1.2 Preparation of standard solution for microwave pre-treatment

10mg of each reference standard Gallic acid were dissolved 50ml of distilled water. Then 0mg/l, 25mg/l, 50mg/l, 150mg/l, 200mg/l of the standard solution was transferred to 250ml volumetric flask. Finally, 0.2ml of this solution, 0.6ml folin-ciocalteu(1:1), and 1ml of distilled wate,r and the volume was with solution 1 ml of Na2co3. After 30minutese the absorbance of the standard solution was measured at 760nm using distilled water for adjustments.

Table 4. 18 Preparation of standard solution

Stock solution	Sample weight	Distilled Water(ml)	Folin(1:1) reagent	Na2Co3 (ml)	Distilled Water
0mg/l	0.2l	0.6ml	0.2ml	1ml	1ml
25mg/l	0.2ml	0.6ml	0.2ml	1ml	1ml
100mg	0.2ml	0.6ml	0.2ml	1ml	1ml
150mg/l	0.2ml	0.6ml	0.2ml	1ml	1ml
200mg/l	0.2ml	0.6ml	0.2ml	1ml	1ml
0 second	0.2ml	0.6ml	0.2ml	1ml	1ml
60 seconds	0.2ml	0.6ml	0.2ml	1ml	1ml
120 seconds	0.2ml	0.6ml	0.2ml	1ml	1ml
240 seconds	0.2ml	0.6ml	0.2ml	1ml	1ml
480 seconds	0.2ml	0.6ml	0.2ml	1ml	1ml
560 seconds	0.2ml	0.6ml	0.2ml	1ml	1ml

Table 4. 19 Total phenolic content of microwave pre-treated sample

Time	**Absorbance**	**µg/0.2ml**	**µg/50ml**	**mg/10mg**	**TPC**
Control	0.6535	16.337	4084.375	4.084375	35.382
60 seconds	0.6861	17.525	4288.125	4.288125	47.645
120 seconds	0.5515	13.785	3446.875	3.446875	48.298
240 seconds	0.7067	17.667	4416.875	4.416875	76.076
480 seconds	0.5506	13.765	3441.250	3.441250	38.236
560 seconds	0.5794	14.485	3621.250	3.621250	33.236

The total phenolic content was significantly affected by microwave pretreatment. When the irradiation power was increased from 0 to 110W for 240 seconds, the total phenolic content more than doubled from 35.382 to 76.076 mg GAE/g; however, when the irradiation power was

increased to 700W, the total phenolic content significantly decreased. When raising the irradiation power from 0 to 110 W and extending the irradiation time to 240 seconds, the total phenolic content increased by 60%. When the irradiation power was increased beyond 330 W, however, the total phenolic content dropped from 76.076 to 38.236 mg GA/g. These results are supported with of Xu (2007), who found that heat treatment of wattle powder increased levels of phenolic compounds. However, due to heat degradation, higher-power irradiation may result in reduced phenolic content. Irradiation at 240 seconds with a result increase than 330 W or 480 seconds with a power greater than 110 W resulted in reduced levels of phenolic content in the current investigation. When the microwave power was increased from 125 to 500 W, Hayat (2010) discovered that the levels of phenolic content in mandarin pomace reduced. It should be emphasized, however, that the effect of irradiation power varies per species due to differences in phenolic chemicals and their heat stability (Jeong 2004).

4.9.2 Physical testing of leather

The physical test results of crust leather for control and experimental are given in the table below.

Table 4. 20 Tensile strength and % elongation at break test results

Sample type	Tensile strength, [N/mm2]		% elongation at break, [%]	
	Values	Average	Values	Average
Wattle tannin(E1)	21.2	18.85	40.9	53.3
Wattle tannin(E2)	16.5		65.7	
Conventional (C1)	14.0	8.5	69.6	60.7
Conventional (C2)	3.1		51.8	

According to the ISO 3376: 2011 test method, the tensile strength must be greater than 15 N/mm2 and the percentage of elongation must be greater than 40%. When this minimal requirement was compared to the test results, it was discovered that the conventionally produced leather did not meet the stipulated minimum tensile strength requirement. In all leather kinds, a high tensile strength value is sought, and this attribute is a key indicator of leather quality (Venkatachalam,

1962). The average tensile strength in this investigation was 18.85 N/mm2.

The fullness attribute of leather is determined by its elongation ability during the break. If the elongation at break value is less than the standard, the leather has a less elastic property. The leather processing business dislikes less elastic leather because it cannot resist the applied force. (Altan, 2010). However, in both tests, wattle bark extracted leather met the stipulated minimum performance criteria.

Table 4. 21 Tear load test results

Sample type	Mean tear load (parallel to backbone), [N]	Mean tear load (perpendicular to the backbone), [N]	Average tear load (Arithmetic mean of 1&2,	Tear load [N/mm]
Experimental	34.8	46.1	38.2	25.35
Conventional	29.1	32.95	31.0	16.9

The minimum average tear load of the upper should be larger than 50 N, according to the ISO 3377-2:2011 test procedure. When this standard requirement was compared to the test results, it was discovered that both wattle bark and conventional were performing poorly (38.2, 31.0, respectively). As a result, neither of them meets the tear load's minimum criterion. The higher level of supplement in the animals' meals improved tear strength. This is consistent with the findings of (Passman and Summer, 1983), who found that leathers produced from better-fed wethers had a stronger and more extensible grain layer and were more resistant to tearing than leathers generated from poorer-fed wethers.

Table 4. 22 Ball burst test results

Type of process	**Distention at burst, [mm]**	**Load at burst, [N]**
Experimental	12.75	534.3
Conventional	12.9	428.0

According to the ISO 3379/2015 standard, the minimum performance requirement for leather

uppers for crack detention is larger than 8mm. When compared to the test results, all-grain strength samples are higher than the leather's minimal performance.

When compared to conventional re-tanning, the strength characteristics of the crust leather produced by wattle powder as re-tanning material, such as tensile strength, percent of elongation at break, tear load (double edge tear), and ball burst (distension at the crack and burst and load at burst), are found to be good.

4.9.3. Organoleptic properties study

Control experimental trials were carried out based on the recipe, starting with wet blue and ending to crust. The organoleptic qualities of the experimental leathers developed were assessed.

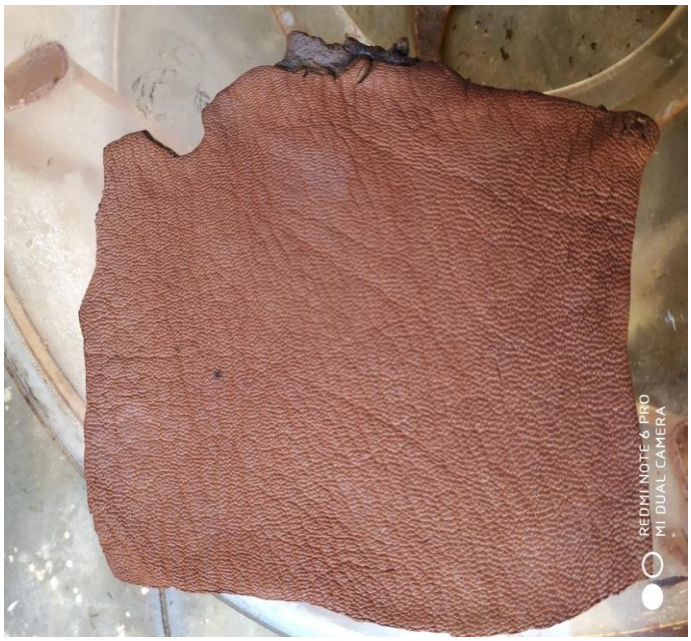

Figure 4. 19 Leather sample re-tanned with enzyme pretreated powder

By hand and eye evaluation, several organoleptic properties of crust leather from both control and experimental methods were assessed. For each functional quality, the average of the ratings for the leathers related to the experiment has been determined and is shown in Fig. 4.22. The higher value, the better its property. In comparison to control leathers, wattle tanned experimental crust leathers had good fullness, as seen in figure 4.22. When compared to control crust leathers, the organoleptic

properties of wattle tanned crust leathers are higher. This is because of better wattle penetration and fixation in the experiment design when compared to the control process. Softness, grain tightness, smoothness, dye consistency, and overall look are all also better than conventionally processed leathers. The appearance of optimized experimental leathers is better than that of control leathers. The conventional one, on the other hand, had more fullness.

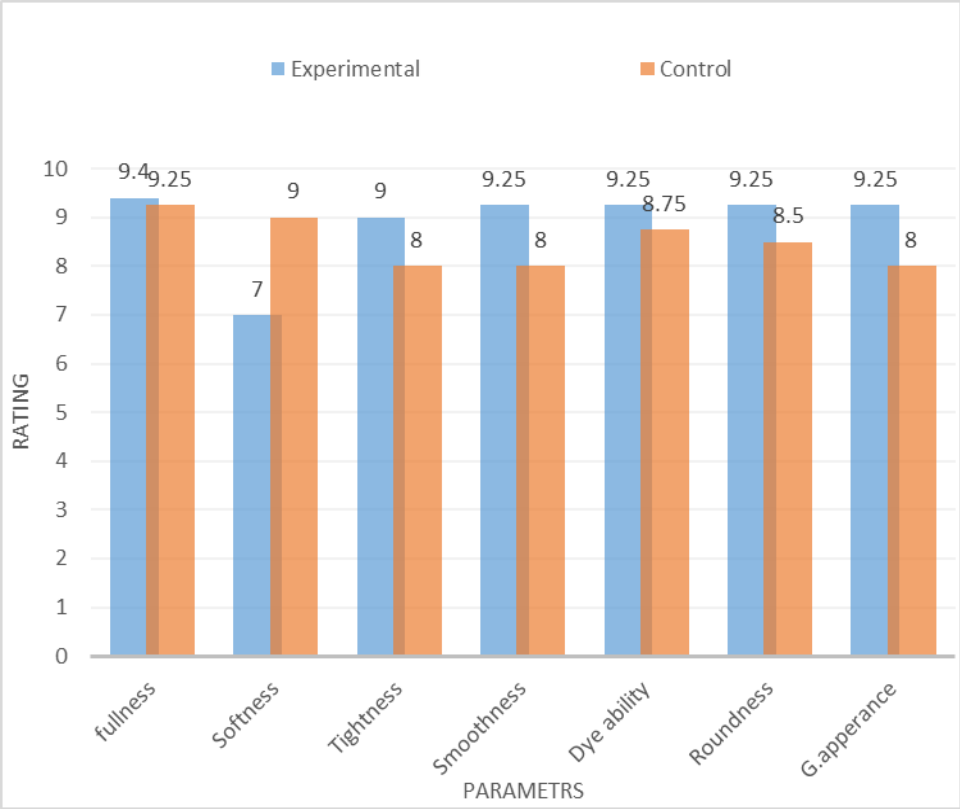

Figure 4. 20 Organoleptic re-tanned leather grading

5. CONCLUSION AND RECOMMENDATION

5.1. Conclusion

Based on the finding in this work, the wattle tannin was successfully extracted from the wattle bark using enzyme and microwave pretreatment. The optimum process condition for enzyme pretreatment method temperature, time, and enzyme concentration are 50℃, 60 $min.\ and$ 2 % respectively. And the extracted tannin yield was obtained at 64.585%. The Characterization of extract tannins which include spectrophotometer and Minolta CR400 Chroma meter were investigated. The maximum total phenolic content of enzyme and microwave pretreatment was78.1669 mg GAE/g dry extract and 76.076 mg GAE/g respectively. The particle size analysis by DLS observed monomial and bimodal particle size by using 2% and 3% enzyme concentration due to different parameters. Based on these monomial graphs show, the average particle size was found to be 167.0 nm with a poly disparity index of 0.0369. The cost of the enzyme could be comparatively expensive if processed with industrial-scale production. Using enzyme pre-treatment in combination with convention extraction methods can we minimize the cost of enzyme and able to achieve extracts of better quality. The tannins were applied to leather after characterizing the tannin extract. it is observed that the strength characteristics like tensile strength, % of elongation at break, tear load (double edge tear), ball burst (distention at the crack and burst and load at burst) of the crust leather produced by wattle powder as re-tanning material is found to be good when compared to the conventional re-tanning process. Then, in comparison to locally available commercial tannins, organoleptic qualities were studied, producing a positive outcome.

5.2 Recommendation

Other alternative extraction methods, such as pre-treating with enzyme, and microwaves, are recommended for leather processing industries to save a large amount of money and time, achieve moderately high recoveries, minimize sample manipulation during the extraction process, reduce solvent consumption, and reduce pollution.

Considering many different aspects in terms of cost, time, and environmental issues. Important future works that are not included in this paper are

- ✓ From different parts of wattle bark such as roots and leaves, only bark wattle were used as a raw material. But, root and leaves have also other components which can be used as raw materials So, it is recommended that other parts of this wattle bark should also be tested for better extraction efficiency.
- ✓ In my study I have used acid enzyme and microwave pretreatment method for extraction of wattle bark. But it is recommended that future studies should use another method of wattle extraction such as autoclave and Infrared-assisted extraction methods.
- ✓ It is recommended that other researchers should be done further characterization of wattle tannin that was not used in this work such as mechanical properties of the leather.

The cost of the enzyme could be comparatively expensive if processed with industrial-scale production so it is recommended to study detailed economic feasibility.

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Annex: Photos of Equipment's used in the study

A) Crashing machine

b) Microwave oven

B) Mechanical shaker

D) Air dry oven

E) SpectropHotometer

F) Digital weighing balance