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Proximate and Elisa Determination of Aflatoxin B1 Levels in *Chrozophora Tinctoria* from Malakand, Khyber Pakhtunkhwa Pakistan

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Abstract: To investigate the proximate and Aflatoxin B1 analysis in *Chrozophora Tinctoria* leaves were shade dried in the lab. The proximate composition of leaves was analyzed by standard methods. Mineral contents in this plant were analyzed by using atomic absorption spectrophotometer. The result revealed that *Chrozophora Tinctoria* leaves contained 86.68% dry matter, 9.80% moisture, 10.01% ash, 21.05% crude protein, Aflatoxin 14%, crude fibers, 14.05% carbohydrate, 44.87% crude fats and energy (243.65 kcal/100g). The analysis of *Chrozophora Tinctoria* revealed that this plant contains high amount of mycotoxin. The type of mycotoxin present in this plant is known as Aflatoxin B1. Aflatoxin caused by high doses in short intervals or low doses in high intervals 1961 caused the death of over 100000 Turkey poult. Naturally produced aflatoxins-B1, B2, G1, G2. *Chrozophora Tinctoria* also contained a high amount of aflatoxin (ppb). Aflatoxin a various poisonous carcinogens and mutagens that are produced by certain molds particularly *aspergillus* species the fungi grow in soil, decaying vegetation and various staple foodstuffs and commodities such as hay, sweetcorn, wheat, millet, sorghum, and various species. This plant contains a huge amount of carbohydrates, and a good source of proteins and fibers.

Keywords: Aflatoxin *Chrozophora Tinctoria*, Proximate and Elisa determination.

1. INTRODUCTION

1.1 CHROZOPHORA TINCTORIA

The genus *Chrozophora* Neck. ex A.Juss. (Euphorbiaceae) contains eight species. *Chrozophora tinctoria* (L.) A. Juss. is known as 'Akbas, (white head) 'dyer's-croton', 'giradol' or 'turnsole'. The plant is used to obtain dye substances including three main colors; red, yellow, and blue. *C. tinctoria* produces dark blue dye due to its high solubility in water and flavonoid content. The plant is used traditionally to treat warts, and it has also been used as an emetic and cathartic and for the treatment of fever (Delazar et al. 2006).

Medicinal plants have been identified and used throughout human history. Plants have the ability to synthesize a wide variety of chemical compounds that are used to perform important biological functions. The World Health Organization (WHO) estimates that 80 percent of the population of some Asian and African countries presently use herbal medicine for some aspect of primary health care. In 2001, researchers identified 122 compounds used in modern medicine which were derived from traditional human use. However, the recent pharmacological and therapeutic studies showed that plants possessed wide range of pharmacological activities and can be utilized to maintain disease-free healthy life. This review was designed to highlight the chemical constituents and pharmacological effects of *Chrozophora Tinctoria* (Saleem 2006).

Turkey has a rich flora due to its geographical position and climatic features. For this reason, many studies of the Turkish flora have been carried out. For deeper knowledge of *Chrozophora tinctoria* (L.) Euphorbiaceae and in order to make use of its economic potential, it should be investigated autecologically, morphologically and anatomically. Ecological investigations of plants distributed naturally in Turkey is important from the point of view of economic evaluation. Besides autecological studies, it has been known for centuries that dye substances can be produced from natural plants existing in the flora of Turkey, and also from stone, soils, mines and animals. Dye

substances can be obtained from different organs such as the flower, fruit, leaf, stem and root of dye-plants. Asibey-Berko (1999).



Fig 01: Chrozophora tinctoria

1.1.1 Scientific Classification

All plants are categorized into seven levels by scientists. Kingdom, Division, Class, Order, Family, Genus, and Species are the different levels. A genus is made up of several species, and the botanical name is made up of Genus (uppercase) and Species (lowercase) (lowercase). A genus is made up of multiple species that are closely related and share numerous characteristics. The lowest level of classification is species, which indicates a group of related plants.

Chrozophora Tinctoria is the botanical name for Kamala. It is a member of the Euphorbiaceae family of plants. The plant's taxonomic classification is listed below.

Kingdom:	Plantae (comprising all living or extinct plants)
Phylum:	Spermapophyta
Subphylum:	Angiospermae
Class:	Dicotyledonae
Order:	Euphorbiales
Family:	Euphorbiaceae – Spurge family
Genus:	chrozophora
Species:	Chrozophora tinctoria

1.1.2 Medicinal uses:

It's used to treat parasitic skin infections, stomach ulcers, tapeworm, and diarrhea. Due to the presence of phytochemicals, it is a medicinal tree. Due to its purgative and anthelmintic characteristics, this medicinal tree is mostly employed in the treatment of intestinal parasites. Tape worm infestations (ascarides, rectal worms) and constipation are treated with it. Internally, it is used to treat bladder and renal calculi, as well as leprosy and sores. Externally, Kamala is used to treat parasite skin infections and wound infections. The fruits and bark have been used to cure stomach ulcers and tapeworms in the past. Kamala powder, made from the fruit's skins, is used as an anthelmintic (Kumar,2020).

The leaves are used to make a decoction that is used to cure diarrhea. Women swallow root scrapings with a betel combination as a contraceptive. Externally, all portions of the tree can be used to treat parasite skin infections. *Chrozophora Tenctoria* is an endangered medicinally significant plant with promising cultivation possibilities in indigenous medicine. It is a significant Ayurvedic medicinal shrub, with secondary metabolites abundant throughout the plant. Various components of the plant are used to cure skin problems, bronchitis, gastrointestinal sickness, jaundice, malaria, and other ailments antifungal, tapeworm, cancer, diabetes, diarrhea, urinogenital infection, and so forth. It also has antioxidant, antimicrobial, anti-filarial, anti-leukemic, antitumor, anti-HIV, anti-tuberculosis, and anti-tuberculosis properties, as well as hepatoprotective properties. The remarkable actions of the *Chrozophora* are highlighted in this review (Kumar,2020).

1-Baldness

Raughan Kamila is mixed with hair oil and massaged on scalp.

2- Cold

The oil prepared from the leaf is used.

3- Intestinal Parasites

In the case of roundworm, giardia, and hookworm, 1-3 gram of powder obtained from the hairs covering the fruit is given in single dose for 2 days.

4- Insect bites

Young fruit ground and mixed with honey is applied.

5- Rheumatism

A decoction of bark is given.

6- Weeping eczema, herpes, scabies, ringworm, and ulcers

In Unani, Raughan Kamila is prescribed for topical use.

All parts of the tree can be applied externally to treat parasitic infections of the skin.

1.1.3 Other Uses

Chrozophora tinctoria produced the blue-purple colorant "turnsole" (also known as katasol or folium used in medieval illuminated manuscripts and as a food colorant in Dutch cheese and certain liquors. Its use was mostly as substitute of the more expensive Tyrian purple, the famous dye obtained from Murex molluscs. The colour comes from the plant's fruit, specifically its dry outer coat. The colorant is also obtained from the translucent sap contained in the plant cells when the leaves of the plant are broken off and exposed to the air. Different shades of blue and purple may also be obtained when the juice extracts are exposed to the vapors emitted from ammonia (NH₃), and which in France, during the late 19th-century, was produced by applying fresh horse manure and urine to the fabric that was soaked with the plant extract. The plant has historically been used throughout the Levant to dye clothing. 100 kilograms (220 lb) of the plant produces 50 kilograms (110 lb) of sap, and with this quantity one is able to dye 25 kilograms (55 lb) of fabric rolls.

In 2020, an interdisciplinary team of researchers from FCT NOVA, University of Porto and University of Aveiro, identified the complex chemical structure of the medieval purple-blue dye extracted from the fruits of *Chrozophora tinctoria*. The chemical structure of the medieval dye was a mystery until now. The extracts obtained showed a novel blue chemical, chrozophoridine as the main chromophore. (Kumar,2020).

1.1.4 Important medicinal properties

Anthelmintic: Ant parasitic, expel parasitic worms (helminths) and other internal parasites from the body.

Antibacterial: Active against bacteria.

Anticancer: Active against cancer.

Antifertility: Reduces fertility

Anti-filarial: Tending to destroy filarial worms or to inhibit their growth.

Antifungal: Fungicide or fungi static used to treat and prevent mycoses such as athlete's foot, ringworm, and candidiasis.

Anti-inflammatory: Reducing inflammation by acting on body mechanisms.

Antimicrobial: Active against microbes.

Antispasmodic: Used to relieve spasm of involuntary muscle.

Cardiac depressant: Depresses the rate or force of contractions of the heart.

Hemostatic: Checking blood flow.

Hypoglycemic: Reducing the level of the sugar glucose in the blood.

Purgative: Strongly laxative in effect

Stimulant: Raises levels of physiological or nervous activity in the body

Wound healing heals wounds.

Anti-proliferative Activity: Anti-proliferative effect was evaluated against Thp-1 cell lines from the isolated compounds of *Chrozophora Tinctoria* fruit extract, in which 4'-hydroxyrottlerin showed 54% growth inhibition of Thp-1 cell line [50]. Other isolated compounds were also tested against different fungi and were found to be very effective IC₅₀ values.

Anti-HIV Activity: Four phloroglucinol derivatives. were tested for their ability to inhibit the activity of human immunodeficiency virus- (HIV-) reverse transcriptase. The mode of inhibition of mallotojaponin was found to be competitive with respect to the template primer, (rA)_n (dT)_{12–18}, and noncompetitive with respect to the triphosphate substrate, dTTP. The K_i value of mallotojaponin for HIV-reverse transcriptase was determined to be 6.1 μM (Kumar,2020).

1.1.5 Traditional Uses

Traditional uses It is an old dye plant widely used in the Middle Ages in the illuminations. Turnsole also called (folium) pigment is more correctly a range of colours from blue through purple to red depending on the PH of the solution. It was considered as another kind of Litmus and sometimes was used for coloring Dutch cheese and certain liquors. Traditionally it is used for the treatment of warts. It was also used as an emetic, cathartic, and for the treatment of fever. The leaves are boiled in water and the obtained juice is given orally to relieve chest burning of digestive origin. , Hakun W.A, Arief. W, Setiyo, Hakun (2017).

1.1.6 Phytochemical constituents:

Chemical constituents The preliminary analysis of *Chrozophora tinctoria* (whole plant, %) showed that it contained 50.00, organic matter 92.73, crude protein 9.13, neutral detergent fiber 31.06 and acid detergent fiber 54.10 Analysis of *Chrozophora tinctoria* showed that it contained dye substances, flavonoids, alkaloids, diterpenoids, xanthenes, coumarins chromones, diterpenoids, and phenylpropanoid glycosides However, analysis of *Chrozophora tinctoria* stems, leaves, and seeds collected during winter from two habitats in Sinai (Egypt), showed the presence of tannins, flavonoids, henoplics, alkaloids, glycosides, reducing sugars, chlorides, and sulfates were detected in samples from both habitats. HPLC analysis revealed the presence of arabinose, ribose, fructose, glucose, and raffinose in the free form, and sucrose in the combined form. Ten amino acids were also isolated from the plant. Phytoanalysis of the plant parts showed that the plant contained alkaloids (stems and roots), saponins (leaves, stems and roots), anthraquinones (leaves, stems and roots), terpenoids (leaves), flavonoids, flavones (leaves and roots), tannins (leaves and roots) and cardiac glycosides (stems). From the aerial parts of the plant, two phenylpropanoid glucosides: 4-O-methyl guaiacylglycerol 9-O-beta- glucopyranoside and 4-O-methyl guaiacylglycerol 8-O-beta-glucopyranoside together with syringin, benzyl alcohol glucoside, isorhamnetin-3-O-beta glucopyranoside-7-O-alpha-rhamnopyranoside and quercetin-3-O-beta-glucopyranoside-7-O-alpha-rhamn op yranoside have been isolated The methanol extract of the aerial parts of *Chrozophora tinctoria* yielded five flavonoid glycosides, quercetin 3-O-rutinoside (rutin), acacetin 7-O-rutinoside , apigenin 7-O-β-D-[(6-p-coumaroyl)]-glucopyranoside, apigenin 7-O- β-D-glucopyranoside and apigenin 7-O-β D-[6-(3,4-dihydroxybenzoyl)]-glucopyranoside (named, *chrozophorin*). Three novel

dolabellane diterpene glucosides and one new dolabellane diterpenoid have been isolated from the plant. The composition of *Chrozophora tinctoria* oil were: eugenyl methy 3.692%, cyclohexyl ketone 13.742% , 2(4H)-benzofuranone, 5,6,7,7 a- tetrahydro - 4,4,7a – trimeth 50.718%, 1H – cycloprop{e} azulene - 7- ol, decahydro - 1,1,7 trimethyl 9.845%, alpha cedrol 3.497%, elemicin 8.558% and capillin 9.948 %. Minerals contents of *Chrozophora tinctoria* were P: 0.15, K: 0.99, Na: 0.11, Ca: 1.38, Mg: 0.32, Fe: 0.010, Cu: 4.40, Mn: 8.30 and Zn: 49.30 %/ dry matter (Akindahunsi, 2006)

1.1.7 Vernacular names / Synonyms:

Scientific name;	<i>Chrozophora Tinctoria</i> (From Latin, Chrozo)
English name;	Giradol or Turnsole
Ayurvedic:	Kampillaka, Kampilla, Kapila, Karkasha, Raktanga, Rechi, Kampilla, : Kamala, Sindur, Rohini, Kambhal
Kannada:	Kampillaka, Kunkumadamara
Malayalam	: Sinduri, Manjana, Kuramatakkku, Kampipala, Ponnagam
Marathi:	Shindur, Shendri, Kapila
Punjabi:	Kumila, Kamal, Kambal, Kamela
Siddha:	Kamela
Tamil:	Kapli, Kungumam, Kurangumanjanatti, Kamala, Manjanai, Kunkumam, Kamala
Telugu:	Kunkuma, Chendra-sinduri, Kapila, Vassuntagunda, Sundari, Vasanta, kumkumamu
Unani:	Kamila

1.1.8 Synonyms

Chrozophora	hierosolymitana,
Chrozophora	verbascifolia Baill
, Chrozophora	oblique,
Croton	tinctorium

1.1.9 Common Adulterants:

Chrozopora tinctoria glandular hair powder is frequently contaminated with Annatto color (*Bixa orellana* Linn.), ferric oxide, brick dust, and ferruginous sand. Adulterants or substitutes for Kampillaka have been reported to include *Casearia tomentosa* (stem bark powder), *Carthamus tinctorius* (flower powder), *Ficus benghalensis* (fruit powder), and *Flemingia macrophylla* (fruit hairs) (Sharma,2011).

1.1.10 Part used for medicinal purpose:

Stem and flower

1.1.11 Side-effects, and Warnings *Chorozopora tinctoria*

For optimal therapeutic dosages, there are unknown hazards and/or adverse effects. Colic, cramps, diarrhea, GI upset, and nausea may occur in large doses. It may cause mild nausea, occasional vomiting, and loose motions. In females, Rottlerin possesses antifertility properties extract decreases serum FSH and LH levels in rats, according to a study, most likely through altering the hypothalamic/pituitary axis. In rats, this lowered level may impact follicular development, ovulated egg quality, corpus luteum formation, estrus cycle, and pregnancy maintenance (Sharma,2011).

1.2 PROXIMATE ANALYSIS

During past decade, much interest has been found in nutraceuticals; however, there is meager information about the presence of Phyto-constituents which derives durable health enhancing or medicinal properties. Nutritional compounds have an important role in the diet that can benefit to human health by continuing use of them (Korver, 1998). Proximate and minerals analysis of fruits and vegetables are playing important role that highlight their nutritional importance. Many medicinal plants are utilized as food due to their medicinal effects and their evaluation may lead significance in understanding their value (Pandey *et al.*, 2006).

It is reported that medicinal plants still used in primary health needs to rural people and support about 80% of the marginal communities round the globe (Prajapati and Prajapati, 2002; Latif *et al.*, 2003).

Proximate analysis provides important information on moisture, ash, and mineral contents. The ash possesses inorganic residue and organic matter resulted after removing by heating. Total ash may vary within species due to variability of nature or physiology of ash. Minerals are normally present in ash and not ruined by heating and they also have relatively low volatility contrasting to rest of food components. Therefore, measurement of mineral content is important, since they may express pharmacological effects (Lee, 2005). Carbohydrates, fats, and protein are important part of complete diet of human being. Protein is richly found in seeds of plants and their quality and quantity assessment may lead in selection of plants for nutritive value, systematic classification, and plant improvement programs (Nisar *et al.*, 2009).

Plants possess various amounts of essential nutrients, minerals, and fibre (Gafar and Itodo, 2011). The leaves and fresh fruits of *Moringa oleifera* are cooked as vegetables and supposed to enhance immune systems. The plant was analyzed for proximate analysis to seek justification of its dietary use (Ogbe and Affiku, 2011).

In Pakistan, very scanty information is available regarding net chemical composition of medicinal oriented plants, which must know for the basis of dietary constituents present in foods. Although the country has 6000 plant species and out of which more than 1,000 species have been reported having medicinal values (Latif *et al.*, 2004; Mushtaq *et al.*, 2009).

From Pakistan, about 6000 species of flowering plants are reported and of which 400-600 are of medicinally importance (Nasir and Ali, 1972).

1.2.1 Ash

The solid remains of the fire is called ash. In the language of analytical chemistry, the ash is the non-gaseous and non-volatile residues that is left behind after the complete combustion of any matter. After the complete combustion, all the organic matter in the sample (e.g., plant) are completely oxidizes and only the mineral left behind. The ash is basically a home for the minerals. The larger the ash concentration in the sample, the more the minerals in the sample will be. According to the world health organization, ash being alkaline, act as a detergent and can be used as alternative to the soap (Saleem, 2017).

1.2.2 Moisture

Moisture contents mean the number of water molecules or the percentage of water in a particular sample. An optimum concentration of water is necessary in every edible product but when the moisture content in a sample deviate from the optimum level, it adversely affect the sample in several ways. The moisture content can affect shape, color, taste, weight, texture, freshness and most importantly the shelf life of a sample. High moisture content in a sample provides a suitable platform for the microbial growth and thus it spoils the product before it reaches to its shelf (Paul, 2015).

1.2.3 Fibers

Fibers are also known as bulk or roughage. Contrary to proteins, fats and carbohydrates, fiber is the part of the food which cannot be digested and absorbed but instead it passes unaffected throughout the digestive tract.

Fibers are classified as soluble and non-soluble.

Soluble fiber. This type of fibre dissolves in water to form a gel-like material. It can help lower blood cholesterol and glucose levels. Soluble fibre is found in oats, peas, beans, apples, citrus fruits, carrots, barley, and psyllium.

Insoluble fiber. This type of fibre promotes the movement of material through your digestive system and increases stool bulk, so it can be of benefit to those who struggle with constipation or irregular stools. Whole-wheat flour, wheat bran, nuts, beans, and vegetables, such as cauliflower, green beans, and potatoes, are good sources of insoluble fibre.

Dietary fibres increase the size and weight of the stools and softens it so that it normalizes the bowel movements and reduces the chances of constipation (Hussain,2010).

The high-fibre diet may also maintain the bowel health by preventing the haemorrhoids formation. High fibrous diet may also reduce the risk of colorectal cancer. High fibres are also best for heart-health. The water-soluble fibres lower the low-density cholesterol (bad cholesterol) which reduces the inflammation and blood pressure and thus improve the heart-health. The dietary fibres especially the soluble fibres slow down the absorption of sugars. Thus, it controls the blood sugar level and reduces the onset of diabetes mellites type 2 (Hussain,2010).

1.2.4 Protein

Protein is constructed from amino acids that are formed by bonds. Protein is the result of folding and linking a polypeptide chain. There are 20 amino acids that make up mammalian peptides and protein. Protein serves a variety of functions within the human body including maintaining body supportive roles through supporting tissue and muscle including the function of collagen in body support, transportation such as through hemoglobin, myoglobin or transportation proteins in phospholipid cell membranes, regulatory functions such as gene transcription and translation as well as in growth factors, regulating and comprising hormones, and comprising enzymes in metabolic events (Stipanuk, 2000). Proteins and amino acids are differentiated by their functional properties and due to the amount of nitrogen that they contain. They contain far more nitrogen (approximately 16%) than other compounds (Stipanuk, 2000).

The 20 amino acids available to synthesize protein and satisfy other metabolic demands are classified as either essential or nonessential amino acids. Essential amino acids are required in the diet because there is no metabolic pathway established to synthesize those amino acids in the body. Nonessential amino acids are not required in the body because they can be synthesized using enzymes and anabolic pathways. The nine amino acids that are essential or that cannot be synthesized in the body are phenylalanine, valine, tryptophan, threonine, isoleucine, methionine, histidine, lysine, and leucine. The remaining amino acids are nonessential due to the body being capable of synthesizing alanine, arginine, asparagine, aspartate, cysteine, glutamate, glutamine, proline, glycine, serine, and tyrosine. However, arginine, proline, glutamine, and glycine are conditionally essential since the rate of use for these amino acids may be greater than the rate of synthesis in the body (Stipanuk, 2000).

Protein and amino acid composition reflect protein quality. Protein quality is defined as the measure of protein bioavailability, which is dependent on digestibility, availability of the amino acids in a protein, and the amino acids that make up the protein. Digestibility refers to the proportion of the dietary protein that is digested and absorbed compared to the amount of protein that passes through the gastrointestinal tract. Digestibility is commonly referred to as the percent of protein digested and utilized (Stipanuk, 2000).

Red meat is approximately 94% digestible compared to beans (78%) and corn (86%) (FAO, 1991). The World Health Organization (WHO) has adopted the protein digestibility corrected amino acid score (PDCAAS) method to further classify digestibility of protein foods. According to WHO, red meat protein foods such as beef have a PDCAAS score of 0.9 compared to other plant protein foods that range from 0.5 to 0.7 on this scoring system. Availability refers to whether individual amino acids can be absorbed, and if so, whether they are incorporated into a metabolic pathway to be utilized. The other factor is determining the amino acid composition in a protein to determine how efficient the protein is. During maintenance, the dietary intake of all amino acids is equal to the amino acid losses that occur from metabolism and digestion resulting in protein balance. Disease, injury, or growth can increase the demand for an amino acid and consequently protein and could affect the rate or if the amino acid is synthesized endogenously (Stipanuk, 2000).

The synthesis and degradation of protein will be affected during these events for the body to try to correct and maintain protein balance. An individual's metabolic state and physical wellbeing determine the demand for various amino acids and protein. The recommended allowance for dietary protein to keep a healthy adult in protein balance is 0.8 grams per kilogram of body weight every day (IOM). On average, this calculates to the average woman requiring 46 grams of dietary protein per day and the average man requiring 56 grams of dietary protein based on normal, healthy body weights across all ages. Dietary protein needs increase when protein synthesis is greater than protein degradation such as during injury, disease, growth, pregnancy, and lactation. The acceptable macronutrient distribution range (AMDR) is another method of measuring protein intake. The AMDR for protein intake is 10-35% of total daily calories for adult men and women (Hussain,2010).

1.2.5 Lipids

Lipids are a category of small molecules essential to biological functions in the human body. Some major functions of lipids within the human body include energy storage, structural roles in cell membranes, signaling molecules, cell signaling properties, receptors, antigens, sensors, electrical insulators, biological detergents, storage, and transportation of lipid soluble vitamins, and serve as membrane anchors for protein. While most media and health publications suggest limiting dietary fat, some level of fat is required in the diet to maintain these lipid functions. Since lipids do not share one unique chemical structure similarity, lipids are categorized into classes based on chemical structure. Classes of lipids include non-esterified fatty acids, glycolipids, glycerophospholipids, steroids, eicosanoids, sphingolipids, isoprenoids, and biological waxes (Stipanuk, 2000).

1.2.6 Fatty Acids

Fatty acids are characterized by a hydrocarbon chain tail and a carboxylic acid head. Fatty acids are commonly classified by chain length; however, this classification is not strictly defined. Short chain fatty acids contain 2 to 6 carbons, medium chain fatty acids contain 8 to 14 carbons, and long chain fatty acids contain greater than 14 carbons. Fatty acids are additionally classified based on the degree of unsaturation or the presence of double bonds within a fatty acid chain. Additional classification from degree of unsaturation results in differentiating fatty acids as either saturated, monounsaturated, or polyunsaturated fatty acids (Stipanuk, 2000).

1.2.7 Saturated Fatty Acids

Saturated fatty acids (SFA) are chains comprised of linear CH₂ (methyl) groups linked with a single, covalent bond without any double or triple bonds in the chain. Physical properties of saturated fatty acids include requiring an increased melting point temperature when compared to unsaturated fatty acids of the same chain length and remaining solid at room temperature (Stipanuk, 2000).

It has been well communicated by health professionals and researchers that there is an association between saturated fat intake and cardiovascular disease. Previous clinical nutrition and epidemiological research studies have concluded that saturated fat is implicated in increasing total and low-density lipoprotein (LDL) blood cholesterol levels. While most research continues to conclude that intake of saturated fat is associated with heart disease, a recent meta-analysis contradicted those results concluding that results did not support reducing saturated fat intake and increasing consumption of polyunsaturated fat as a substitute for the reduction in saturated fat intake (Chowdhury et al., 2014).

1.2.8 Unsaturated Fatty Acids

Unsaturated fatty acids are chains of carbon molecules that contain at least one double bond between two adjacent carbon atoms within the chain. Unsaturated fatty acids are further classified based on the number of carbon double bonds. Monounsaturated fatty acids (MUFAs) contain one double bond linking adjacent carbons, and fatty acids containing more than one double bond between carbon molecules are classified as polyunsaturated fatty acids (PUFAs) (Stipanuk, 2000).

Due to their chemical properties and structure, unsaturated fatty acids include physical properties consisting of having a lower melting temperature than saturated fatty acids of the same chain length, remaining liquid at room temperature, and oxidizing more readily. The 2015 Dietary Guidelines for Americans suggest that 30% or less of an individual's daily caloric intake should be from fat. Of that amount, most calories from fat should be consumed in the form of PUFA's and MUFA's while limiting the amount of SFA's that are consumed (USDA-USDH, 2015).

Endogenous lipogenesis is an enzymatic process that allows the human body to synthesize many unsaturated fatty acids. However, the lacking desaturase enzyme that adds a double bond beyond the ninth carbon of an 18-carbon fatty acid in mammals prevents omega-3 and omega-6 fatty acid synthesis. As a result, omega-3 and omega-6 are essential fatty acids, and these must be consumed in the diet (Stipanuk, 2000).

1.2.9 Carbohydrates:

A carbohydrate is a biomolecule consisting of carbon (C), hydrogen (H) and oxygen (O) atoms, usually with a hydrogen-oxygen atom ratio of 2:1 (as in water) and thus with the empirical formula C_m (H₂O)_n (where

m may or may not be different from n). The term is most common in biochemistry, where it is a synonym of saccharide, a group that includes sugars, starch, and cellulose. The saccharides are divided into four chemical groups: monosaccharides, disaccharides, oligosaccharides, and polysaccharides (Hussain,2010).

Monosaccharides and disaccharides, the smallest (lower molecular weight) carbohydrates, are commonly referred to as sugars. Carbohydrate consumed in food yields 3.87 kilocalories of energy per gram for simple sugars and 3.57 to 4.12 kilocalories per gram for complex carbohydrate in most other foods. Relatively high levels of carbohydrate are associated with processed foods or refined foods made from plants, including sweets, cookies and candy, table sugar, honey, soft drinks, breads and crackers, jams and fruit products, pastas, and breakfast cereals. Lower amounts of carbohydrate are usually associated with unrefined foods, including beans, tubers, rice, and unrefined fruit. Animal-based foods generally have the lowest carbohydrate levels, although milk does contain a high proportion of lactose (Hussain,2010).

Organisms typically cannot metabolize all types of carbohydrate to yield energy. Glucose is a nearly universal and accessible source of energy. Many organisms also have the ability to metabolize other monosaccharides and disaccharides but glucose is often metabolized first. In *Escherichia coli*, for example, the lac operon will express enzymes for the digestion of lactose when it is present, but if both lactose and glucose are present the lac operon is repressed, resulting in the glucose being used first (see: Diauxie). Polysaccharides are also common sources of energy. Many organisms can easily break down starches into glucose; most organisms, however, cannot metabolize cellulose or other polysaccharides like chitin and arabinoxylans. These carbohydrate types can be metabolized by some bacteria and protists. Ruminants and termites, for example, use microorganisms to process cellulose. Even though these complex carbohydrates are not very digestible, they represent an important dietary element for humans, called dietary fibres. Fibre enhances digestion, among other benefits (Hoffman,2001).

The Institute of Medicine recommends that American and Canadian adults get between 45 and 65% of dietary energy from whole-grain carbohydrates. The Food and Agriculture Organization and World Health Organization jointly recommend that national dietary guidelines set a goal of 55–75% of total energy from carbohydrates, but only 10% directly from sugars (their term for simple carbohydrates). A 2017 Cochrane Systematic Review concluded that there was insufficient evidence to support the claim that whole grain diets can affect cardiovascular disease (Hoffman,2001).

1.3 ANALISES OF MYCOTOXIN

1.3.1 Mycotaxin

Fungi are an organism that exists either in single-celled or complex multicellular organisms. This number of the organism may cause diseases by producing toxic substances which known as mycotoxins. Mycotoxins are toxic secondary metabolites of various fungi that significantly impact global food safety and security, from toxin exposure, economic loss of crops, or the salability of said crops. They are a widespread mixture of contaminants in various agricultural and food products, with both acute and chronic toxicological effects on human health. Mycotoxin produced mainly by mycelial structure of filamentous fungi or specifically molds that may cause a harmful effect to animals as well as humans such as carcinogenic, nephrotoxicity, mutagenic, immunosuppressive, estrogenic neurotoxicity, reproductive and developmental toxicity, hepatotoxicity and indigestion.

Mycotoxins including aflatoxins (AFs), ochratoxins (OT), trichothecenes, zearalenone (ZEN), fumonisins (F), tremorgenic toxins, and ergot alkaloids mostly affect the public health and agro-economic significance. Factors affecting the magnitude of toxicity to the living organism are by consuming mycotoxin-contaminated foods or feeds, including species, mechanisms/modes of action, metabolism, and defense mechanisms.

Most of the countries agreed to set the limits of mycotoxins present in food because of the effects of the mycotoxins to human health. The permitted level is slightly different, which depends on the type of food products. The minimum limits for mycotoxins in single ppb (part per billion) and even below (0.05 ppb for infant foods) are established in EU, with similar standards in China and Japan. Guan et al. reported about 98% of the agricultural commodities, including corn, compound animal feeds, silage, cornmeal, puffed corn, wheat, bran, soybean meal, rapeseed meal, cottonseed meal and whole cottonseed contain various group of mycotoxins. Besides, Smith et al. stated that several mycotoxins contaminate approximately 48% of 7049 feedstuffs. Thus, it is essential to detect mycotoxins in the food industry to address the mycotoxin-related health issues to humans and animals effectively.

Conventional techniques such as thin-layer chromatography (TLC), high-performance liquid chromatography (HPLC) and mass spectrometry have been suggested by international organizations as standard approaches to study the occurrence of mycotoxins in food products. Besides, enzyme-linked immunosorbent assay (ELISA) had been widely used to identify different types of mycotoxins. However, it has slight defects of cross-reactivity and possible false-positive or false-negative outcomes.

Also, those techniques usually costly and available in a specialized research laboratory needs highly personnel trained and laborious. Recently, advanced methods used to detect the presence of mycotoxins in food samples, which show high sensitivity, low cost, simple operation, and portable on-field use. Besides, portable and easy-to-use biosensor devices suitable for express, in-field detection of mycotoxins. The development of biosensors for mycotoxins has risen sharply in the last decade with a large number of different bio-sensing technologies application. Zheng et al. (2006) reported biosensor as rapid methods which typically cost-effective, easy to be handled as well as a portable device to be used in an interchanging site compared to laboratory analysis. Gafar, M.K and A.U. Itodo (2014)

1.3.2 Types of Mycotoxin

1.3.2.1 Aflatoxin B1

Fungal toxins are secondary metabolites, which can cause some diseases in living things known as mycoses; meanwhile, dietary exposure to such metabolites produces the disease named mycotoxicoses. Mycotoxins are known as secondary metabolites, produced from microfungi and able to cause effect human health as well as animals. Mycotoxins are commonly used as antibiotics and growth promoters because of their unique characteristics in pharmacological activity. Most of the mycotoxins are found as natural contaminant food, mainly in vegetable and feed. Nut, cereals, oilseeds, dried fruits, spices, and food from animal origins for example milk, egg, and meat are also may contain mycotoxin either outside or inside the product. A mycotoxin is believed no function in the life of a producer cell, unlike primary metabolites. There are few types of mycotoxin such as aflatoxins (AFs), zearalenone (ZEA), deoxynivalenol (DON), ochratoxin (OTA) and T-2 toxin (trichothecene mycotoxin) which are a significant threat to the life and health of human and live stocks. Mycotoxins are low molecular weight and thermal-stable secondary metabolite of toxic molds that belong to genera *Aspergillus*, *Penicillium*, *Alternaria*, and *Fusarium*. These toxins are present in the mycelium and spore of the mold. Mycotoxin may become a biological weapon in bioterrorism because of its acute and chronic toxicities.

2. Types of mycotoxin The established mycotoxins for agriculture and public health concerns including aflatoxins, ochratoxins, zearalenone, T-2 and HT-2 toxin, deoxynivalenol, fumonisins, citrinin, patulin, and ergot alkaloids shown in Figure 1. Aflatoxins B1 and M1 (AFTB1 & M1) produced by *Aspergillus flavus* and *A. parasiticus* species grown on grains and cereals, spices, tree nuts. Aflatoxin B1 (AFB1) is one of the most carcinogenic substances produced by fungi and results in inevitable contamination of food and feed at deficient concentrations. Four main types of aflatoxin naturally contaminate foods which are aflatoxin B1 (AFB1), G1 (AFG1) and their dihydroderivatives B2 (AFB2) and G2 (AFG2). Others without additional metabolites known as Aflatoxin M1 and Aflatoxin M2. AFT M1 being a 4-hydroxylated metabolite of AFT B1, is found in cow and sheep milk and milk products. Some studies had been identified there is 20 aflatoxins that belongs to a group called highly substituted difuranocoumarins. The International Agency for Research on Cancer (IARC) had been classified aflatoxin as very toxic compounds in group 1 due to evidence that shows the carcinogenicity in human. Ochratoxin A (OTA) produced by *Aspergillus ochraceus*, *A. carbonarius*, and *Penicillium verrucosum* is one of the most abundant contaminants in grain and pork products, coffee, dried grapes, as well in wine and beer at humidity around 15–19%.

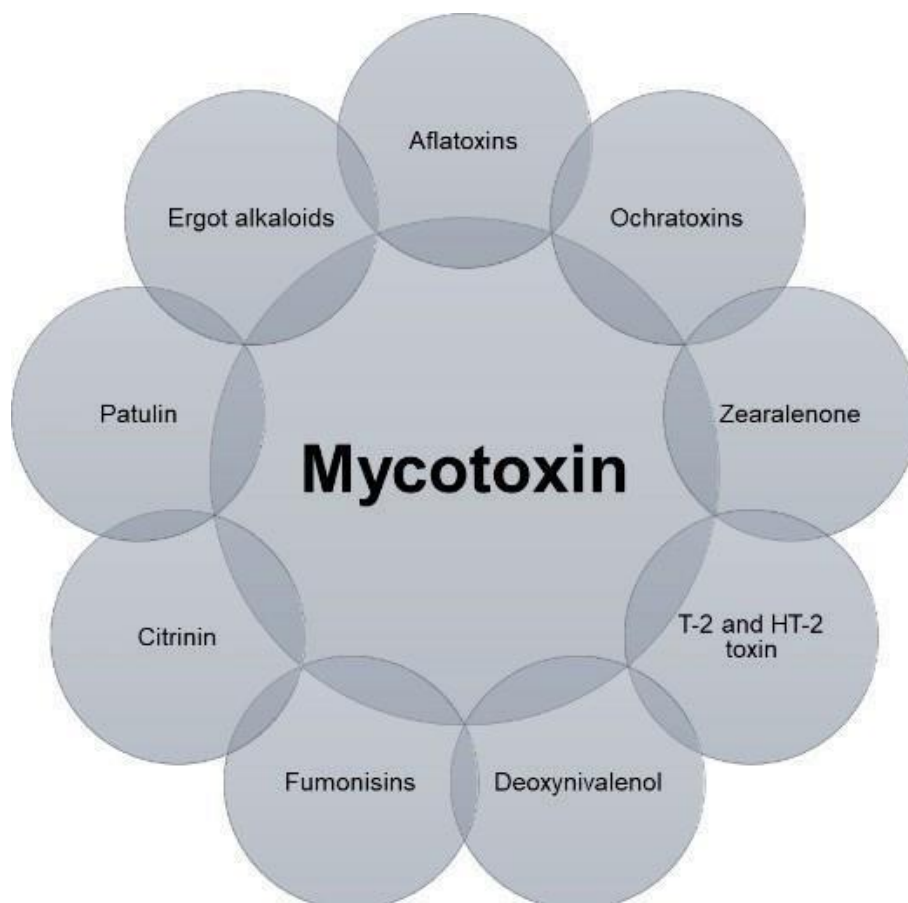


Figure 2. Mycotoxin

Primary groups of mycotoxins in various food products, and temperature $\geq 15^{\circ}\text{C}$. OTA is carcinogenic and neurotoxic for humans, and immunotoxic for animals. OTA can cause various forms of kidney, liver, and brain diseases in both humans and animals, although the trace amount of OTA usually is present in food. Zearalenone (ZEN) produced by *Fusarium* or *Gibberella* species grown on crops (maize, barley, oats, wheat, rice, also bread) is a potent estrogen metabolite causing infertility in swine and poultry. Aflatoxins, a group of highly toxic and carcinogenic difuranocoumarin compounds, are secondary metabolites produced by *Aspergillus flavus* and *Aspergillus parasiticus*. Of more than 20 different aflatoxins identified, those belonging to the two major groups, B and G (Figure 1), are frequently found to contaminate food and feed, especially peanuts and maize. Due to their extremely toxic and carcinogenic effects, legal limits have been imposed on various agri-food products in different countries and regions of the world. For example, the Codex Alimentarius Commission, Joint FAO/WHO Food Standards Program has adopted a limit of $15\ \mu\text{g}\cdot\text{kg}^{-1}$ for total aflatoxins. The European Commission sets the maximum level of $0.1\text{--}12\ \mu\text{g}\cdot\text{kg}^{-1}$ for aflatoxin B1 and $4\text{--}15\ \mu\text{g}\cdot\text{kg}^{-1}$ for total aflatoxins in certain foodstuffs for human consumption.

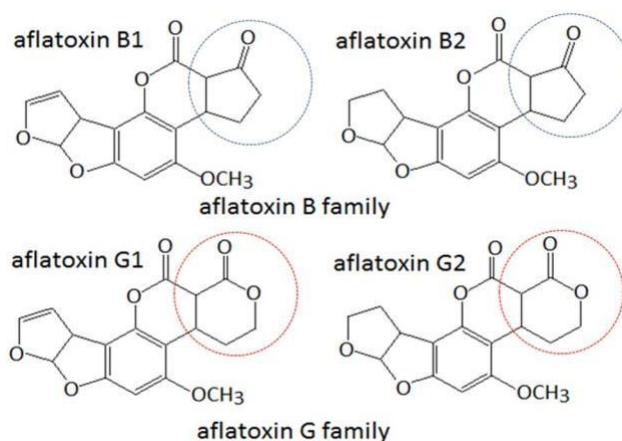


Fig 03: Aflatoxin

Chemical structures of main aflatoxins divided into two groups, B and G family. The moiety in blue or red Circle shows the difference between the two groups. To date, many analytical methodologies have been well-established for the determination of aflatoxins, such as thin layer chromatography (TLC), high-performance liquid chromatography (HPLC), and high performance liquid chromatography-mass spectrometry (HPLC-MS) However, these techniques are time-consuming and not cost-effective, and therefore are not suitable for routine analyses of large numbers of samples. Over the last two decades, the application of immunological methods in aflatoxin detection, particularly enzyme-linked immunosorbent assay (ELISA) and immunochromatographic assay (strip), has been favorably accepted ELISA not only is a tool for rapid and sensitive detection with high sample throughput capacity, but also is relatively inexpensive. Although the toxicity of G-group aflatoxins is lower than that of aflatoxin B1, there is still a need to monitor this group of aflatoxins in food and feed to protect health of humans and animals. Ene-Obong H. N. and Carnovale E (1992)

Aims and objective

- 1 To evaluate the Proximate analysis of *Chrozopora tincatoria*
- 2 To carry out Aflatoxin analysis of *Chrozopora tincatoria*

2 LITERATURE REVIEW

Proximate analysis in plants gives valuable information and help to access the quality of the sample. It provides information on moisture content, ash content, volatile matter content, ash, fixed carbon etc. Different research has carried out proximate analysis on different plants for nutraceutical studies.

Chrozopora tincatoria is a plant belonging to a family Euphorbiaceae. Ngaha and his colleagues conducted research to determine and compare the nutritional value of the leaves and bark of *Chrozopora tincatoria* using standard analytical method (Proximate analysis). Three samples of leaves and bark of were taken and analyzed for its nutritional content. The result of leaves revealed the presence of 13.19% of Crude Protein, 1.12% of Ether Extract, 33.58% of Crude Fiber, 8.72% of moisture, 91.27% of dry matter and 96.36% of Organic matter (Ngaha et al. 2016):

The proximate analysis on the bark of the same plant also revealed the presence of crude protein (5.61%), Ether Extract of (0.72%), Crude Fibres (31.37%), and 10.06% of moisture, 89.93% of dry matter, and 89.65 % of organic matter for bark. The results confirmed that there is variation in nutritional value of *Chrozopora tincatoria* leaves and bark. However, the results indicated that *Chrozopora tincatoria* is a good source of nutritional elements which supports their use as food and medicinal plant (Alagbe, J.O. et al: 2020)

(David Susanto published the research work on the proximate analysis of the leaves of *Calophyllum inophyllum* in 2017. According to the result of this research work, the dried leaves of *C. inophyllum* contained crude fibers which is the highest content (23.96%), the second highest is the moisture content which is (11.24%), followed by carbohydrate (9.91%), protein being (6.43%) and the last is the ash content which came to be (4.75%). The caloric value of the leaves of the *C. inophyllum* was came to be (79.17 kcal/100 g) David Febrilliant Susanto et al, 2017).

The leaves also contained mycotaxin .Aflataxin which is the highest concentration, (14%) .

Delonix regia is also one of the potential medicinal plants, therefore this study was carried out to determine the proximate and mineral composition in the plant (leaf and root). The result on proximate analysis of *Delonix regia* leaf (DRL) showed that it contained moisture (8.10 %), dry matter (91.90 %), crude protein (18.77 %), crude fibre (9.85 %), ether extract (1.33 %), ash (5.21 %), carbohydrates (78.54 %) and energy (1703.5 kJ/100 g). *Delonix regia* leaf (DRL) shows the mineral composition of calcium (77.31 mg/100g), phosphorus (40.35 mg/100g), potassium (51.60 mg/100g), zinc (3.21 mg/100g), magnesium (10.35 mg/100g), sodium (33.18 mg/100g), copper (7.35 mg/100g) and iron (1.89 mg/100g) (Alagbe, J.O. et al: 2020)

The roots of *Delonix regia* contained moisture content 9.60 %, dry matter 90.40 %, crude protein concentration 10.63 %, crude fibre 7.44 %, ether extract 2.71 %, ash 9.30 %, carbohydrates content 90.18 % and energy 1814 kJ/100g respectively. Mineral analysis of the roots of *Delonix regia* contained higher concentrations of calcium, phosphorus, potassium, zinc, magnesium, sodium, copper, iron, and cobalt at 95.43 mg/100g, 40.77 mg/100g, 100.4 mg/100g, 7.21 mg/100g, 14.21 mg/100g, 41.22 mg/100g, 10.2 mg/100g, 1.10

mg/100g and 0.05 mg/100g. It was concluded that the leaves and roots of *Delonix regia* contains various nutrients and minerals that produce definite physiological action on the body of animals (Khalil *et al.* 1986).

Cajanus cajan (Pigeon pea) leaves from, Ogun state, Nigeria, were collected and investigated for proximate and mineral composition. The proximate analysis of the leaves revealed a high composition of 22.4% crude protein, followed by 11.20% moisture, 8.22% ash, 7.25% crude fibre. The lowest concentration being found was 2.74% crude fat which is followed by 0.65% acid insoluble ash value. The significance of this plant was discussed in relation to the presence of these metabolites as well as the proximate values (David G. Oke. 2014).

Carried out Proximate analysis on *Cajanus cajan* (Pigeon pea) reported that mature, whole pigeon pea seed contained 10.13% (moisture), 19.2% (protein), 57.3% (carbohydrate), 1.5% (fat), 8.1% (crude fibre) and 3.8% (ash). These values were like those of Khalil *et al.* (Salunkhe *et al.* 1986): (Salunkhe *et al.*,)

Who reported the values of six components of proximate analysis from the investigated plant *Cajanus cajan*. He reported high content of 54.8% (NFE), followed by 21.3% (crude protein), 10.0% (moisture), crude fibre (8.2%). The low content was of ash (4.5%) followed by 1.2% (fat) (Khalil *et al.* 1986).

From the report of Hardallon *et al.* pigeon pea from Sudan contained 6.1% (moisture), 19.3% (crude protein), 2.0% (fat), 3.6% (ash), 6.4% (crude fibre) and 62.7% (carbohydrate) (Hardallon *et al.* 1980).

Oshodi *et al.* reported that the pigeon pea from parts of western Nigeria contained 10.1% (moisture), 23.11% (crude protein), 1.5% (fat), 57.3% (carbohydrates), 8.1% (crude fibre) and 3.8% (ash). Other reports revealed that percent crude protein varied from 20.425, 20.626 to 21.22(Oshodi *et al.* 1993).

3 MATERIALS AND METHOD

3.1 COLLECTION AND IDENTIFICATION OF PLANT SAMPLE

The selected plant species was collected from the Maina Kot Malakand Agency Khyber Pakhtunkhwa Pakistan and brought in the Taxonomy & Phytomedicine Lab Department of botany university of Malakand. These were identified by Dr. Gul Rahim. Fresh material of plants was examined and the old, insect and fungus-infected leaves were removed. All materials were washed with tap water and all plant parts were made into small pieces. These were dried in open air/under shade. Exposure of plant material to sun light was avoided to prevent the loss of bioactive compounds. The plant materials were washed thoroughly and air dried in the laboratory at room temperature for three weeks. After being fully dried, the plant samples were finely grinded to a coarse powder using laboratory grinding mill and stored in a glass container for further use.

3.2 PROXIMATE ANALYSIS

The proximate analysis of the powdered plant samples for dry matter, moisture and ash content, protein, fat, fibre, was undertaken by using the various analytical method of Association of Analytical Chemist (AOAC 1999). The following are all the methods of proximate analysis.

3.2.1 Determination of Dry Matter

The plant sample was taken in a bottle and mixed with water. The water content was determined by weighing out 2 g of the sample into a silica dish which has been previously heated and weighed. The same was dried in the oven for 24 hrs. at 100°C and then allowed to cool for 10 minutes in desiccators before weighing. Following formula was used to calculate the dry matter.

$$\text{Dry matter} = 100 - \% \text{ of moisture}$$

3.2.2 Determination of Moisture Content

The moisture content in the sample was determined by using the sample drying method in an oven. Take ten grams of accurately weight plant sample (W1). Put the sample in a clean and dried crucibles and weigh it (W2). The sample was put into a moisture extraction oven at 135°C and heated it for 2h. Transferred the dried sample from the oven into the desiccators and then allowed to cool and reweighed. This process was repeated

until a constant weight was obtained (W3). The difference between the weights were calculated as percentage of the original sample.

$$\text{Moisture (\%)} = \frac{W_2 - W_3}{W_1} \times 100$$

Where:

W1= weight (grams) of sample

W2= weight (grams) of dish and sample before heating

W3= weight (grams) of dish and sample after heating

3.2.3 Determination of Ash Content

First, weigh 2gm of the sample as the initial weight (W1). Put the sample in the dried and clean crucible and weigh it (W2). Now burn the sample in fire until the sample become dark in color. This indicate that the sample is free of carbon. Now shift the crucible to the Muffle furnace. Ignite the sample at 600 °C in the Muffle furnace for at least 4 hours until it turns to greyish white color. This white ash indicates that all the organic matter in the sample had been completely oxidized. Cool down the crucible to 200 – 300 °C in Muffle furnace and transfer it to desiccators. Keep it in the desiccators for one hour to cool down to room temperature. Now weigh the Ash present in the crucible (W3). Calculate the total ash by the following formula:

$$\text{Total Ash (\%)} = \frac{W_2 - W_3}{W_1} \times 100$$

Where:

W1 = weight of the sample

W2 = weight of crucible + sample before treatment

W3 = weight of crucible + sample after treatment

Figure 4: Muffle Furnace for determination of Ash content

3.2.4 Determination of Crude protein:

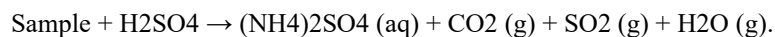
In the presence of sulfuric acid and a catalyst, the amino group of protein is converted into ammonium sulphate. From the ammonium sulphate, ammonia is distilled from an alkaline medium and absorbed in boric acid to form ammonium borate. The ammonia is determined by back titration with a standardized sulphuric acid solution.

The crude protein was determined by using the micro-Kjeld Hal method according to AOAC (1984) as follows:

3.2.4.1 Digestion:

1g of the sample was weighed and taken in small digestion flask. 5g of digestion mixture was mixed with this sample in the same khjeldal flask. Digestion mixture is basically the combination of 3 compounds i.e., K₂SO₄, CuSO₄, FeSO₄ in the ratio of 90, 7 and 3 respectively. 30ml of conc. H₂SO₄ (98% v/w of H₂SO₄) was added. Shake and whirl the khjeldal flask for thoroughly mixing. The contents of the flask were then heated on an electrical heater till the color changed to light green. At this point, all the organic matter has been oxidized. The tubes were then removed from the digester and allowed to cool the flask.

Degradation:

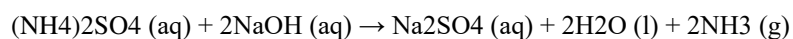




3.2.4.2 Distillation:

The digested sample was transferred to the distillation unit and 20ml of 40% sodium hydroxide (NaOH) were added. The ammonia was received in 100ml conical flask containing 10ml of 2% boric acid plus 3-4 drops of methyl-red indicator. The distillation was continued until the volume reached 50ml.

Neutralization (Liberation of ammonia):



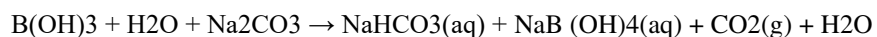
Capture of ammonia:



3.2.4.3 Titration:

The content of the flask was titrated against 0.02 N HCl. The titration reading was recorded. The Kjeldahl method is summarized below.

Back-titration:



The crude protein was calculated using the following equation:

$$CP \% = \frac{(T-B) \times N \times 14 \times 100 \times 6.25}{W_s \times 1000}$$

Where:

CP = Crude protein
T = Titration reading
B = Blank titration reading
N = HCl normality
Ws = Sample weight
1000 = To convert to mg

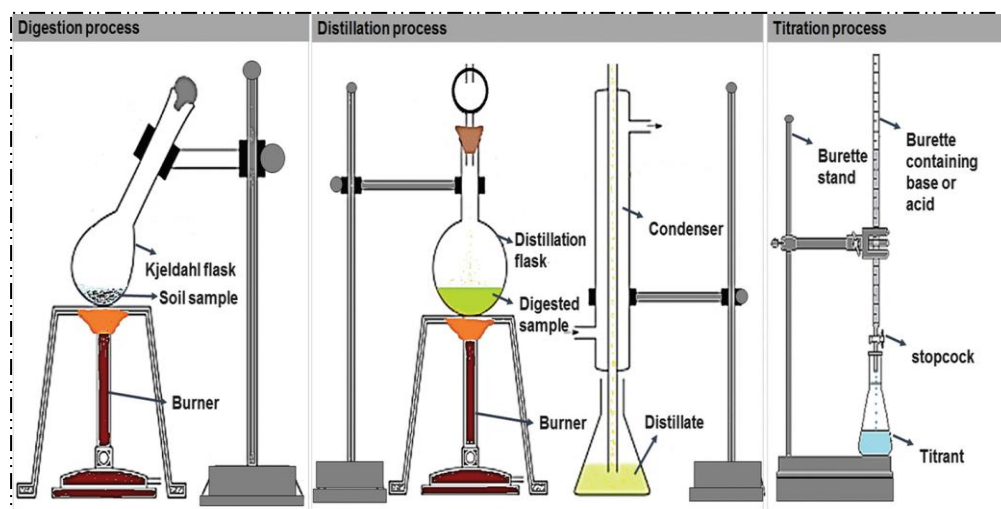


Figure 5 Diagrammatic overview of Kjeldal mechanism

Titration (Ml)	% Of crude protein	Titration (Ml)	% Of crude protein	Titration (Ml)	% Of crude protein
0.1	0.880	3.6	31.50	7.1	62.13
0.2	1.750	3.7	32.38	7.2	63.00
0.3	2.630	3.8	33.25	7.3	63.88
0.4	3.500	3.9	34.13	7.4	64.75
0.5	4.380	4.0	35.00	7.5	65.63
0.6	5.250	4.1	35.88	7.6	66.50
0.7	6.130	4.2	36.75	7.7	67.38
0.8	7.000	4.3	37.63	7.8	68.25
0.9	7.880	4.4	38.5	7.9	69.13
1.0	8.750	4.5	39.38	8.0	70.00
1.1	9.630	4.6	40.25	8.1	70.88
1.2	10.50	4.7	41.13	8.2	71.75
1.3	11.38	4.8	42.00	8.3	72.63
1.4	12.25	4.9	42.88	8.4	73.50
1.5	13.13	5.0	43.75	8.5	74.38
1.6	14.00	5.1	44.63	8.6	75.25
1.7	14.88	5.2	45.5	8.7	76.13
1.8	15.75	5.3	46.38	8.8	77.00
1.9	16.63	5.4	47.25	8.9	77.88
2.0	17.50	5.5	48.13	9.0	78.75
2.1	18.83	5.6	49.00	9.1	79.63
2.2	19.25	5.7	49.88	9.2	80.50
2.3	20.13	5.8	50.75	9.3	81.38

2.4	21.00	5.9	51.63	9.4	82.25
2.5	21.88	6.0	52.50	9.5	83.13
2.6	22.75	6.1	53.38	9.6	84.00
2.7	23.63	6.2	54.25	9.7	84.88
2.8	24.50	6.3	55.13	9.8	85.75
2.9	25.38	6.4	56.00	9.9	86.63
3.0	26.25	6.5	56.88	10	87.50
3.1	27.13	6.6	57.75		
3.2	28.00	6.7	58.63		
3.3	28.88	6.8	59.50		
3.4	29.75	6.9	60.38		
3.5	30.63	7.0	61.25		

Table 1: % Of crude protein

3.2.5 Determination of the Crude Fats

The crude lipids in the selected plant part were determined using Soxhlet apparatus. The Soxhlet extractor is equipped with a condenser system. First weigh accurately 3g (initial weight W1) of a moisture free sample into a clean previously dried extraction thimble or filter paper. If it's a thimble then plug it with absorbent cotton wool. If it's a filter-paper, then just wrap it tightly. Place the thimble/filter paper in extractor and fix under the condenser of the extraction apparatus. Hexane (C_6H_{12}) is used as the solvent for the extraction of neutral lipids from the leaves. 70mL of hexane was put in the 500mL rounded-bottom flask of the extractor. Turn on the heater of the Soxhlet apparatus. The heater will vaporize the hexane which will move up through the ankle of Soxhlet apparatus. There the vapors will produce pressure and will push down the lipids to the bottom of the apparatus. Extract for 4 hours at the rate of condensation of 5-6 drops/sec. Remove the thimble from extractor. Continue the process to recover the solvent for future use. Evaporate and dry the thimble. Dry and weigh the thimble after extraction (Greenfield H *et al*).

Calculate the crude fats % by the following formula.

$$\text{Crude Fat (\%)} = \frac{\text{Initial weight} - \text{Final weight}}{\text{Initial weight}} \times 100$$

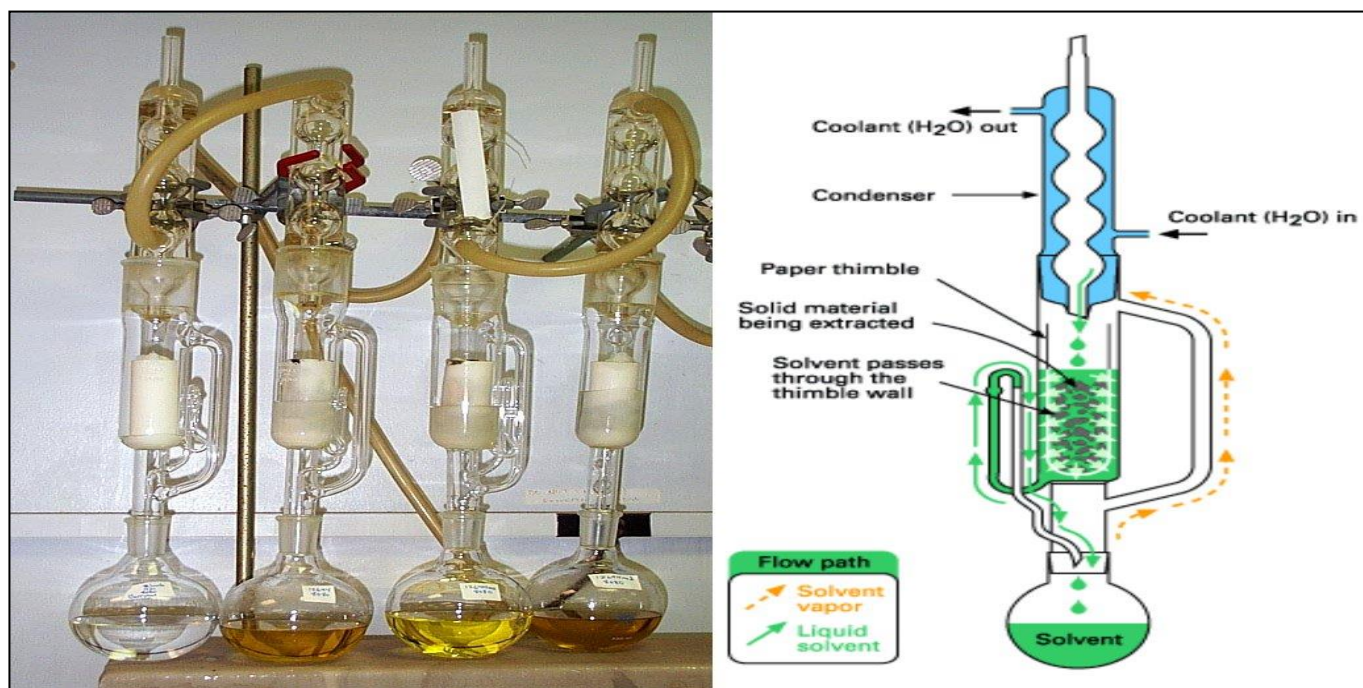


Figure 6 Soxhlet extractor for fats.

3.2.6 Determination of the crude fibers:

Fibers contents in the sample was determined by gravimetric method. First, weighs accurately 2g (W_1) of moisture free and ether extract sample. Place sample in tall form beaker. Add 200 mL of 1.25% H_2SO_4 solution sample. Digest the sample on crude fiber extraction apparatus for exactly 30 minutes. Filter it through sintered glass Buchner funnel with an aid of suction air pump. Wash the beaker with hot water until it is acid-free. Now collect the wet sample from the filter and transfer it to tall form beaker again. Now add 200 mL of 1.25% NaOH solution to the sample and digest it again in the crude fiber's digestion apparatus for exactly 30 minutes. Filter it again through sintered glass Buchner funnel with an aid of suction air pump. Now transfer it to a prepared Gooch crucible. Dry the filtrate in oven at $135^{\circ}C$ for 2 hours. Cool it in desiccators for 30 minutes and weigh (W_2). This is the weight before incineration, here it contains ash and fibers. Ignite the filtrate in muffle furnace at $600^{\circ}C$ for 30 minutes. Muffle furnace will destroy fibers content and left the ash behind. Cool it in desiccators for an hour and weigh (W_3) (Greenfield H *et al*).

Calculate the crude fiber (%) by the following formula.

$$\text{Crude fiber (\%)} = \frac{W_2 - W_3}{W_1} \times 100$$

Where:

W_1 = weight of the sample

W_2 = weight of crucible + filtered dry residue

W_3 = weight of crucible + Ash



Figure 7 Crude fibers extraction apparatus

3.2.7 Determination of Carbohydrate Content

The carbohydrate content was established by using nitrogen free method as described by Ai- Khalifa (1996) and Moses *et al.* (2012). The carbohydrate is calculated as weight by difference between 100 and the summation of other proximate parameters such as crude protein, crude lipid, crude fiber, ash, and moisture. The formula is given below:

$$\text{Carbohydrate (\%)} = 100 - (\% \text{ moisture} + \% \text{ protein} + \% \text{ crude lipid} + \% \text{ crude fiber} + \% \text{ ash})$$

3.2.8 Determination of total energy

Energy/Nutritive values of our sample was obtained by multiplying the carbohydrate, protein, and fat by the factors of 3.5, 4.0, and 9.0 respectively. The nutritive value is expressed in kcal/100g while the proximate values are expressed in percentage (Greenfield H *et al.*).

RESULTS

3.3 PROXIMATE ANALYSIS:

The proximate analysis of the medicinal plant *Chrozophora Tinctoria* leaves is provided in the table below. The result of analysis was in the order of Carbohydrates > Fibers > Proteins > Moistures > Fats > Ash. The percentage of dry matter in this plant was **86.68 %**.

The total moisture in *C.Tinctoria* leaves was **10.1 %**. The total ash percentage in the leaves of this plant was recorded as 5.00%. In the case of crude protein, the percentage was **21.88 %**. Apart from this, the total crude fats analyzed was **4.7 %**.

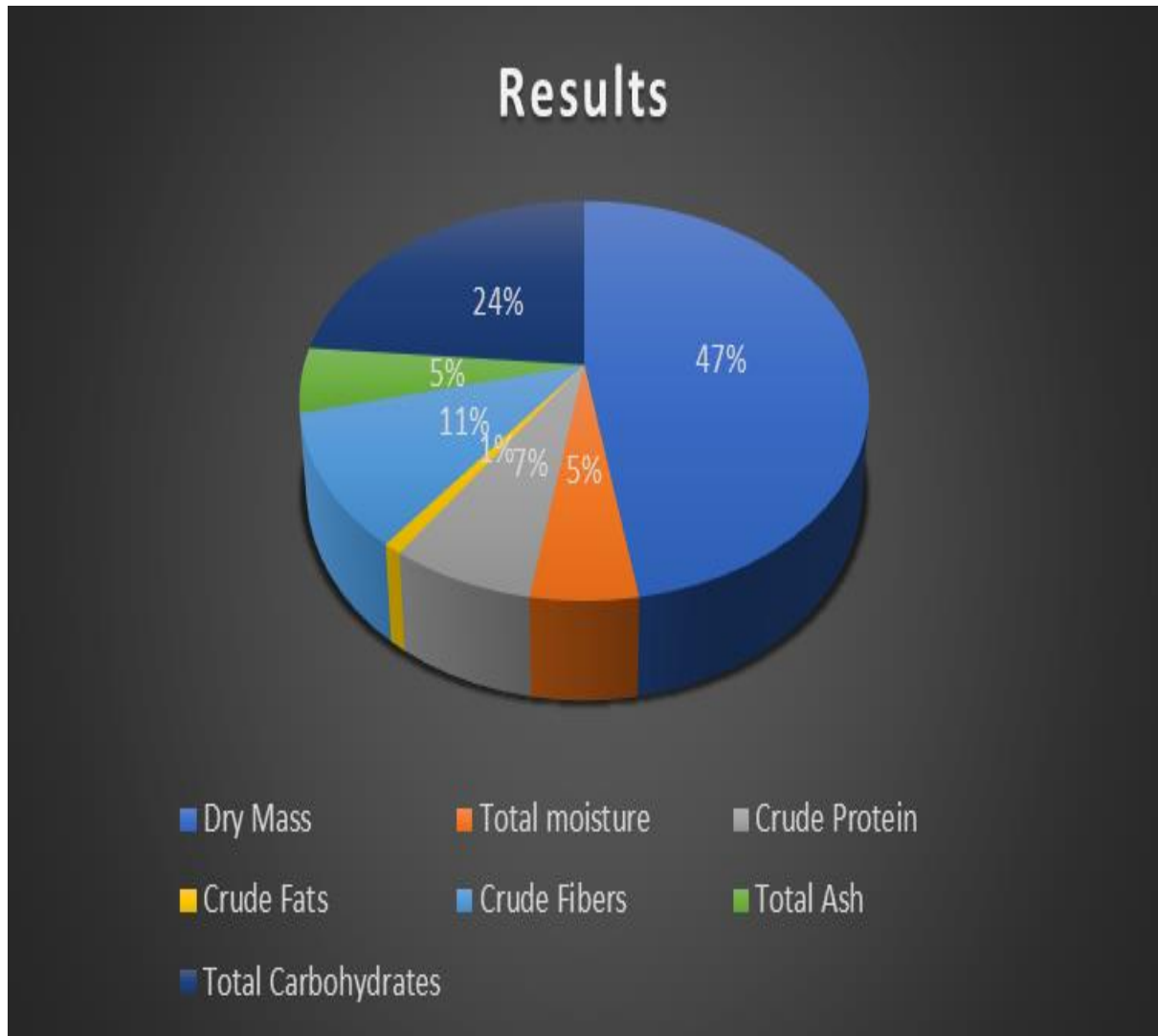
The total fibers in the leaves of *C.Tinctoria* was **14.5 %** while the total carbohydrates recorded in this medicinal plant was **44.87%**.

Table 1: Proximate Analysis (%) of plant sample.

	Dry Mass	Total moisture	Crude Protein	Crude Fats	Crude Fibers	Total Ash	Total Carbohydrates
<i>Chrozopora tinctoria</i>	86.68	10.1	21.88	4.7	14.5	21.5	44.77
	86.9	9.92	21.83	3.98	14.15	21.2	44.94
	86.11	9.94	21.84	3.96	14.12	21.8	44.90
Results	86.12	9.96	21.85	4.00	14.11	21.5	44.87

Table 2 Proximate Analysis (%) of plant sample.

Figure 8 Proximate Analysis (%) of plant sample.



3.4 MYCOTOXIN ELISA TEST KITS



Figure 09: MYCOTOXIN ELISA TEST KITS

3.3.2 How the test works

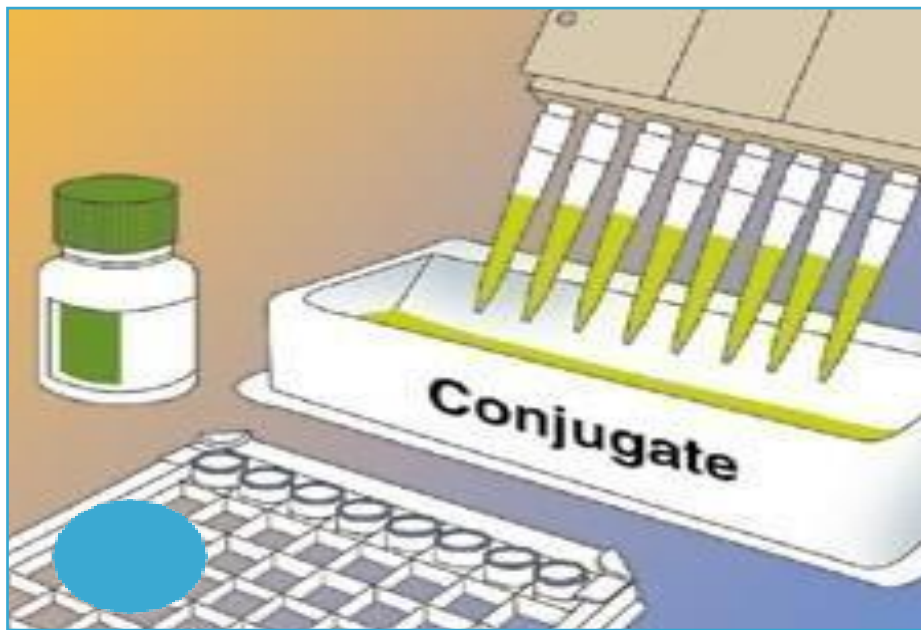
Mycotoxin products are direct competitive enzyme-linked immunosorbent assays (ELISA). One universal sample extraction using a 70% methanol solution can be applied to analyze six classes of mycotoxins with eight different kits. Some kits apply a dilution of the sample extract to reduce matrix effects. After combining sample extract or calibrators and enzyme conjugated toxin in the antibody coated well the competitive reaction takes 10 – 60 min (first incubation). Subsequent washing removes unbound reactants followed by the addition of substrate starting a color reaction that takes 5 – 20 min (second incubation). The intensity of the color is inversely proportionate to the concentration of mycotoxin in the sample or standard. A stop solution prevents further development of the color reaction enabling reproducible measurements of the intensity using a microwell reader. Results can be interpreted by using Romer Labs spreadsheets

Kit contents

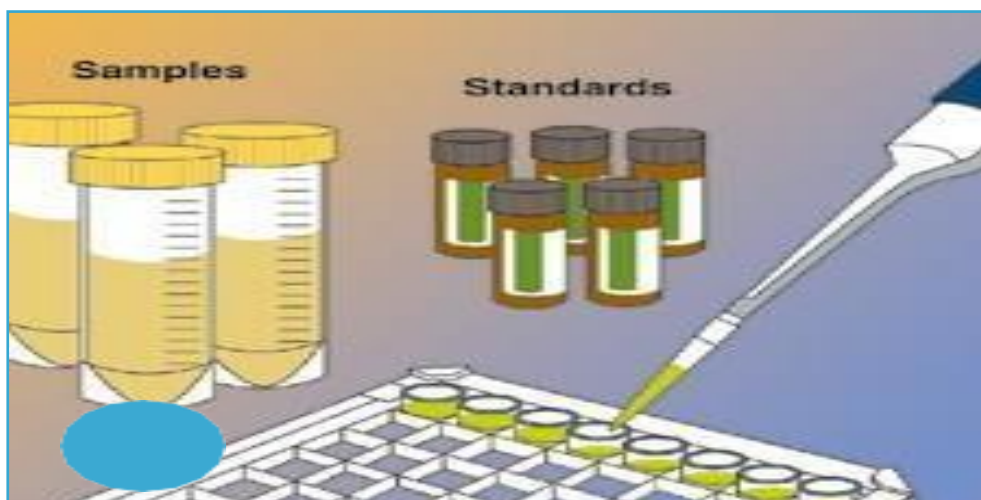
Each kit comes complete with 5 to 6 standards, 48 or 96 antibody-coated microwells, 48 or 96 color-coded dilution microwells, conjugate, substrate and stop solution

3.3.3 Mycotoxin ELISA Test

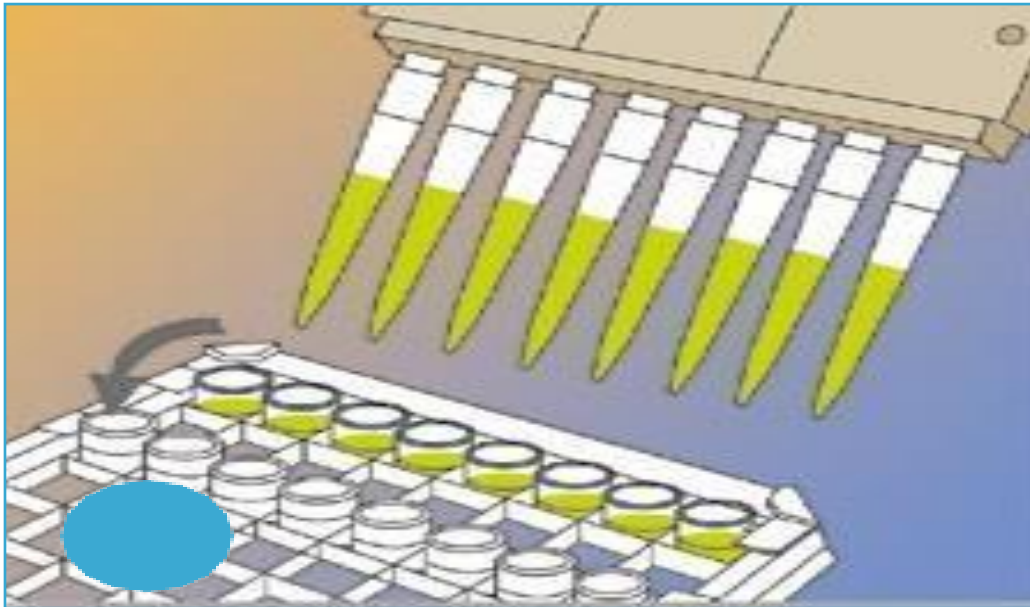
Step.1. Add 200 μ L conjugate into each color-coded dilution well.



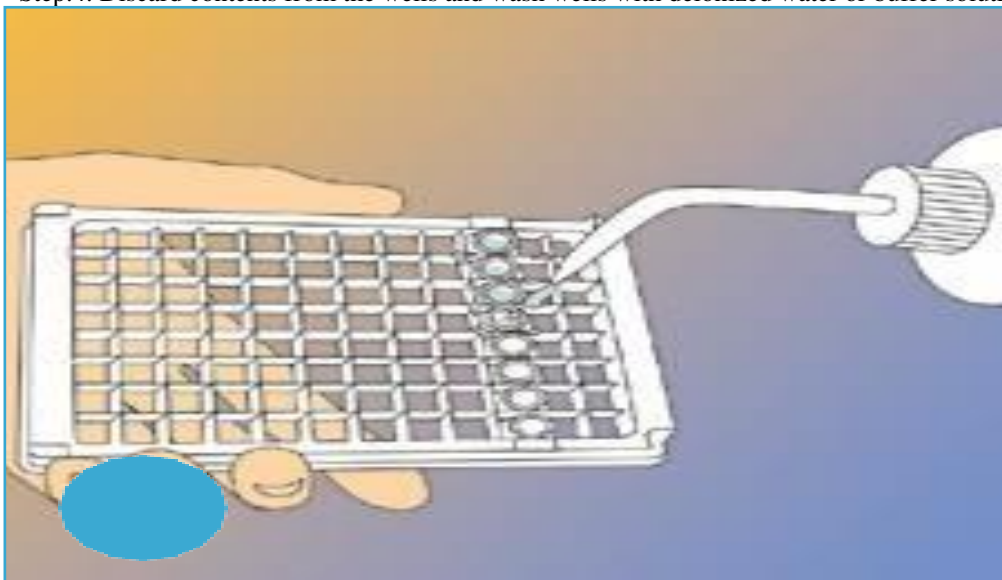
Step.2. Add 100 μ L standards or samples to the conjugate



Step.3. Mix well. Transfer 100 μ L content to antibody-coated wells. Incubate for 5 – 60 minutes.



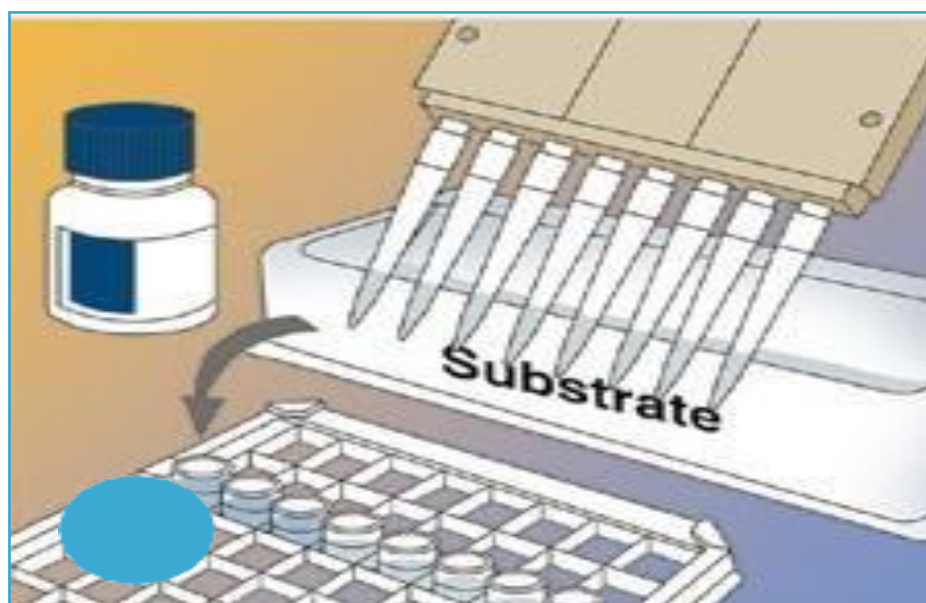
Step.4. Discard contents from the wells and wash wells with deionized water or buffer solution (5x).



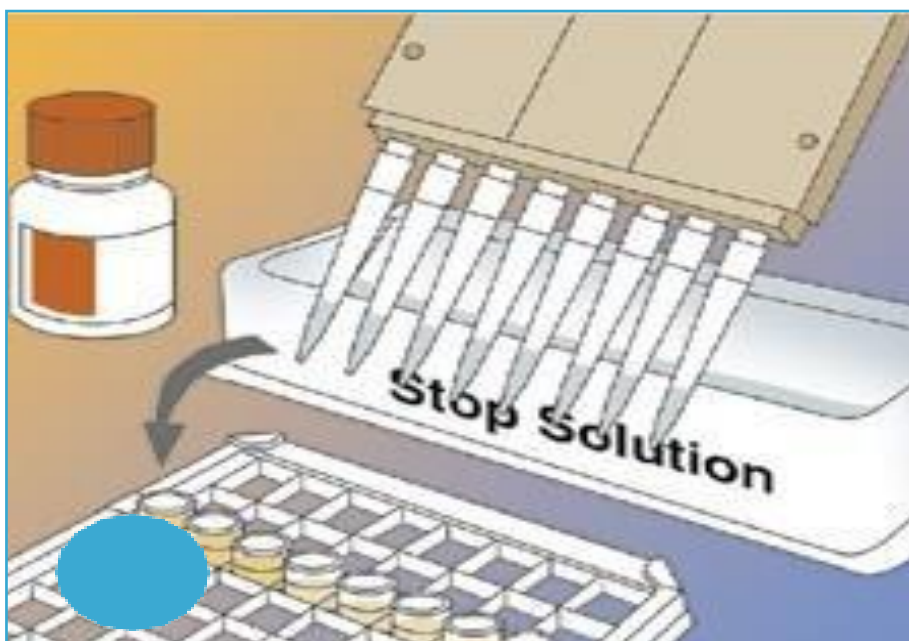
Step.5. Tap dry the wells on absorbent paper towel.



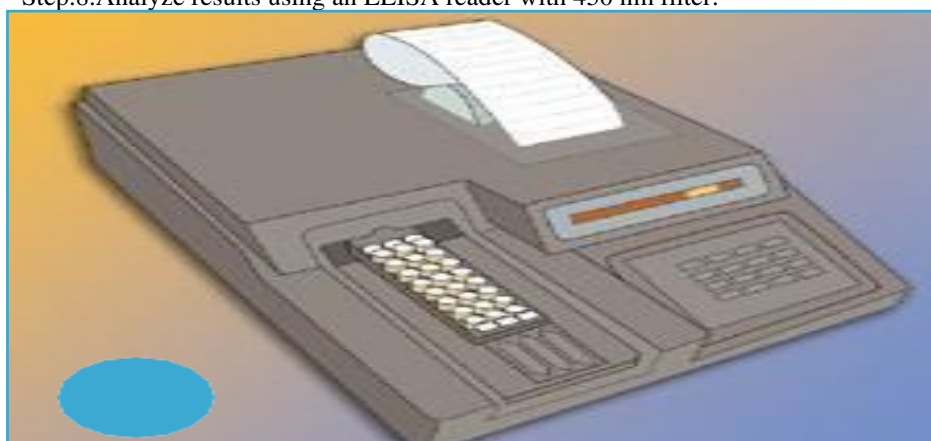
Step.6. Add 100 μ L substrate into each well. Incubate for 5 – 20 minutes



Step.7. Add 100 μ L stop solution into each well.



Step.8. Analyze results using an ELISA reader with 450 nm filter.



RESULTS

Plant Name	Aflatoxin B1 Value (ppb)
<i>Chrozopora tincatoria</i>	14%

3.3.4 STATISTICAL ANALYSIS:

The analyses were done in triplicates and the data obtained were expressed as mean $\pm$ standard error of the means (mean $\pm$ S.E.M). The data were subjected to one way analysis of variance (ANOVA) and differences between samples were determined by Tukey HSD, LSD, Sidak, Bonferroni, Scheffe, Duncan multiple range test (Duncan, 1955). Significant was declared if $P \leq 0.05$.

4 DISCUSSION

The plant's leaves in its dried form have moisture contents of 9.80% which is comparably equal to the moisture content of *Carica papaya* 10.40% (Rita Nath *et al*: 2016), *Jatropha curcas* 11.09% (Asuk AA *et al*: 2015), *Atheris hispida* 11.02%, and *Acalypha manginata* 10.82% reported by (Iniaghe OM *et al*: 2009), and this value is higher than 6.65%, 4.36%, 3.71% of *Corriandrum sativum*, *Pistacia vera* and *Spharanthus hirtus* respectively reported by This value is however much lower than the moisture content of *Manihot esculenta* 51.6% (Lawal *et al*: 2017) and 70.5% reported in the leaves of *Ipomoea specie* The high moisture concentration provides a way for the microbial contamination because of the greater activities of the water-soluble enzymes and co-enzymes in high moisture concentration in these leaves. Therefore, to hinder the microbial contamination, it must be stored in cool condition (Umer *et al*, 2007).

When organic material is incinerated at high temperature (500-600°C), the remaining residue is the ash which consists of salts and oxides of anions like chlorides, phosphates, sulphates and other halides and cations like iron, sodium, calcium, potassium, magnesium, and manganese. These are important constituents of animal diet. Present ash analysis showed that average ash contents of herbs were 10.16% (*Convunvulus Prostratus*), 8.2% (*Portulaca quadrifida*), 7.5% (*Taraxacum officinale*) while woody plants like *Albizia lebbeck* had (10.4%), *Olea ferruginea* (9.3%), *Salvadora oleoides* (9.56%), *Suaeda fruticosa* (8.26%) while in *Vitex negundo* 08% ash content (Table 14). Statistical analysis (t.test) showed little amount of ash contents at three phonological stages of herbs and woody species (Offor C.E *et al*, 2015).

Proteins are fundamental components of all living cells because it is the building unit of enzymes, hormones, and antibodies which are necessary for the proper functioning of an organism. For growth and repair of tissue proteins are essential in the diet of animals. All the nitrogenous compounds present in forage feed is reliable source of overall nutritional status referred as crude protein. They are directly related to digestibility, calcium, vitamins, and phosphorus requirements of body (Ganskopp & Bohner, 2003; Hussain & Durrani, 2009a). Present studies showed that crude proteins in herbs were 6.9% (*Convunvulus Prostratus*), 6.4% (*Portulaca quadrifida*), 10.26% (*Taraxacum officinale*), while in woody plants like *Albizia lebbeck* were 8.23%, *Olea ferruginea* (8.5%), *Salvadora oleoides* (9.6%), *Suaeda fruticosa* (10.86%) and in *Vitex negundo* 11.16% (Table 14). Statistical analysis (t.test) showed that little amount of crude protein at three phonological stages of herbs and woody species (Offor C.E *et al*, 2015).

The crude fats in the *Chrozopora tincatoria* leaves is 1.70% and this is nearly equal to the findings of (Akindahunsi and Salawu, 2005) from *Talinum triangulare* 1.09% but this value is higher than the *Zizyphus vulgaris* 0.41%, *Ammonum sulbulatum* 2.07%, *Spharanthus hirtis* 3.68% as reported by (Hussain *et al*, 2009) and from the leaves of *Ipomoea batatas* 0.74% reported by (Asibey-Berko and Tayie, 1999). However, this value is lower than the findings of Hussain *et al* (2009) from *Corriandrum sativum* 9.83%, from the findings of *Parquetin nigrescen* 11.30%, *Morinda lucida* 10.20% as reported by (Abiodun Bukunmi Aborisade *et al*, 2017) and from *Valeriana officinalis* 14.35% as reported by (Adnan *et al*: 2010). According to the report of (Antia *et al*: 2006) the dietary fats increase the palatability of food by absorbing and retaining flavour but excess fats consumption causes certain cardiovascular disorders and aging (Ngaha *et al*: 2016).

Fibres are the indigestible part of the diet. The fibres cause the peristaltic motion in the gastro-intestinal tract and normalize and ease the bowel movement in GIT. The plants and the plants product are the good source of dietary fibres when consumed. The intake of adequate fibres can lower the cholesterol level, hypertension, heart diseases, diabetes, constipation, breast cancer and colon cancer. (Ishida *et al*: 2000). The total fibres in the leaves of *M.phillipensis* was 20.5% which is relatively very high when compared with *Gongronema latifolium* 4.20% reported by (Offor C.E *et al*, 2015), *Atheris hispida* 10.25% reported by (Iniaghe O.M *et al*, 2009), *Manihot esculenta* leaves 7.87% reported by (Etong *et al*: 2006), *Mentha sylvestris* 6.47% and *Punica granatum* 4.44% as reported by (Hussain *et al*: 2009). The studied plant sample have a comparable fibres content with that of *Phlomis bracteosa* 24.50%, *Phlomis cashmeriana* 23.96% reported by (Hussain *et al*: 2010) and *Ceratotheca sesamoides* 25.75% reported by (Fasakin K: 2004). However, the sample has low proportion of crude fibres

compared to that of *Piliostigma thionningii* 35.03% as reported by (Ene-Obong and Carnovale: 1992) and *Alchornea cordifolia* leaves 33.58% reported by (Ngaha *et al*: 2016).

Carbohydrate is the main and primary source of energy for all living organisms and an essential nutrient required for adequate diet (Emebu and Anyika, 2011). Carbohydrates supplies energy to the cells such as brain, muscles, and blood (Ejelonu *et al*: 2011). Low level of carbohydrates in the diets of animals could lead to hypoglycaemia (Vasudevana and sreekumari, 2007). Our tested sample of *Chrozophora Tinctoria* leaves contain 44.87% carbohydrates however various researches have reported higher carbohydrates content in different plants. For instance, (Lawal *et al*: 2017) reported 68.83% carbohydrate content in *Manihot esculenta*, (Hussain *et al*: 2010) reported 63.64% and 62.85% in *Dalbergia sisso* and *Phlomis cashmeriana* respectively and (Achi *et al*: 2017) reported 73.77% carbohydrates in the *Ficus capensis* leaves. But our value is higher than *Chrozophra Tinctoria* fruit 13.6% reported by (Khattak K.F: 2012) and *Pistacia vera* 21.49% reported by (Hussain *et al*: 2009). However, our sample value is nearly equal to *Acalypha marginata* 38.24% reported by (Iniaghe *et al*: 2009) and *Parquetin nigrescen* 36.03% reported by (Abiodun Bukunmi Aborisade *et al*: 2017).

CONCLUSION:

The analysis of the studied plants *Chrozopora tincatoria* revealed that this plant contains high nutritional composition. This plant contains a huge amount of carbohydrates (as a primary source of energy) and a good source of proteins (secondary source of energy). The high fiber content makes it medicinally important as the fibers helps in elimination of various chronic and serious disorders. Kamala powder, obtained from the skins of the fruits, is used as anthelmintic. Kamala powder, obtained from the skins of the fruits, is used as a preservative for vegetable oils and dairy products. All parts of the tree can be applied externally to treat parasitic infections of the skin. The fruits and bark have been reported to be used medicinally to treat stomach ulcers and tapeworm. A decoction of the leaves is used in the treatment of diarrhea. Root scrapings are chewed with a betel mixture as a contraceptive for women.

RECOMMENDATION

The given research study included proximate and ELISA determination of aflatoxin B1 analysis. Aflatoxin is the type of mycotoxin. I worked only one type of mycotaxins (Aflatoxin B1) they are found in agricultural crops such as maize,peanuts,cotton seed etc.For further research one may also study on other types of mycotoxins such as Deoxynivalenot (vomitoxin), fumonisin (FB1,FB2 FB3), zearalenone, ochratoxine, ochratoxi (ochratoxin A), patulin, citrinin and trichothecenes.

Conflict of Interest: All authors declared no conflict of interest.

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APPENDIX A

ANOVA for result of proximate and ELISA analysis

Analysis of Variance Table for V001 Statistix 10.0 (30-day

Source	DF	SS	MS	F	P
V003	7	109.500	15.6429	15.17	0.0000
Error	16	16.500	1.0313		
Total	23				

Note: SS are marginal (type III) sums of squares

Grand Mean 4.8750
CV 20.83

Analysis of Variance Table for V002

Source	DF	SS	MS	F	P
V003	7	1.2500	0.17857	0.19	0.9825
Error	16	14.7500	0.92188		

Total 23

Note: SS are marginal (type III) sums of squares

Grand Mean 2.0937
CV 45.86

Statistix 10.0 (30-day Trial)

1/3/2022, 10:36:37 AM

Tukey HSD All-Pairwise Comparisons Test of V001 for V003

V003	Mean	Homogeneous Groups
27	8.0000	A
13	7.0000	AB
15	7.0000	AB
14	5.5000	AB
21	4.5000	B
4	4.0000	BC
10	2.0000	CD
89	1.0000	D

Alpha	0.05	Standard Error for Comparison	0.6555 TO 1.4361
Critical Q Value	4.903	Critical Value for Comparison	2.2726 TO 4.9789
There are 4 groups (A, B, etc.) in which the means are not significantly different from one another.			

Tukey HSD All-Pairwise Comparisons Test of V002 for V003

V003	Mean	Homogeneous Groups
15	3.0000	A
27	2.0000	A
13	2.0000	A
21	2.0000	A
4	2.0000	A
10	2.0000	A
89	2.0000	A
14	1.7500	A

Alpha	0.05	Standard Error for Comparison	0.6198 TO 1.3578
Critical Q Value	4.903	Critical Value for Comparison	2.1487 TO 4.7075
There are no significant pairwise differences among the means.			

Statistix 10.0 (30-day Trial)

1/3/2022, 10:37:08 AM

LSD All-Pairwise Comparisons Test of V001 for V003

V003	Mean	Homogeneous Groups
27	8.0000	A
13	7.0000	AB
15	7.0000	AB
14	5.5000	BC
21	4.5000	C
4	4.0000	C
10	2.0000	D
89	1.0000	D

Alpha	0.05	Standard Error for Comparison	0.6555 TO 1.4361
Critical T Value	2.120	Critical Value for Comparison	1.3896 TO 3.0445
There are 4 groups (A, B, etc.) in which the means			

are not significantly different from one another.

LSD All-Pairwise Comparisons Test of V002 for V003

V003	Mean	Homogeneous Groups
15	3.0000	A
27	2.0000	A
13	2.0000	A
21	2.0000	A
4	2.0000	A
10	2.0000	A
89	2.0000	A
14	1.7500	A

Alpha	0.05	Standard Error for Comparison	0.6198 TO 1.3578
Critical T Value	2.120	Critical Value for Comparison	1.3139 TO 2.8785

There are no significant pairwise differences among the means.
Statistix 10.0 (30-day Trial) 1/3/2022, 10:37:34 AM

Scheffe All-Pairwise Comparisons Test of V001 for V003

V003	Mean	Homogeneous Groups
27	8.0000	A
13	7.0000	AB
15	7.0000	AB
14	5.5000	AB
21	4.5000	B
4	4.0000	BC
10	2.0000	BC
89	1.0000	C

Alpha	0.05	Standard Error for Comparison	0.6555 TO 1.4361
Critical F Value	2.657	Critical Value for Comparison	2.8271 TO 6.1938

There are 3 groups (A, B, etc.) in which the means are not significantly different from one another.

Scheffe All-Pairwise Comparisons Test of V002 for V003

V003	Mean	Homogeneous Groups
15	3.0000	A
27	2.0000	A
13	2.0000	A
21	2.0000	A
4	2.0000	A
10	2.0000	A
89	2.0000	A
14	1.7500	A

Alpha	0.05	Standard Error for Comparison	0.6198 TO 1.3578
Critical F Value	2.657	Critical Value for Comparison	2.6730 TO 5.8561

There are no significant pairwise differences among the means.
Statistix 10.0 (30-day Trial) 1/3/2022, 10:37:55 AM

Sidak All-Pairwise Comparisons Test of V001 for V003

V003	Mean	Homogeneous Groups
27	8.0000	A
13	7.0000	AB
15	7.0000	AB
14	5.5000	AB

21	4.5000	B
4	4.0000	BC
10	2.0000	BC
89	1.0000	C

Alpha 0.05 Standard Error for Comparison 0.6555 TO 1.4361
Critical F Value 2.657 Critical Value for Comparison 2.8271 TO 6.1938
There are 3 groups (A, B, etc.) in which the means are not significantly different from one another.

Sidak All-Pairwise Comparisons Test of V002 for V003

V003	Mean	Homogeneous Groups
15	3.0000	A
27	2.0000	A
13	2.0000	A
21	2.0000	A
4	2.0000	A
10	2.0000	A
89	2.0000	A
14	1.7500	A

Alpha 0.05 Standard Error for Comparison 0.6198 TO 1.3578
Critical F Value 2.657 Critical Value for Comparison 2.6730 TO 5.8561
There are no significant pairwise differences among the means.
Statistix 10.0 (30-day Trial) 1/3/2022, 10:38:17 AM

Bonferroni All-Pairwise Comparisons Test of V001 for V003

V003	Mean	Homogeneous Groups
27	8.0000	A
13	7.0000	AB
15	7.0000	AB
14	5.5000	AB
21	4.5000	BC
4	4.0000	BCD
10	2.0000	CD
89	1.0000	D

Alpha 0.05 Standard Error for Comparison 0.6555 TO 1.4361
Critical T Value 3.740 Critical Value for Comparison 2.4515 TO 5.3709
There are 4 groups (A, B, etc.) in which the means are not significantly different from one another.

Bonferroni All-Pairwise Comparisons Test of V002 for V003

V003	Mean	Homogeneous Groups
15	3.0000	A
27	2.0000	A
13	2.0000	A
21	2.0000	A
4	2.0000	A
10	2.0000	A
89	2.0000	A
14	1.7500	A

Alpha 0.05 Standard Error for Comparison 0.6198 TO 1.3578
Critical T Value 3.740 Critical Value for Comparison 2.3178 TO 5.0781
There are no significant pairwise differences among the means.