The Effect of *Andrographis paniculata* leaves extract on, Colour Enhancement and Caudal fin Regeneration in Wild Guppies (*Poecilia reticulata*).

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DECLARATION BY STUDENT

I hereby declare that the data presented in this dissertation report entitled , "The Effect of *Andrographis paniculata* leaves extract on ,Colour enhancement and Caudal fin Regeneration in Wild Guppies (*Poecilia reticulata)*" is based on the results of investigations carried out by me in the M.Sc. Zoology at the School of Biological Science and Biotechnology, Goa University under the Supervision of Ms .Gandhita V Kundaikar and the same has not been submitted elsewhere for the award of a degree or diploma by me. Further, I understand that Goa University or its authorities will not be responsible for the correctness of observations/ experimental or other findings given in the dissertation.

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COMPLETION CERTIFICATE

This is to certify that the dissertation report "The Effect of *Andrographis paniculata* leaves extract on ,Colour enhancement and Caudal fin Regeneration in Wild Guppies (*Poecilia reticulata)*" is a bonafide work carried out by Ms. Anishka A Khandeparkar under my supervision in partial fulfilment of the requirements for the award of the degree of Masters in the Zoology discipline at the School of Biological Science and Biotechnology , Goa University.

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PREFACE

Ichthyology and ornamental fisheries have always been an interest of my topic since my graduation. The aspiration to be well –versed with knowledge of this field has always inspired me to keep taking up minor research works. While studying my academic papers brought to my realisation, how relatively less focus is developed with respect to ailments in fishes and how most of the diseases cause a huge mortality in them. Since, the recent trend is organic, naturopathy, my idea was to research a similar kind of treatment basis for fishes as well, where a potent drug from medicinal plants can be used for its potency to treat fishes. Being from state, where very less explorations have been done with respect to our cultural science, I decided to choose one of the well known medicinal plant in Goa, *Andrographis paniculata* as my model plant for study. I am most grateful, to my guide who always encouraged me and also made me realise and explore my inner capabilities.

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Tables and Figures

Tables

Figures

ABBREVIATION USED

Entity	Abbrevation
Andrographis paniculata	AP
Ash Content	AC
Astaxanthin	AX
Control	C
Control positive	C+
Concentration	conc
Crude Proteins	CP
Crude Fats	CF
Crude Fibres	Cf
Days post amputation	dpa
Dichloromethane	DCM
Experiment I	EI
Experiment II	EII
Experiment III	EIII
Grams	g
Lambda maximum	λ max
Litre	L
Methanol	Met
Micrograms	µg
Milligrams	mg
Millilitre	ml
Minutes	mins
Optical Density	OD
Moisture Content	MC
Thin Layer Chromatography	TLC
Total Carbohydrates	TC
Ultraviolet	UV
Unknown	Uk

ABSTRACT

Andrographis paniculata is a medicinal plant Indigenous to South and South –East Asia. It is well renowned as '*kalmegh* . A plant with exemplary antioxidant properties, has not been fully researched in field of Aquaculture to its potential. The current study, focuses on to replacing the market's high demanded synthetic pigments with *Andrographis paniculata* both as source of pigmentation and source of antioxidant in ornamental fishes. The study conducted shows the effective nature of this plant leaves extract in inducing high rate of regeneration in amputated caudal fin of wild guppies, and its effect of colour enhancement in integument of Guppies, compared to Astaxanthin, which was used as positive control. The dose of 45mg/100g *Andrographis paniculata* fish feed concludes to be the effective dosage in inducing regeneration and the dose of 90mg/100g and above of *Andrographis paniculata* as beneficial colour enhancer in Guppies. This suggests a use of medicinal plants in field of Ichthyology in treating ailments not only a natural but more environmental friendly solutions in Ornamental fishkeeping.

CHAPTER 1: INTRODUCTION

1.1BACKGROUND

Since the beginning of time, humans have raised animals. One of the most adored and desired pet animals are fish. One of the earliest gnathostomes, fish are aquatic, limbless vertebrates. They are raised for their stunningly altered fins and the range of colours they exhibit. Fishkeeping has been documented archaeologically as early as 2500 BC by the Sumerians and 500 BC by the Babylonians. In addition to being raised for their auspiciousness, fish are raised for their aesthetic value in homes. The Nile perch was said to be revered by Egyptians. The Chinese (960–1279) were the ones who first popularized fish breeding. Tullock reported seeing them in a bowl. Florida was amongst the first to host the commercial fish culture in the 1920s. Many Asian cultures view fish as sacred and auspicious; Japan, for instance, views the Koi fish as lucky and recognizes it as their national animal. Similar to this, fish is central to many cultures and traditions in India's Puranic traditions, *Matsya* is an important incarnation of Lord Vishnu. They are also considered one of the *Ashtamangala* in many religions, like Jainism, Buddhism, and Hinduism (Belcher, 2018).

Based on their purposes, fish can be broadly categorized into two groups. Fish that are ornamental and edible. In aquariums, ornamental fish are solely kept as pets and are not consumed. Because of the fascination and market demand for ornamental fish, one of the most successful industries in the world is fishkeeping, which encompasses aquaculture techniques like breeding, culturing, and hatcheries. A wide variety of ornamental fish can be found in India's marine and inland waters. Around 155 varieties were reported from North-East and and around 40 reported from the Western ghats (NFDB, 2014). Exotic ornamental

fish are also in high demand in India. Examples include goldfish (*Carassius auratus*), Discus (*Symphysodon sp*.), Mollies (*Poecilia sphenops*), Guppies (*Poecilia reticulata*), Tetras (*Paracheirodon axelrodi*), Flowerhorn (*Amphilophus sp*.), Oscar (*Astronotus ocellatus*), etc. There is another well known fish *Gambusia affinis* (Mosquito fish) indigenous to North America commonly mistaken for guppies. But they are completely two different genus , belonging to same family.

Table 1.1.1: Showing names of some indigenous ornamental fishes of India.

Indian ornamental fish	**Names of the fishes**
Freshwater fishes	*Channa orientalis, Nandus nandus,Notopterus notopterus, Garra gotyla gotyla , Puntius filamentosus,* etc
Marine Fishes	C*helonodon patoca, Ostracion cubicus, Pomacentrus caeruleus, Narcine timlei , Thalassoma lunare*
Brackishwater	*Scatophagus argus, Monodactylus argentus, Orange chromid, Ambassis nama*

One percent of the ornamental fishes traded worldwide comes from India. In 2020–21, 54 tons of these fishes were exported, valued at ₹13.08 crore. It showed an increase of 20.59% in value in Indian rupees and 66.55% in quantity (Ahilan et al., 2008). One of the most common aquarium fish kept in India are **Guppies *(Poecilia reticulata)*.** *The Greek word 'Poecilia' means speckled, while the Latin word 'reticulata' means a pattern resembling a net.* This fish is tropical and indigenous to South America. It is a live bearer with a life span of about two years (MPEDA, 2023). Their caudal fin exhibits remarkable phenotypic colours. In comparison to the females, the males have more vivid colours. Due to their compact size, low maintenance requirements, and hardy behaviour , guppies are currently

employed as model organisms in scientific research. Their IUCN status is least concerned. Guppies are categorized based on the colour, pattern, and structure of their scales. Examples of these include cobra, fancy, fantail, and lyre tail Guppies. Whereas wild Guppies are ones without any colour. They are *Poecilia reticulata* strains rather than distinct species. They have an average weight of 0.7–1 grams. They grow up to an average length of 0.6- 1 inch, maximum being 2.4 inches. They are omnivores and feed on anything ranging from algae to small plants; they even feed on mosquito larvae, hence the name mosquito fish. They mostly prefer live fish feed. They are also called million fishes, rainbow fishes due to their exemplary showcase of vivid colours (Hall, 2023).

1.1.1 IMPORTANNCE OF ORNAMENTAL FISHES

- Ornamental fish keeping is not just adding aesthetic touch to various houses
- It is a source of foreign exchange earnings.
- It helps to raise the economies of developed and developing countries, with export trade about 0.38 million US dollars in India, with India having perfect climate and labour availability (NFDB, 2014).
- The total estimated ornamental fish-keeping industry is worth of 14 Billion US dollars (Thomas, 2008)
- Generates employment opportunities and also self-employment opportunities.
- In the International market, Guppies are the dominant fish, followed by tetras.
- Multiple studies have shown gazing at aquarium fishes have health benefits to humans like, reduce blood pressure, therapeutic effects like reduced stress and anxiety level, and have also shown to be beneficiary to Alzheimer patients (Sharpe, 2022).

1.1.2 Problems faced in the aquaculture sector of ornamental fish keeping.

Due to the ever-increasing demand for ornamental fishes in market, the number of fish in the rearing business has to double. Culturing double-fold number of fish on the limited land available makes it a challenging situation. These lead to overstocking of fish in the tanks, so more profit can be made with the minimal available resources. Also, fish are artificially bred again and again to avail large amount of fish seed, as possessing the fish seed in wild is a problem due to mixing and deteriorating conditions of aquatic habitats in wild. Many experiments are conducted to increase the efficiency of fish feed, leading to a shift of field of research towards fish feed. Also, the intensive fish cultivation method has high involvement of artificial systems, leading to less exposure of fishes to natural products and natural environment.

This has led to three main problems in fisheries in culture

- Diminishing colour is seen in fish due to lack of nutritional and natural feed, continuous artificial breeding, and inbreeding depression done to maintain homogeneity in the genome and produce purebred.
- The second problem faced is due to competition and over-stocking of fishes, which leads to injuries, loss of fins during fight, lesions, etc. Though, fish have the natural ability to regenerate their fins, but stress and other abiotic factors can slow down this process.
- The third problem to be focused upon is, due to poor water quality, poor feed, and injuries, the growing bacteria, fungus, and parasites can invade the body of fish and cause serious illness. Which will cause loss not only to fishes but also cause loss in fish businesses, and the transfer of such pathogens to humans can also pose the next level of threat.

1.1.3 Colour Physiology

Colour is one of the many factors that add beauty to ornamental fish. Ornamental fish come in various colours, and this determines their economic value in the market. Colouration in fish is important for their camouflage, courtship behaviour, and flash warning signs to predators (Hubbard, 2010). Fish have specialised star-shaped cells embedded in the skin or on the surface of the skin below scales. These star-shaped cells are called chromatophores. Chromatophores store different pigments within them, and based on that, they have been assigned various names. Pigments are the compounds responsible for imparting colour in organisms. Pigments are naturally found in algae, plants, and fungi. No animal can synthesise the pigments and therefore derive them from their diet. Fish feed on algae, crustaceans, and small other fish, which become the source of pigment for them. The types of pigments found in nature are mainly two types:

- Photosynthetic
- Non-photosynthetic

Photosynthetic pigments: Chlorophyll, the one responsible for the green colour in plants, is responsible for photosynthesis. There are four types of chlorophyll pigments: a,b, c, and d.

Non-photosynthetic pigments: Carotenoids

Carotenoids comprise a huge group of 600 pigments, further divided into Carotenes and Xanthophylls. Hydrocarbons with 40 carbon atoms, carotenoids are made up of two terminal ring systems connected by a conjugated double bond chain, or poliene system (Urich, 1994). Among these, the oxygenated species are called Xanthophylls; Zeaxanthin, Canthaxanthin, and Astaxanthin are some well-known examples of xanthophylls. And non- oxygenated species are Carotenes, e.g., Beta-carotene (Higuera-Ciapara et al., 2006). Colour enhancement is this property that increases the pigment concentration in the chromatophores. The process of colour

change in fish is controlled by a combination of hormonal, neural, and environmental factors. Nerve impulses originating from the brain can stimulate or inhibit chromatophores. Nerve signals are transmitted to the chromatophores by fish when it detects changes in its surroundings or reacts to specific stimuli, which causes the fish to change colour. Endocrine system-produced hormones have the ability to enter the bloodstream and influence chromatophore's activity either directly or indirectly. Chromatophores contain pigments such as melanin (black or brown), carotenoids (red, orange, or yellow), and purines (reflective or iridescent). When a colour change is initiated, the chromatophores undergo a process called **pigment migration.** This involves the movement of pigment granules within the chromatophores, altering the colour displayed on the fish's body. In some fish species, there are specialised muscles associated with chromatophores. These muscles can stretch or contract the chromatophores, influencing the extent of pigment exposure on the fish's surface. By controlling the degree of pigment exposure, fish can create different patterns and colour intensities (Plate1.1). Fish mostly retains pigments in skin in form of esters. The amount of carotenoids a fish can retain in its body depends on various factors like size of the fish, sex, species etc. Uptil now pigment retension % has been reported in Salmonids and Rainbow trout (Torrisen et al., 1989). Carotenoids are hydrophobic and therefore , maximum digestion occurs in small intestine , by bile juices. The absorbed digested carotenoids are stored in liver. And transported as lipoproteins to skin (Torrisen et al., 1989). They are either directly deposited in the fish chromatophores, or first converted through cellular metabolism (Sathyaruban et al., 2021).

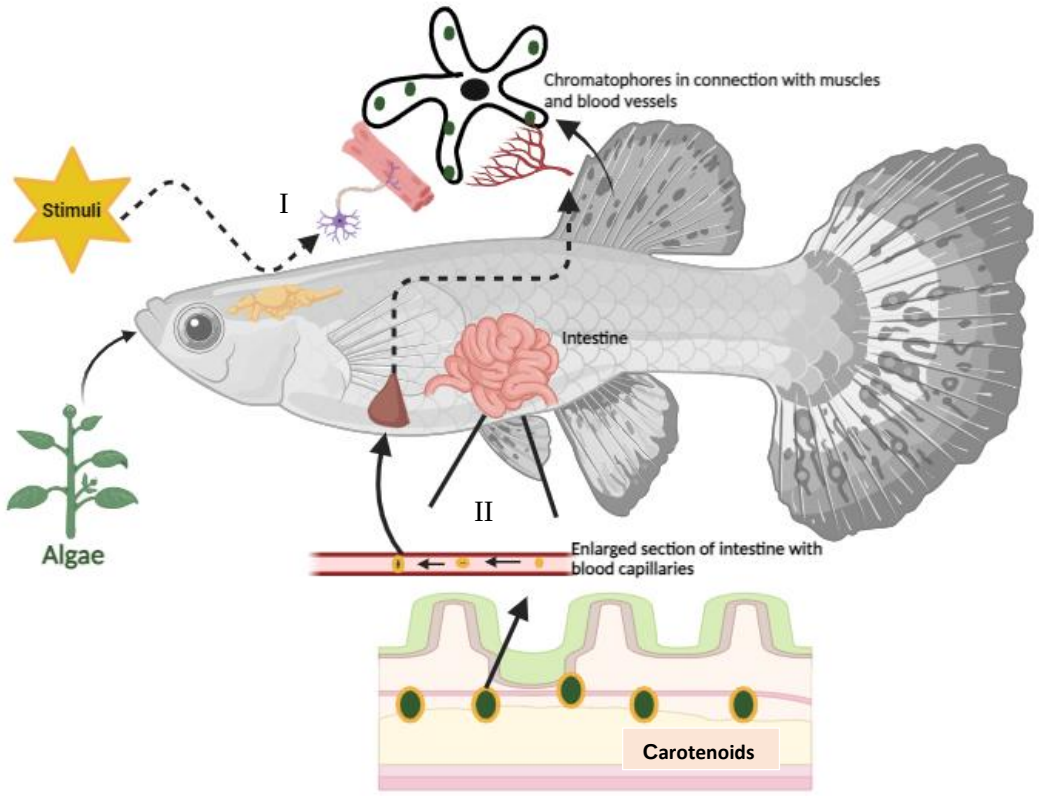


Fig 1.1.1: Pictorial representation of pigment migration in guppies. The picture depicts the pigment migration process in fishes, via (I) neural-hormonal mode and via (II) pigment digestion mode.
Designed using BioRender app.

1.1.3.a Sources of pigments in Ornamental industry.

The most widely used pigment for colour enhancement in fish is Astaxanthin. Synthetic astaxanthin is known to be the most traded carotenoid, accounting for 25.70% of the global market for pigments in 2017. (FIORMARKETS global industry market analysis, 2017). It is responsible for the red colour and is widely used in the fish feed industry for fish like Cichlids (Kop & Durmaz, 2008), Koi carp (Sun et al., 2012), and Dwarf gourami (Baron et al., 2008).

For economical use of astaxanthin, they are highly extracted from the red algae ,the crustaceans waste,and yeast. Shrimp meal is the most widely used source of astaxanthin. Because of this, a low-cost method of extracting the pigments that can be used to provide fish feed that enhances colour is required.

Fish acquire their natural colouration through the ingestion of Astaxanthin, which is biosynthesized by microalgae in the aquatic environment and subsequently consumed by zooplanktons, insects, or crustaceans (Lorenz, 1998). Since farmed fish and crustaceans lack access to natural sources of Astaxanthin, their diet must provide all of the Astaxanthin they require, or an alternative source.

1.1.3.b Astaxanthin

Astaxanthin is a pigment belonging to the group xanthophylls, which are synthesised in plants from lycopene (Higuera-Cipara et al., 2006). They are known for their colour enhancement properties in fish and also have antioxidant properties surpassing those of carotene and a-tocopherol (Miki, 1991). Due to its outstanding antioxidant activity, it has been attributed with extraordinary potential for protecting the organism against a wide range of ailments, such as cardiovascular problems, different types of cancer, and some diseases of the immunological system. (Higuera-Cipara et al., 2006).

1.1.3.c Problems associated with use of synthetic or natural astaxanthin.

Due to its vivid properties like antioxidant, colour-enhancing, and growth-enhancing properties, the demand for astaxanthin in the market is very high. The astaxanthin market is projected to reach 965.8 million US $ in 2006, with CAGR of 8.3%. (Markets and Markets: Global forecast to 2026**,** 2012).

They are present naturally and synthetically, but due to high cost of production involved in extracting them from natural sources , now they are synthetically produced using petrochemicals. Also, the whooping rate of natural astaxanthin is nearly 7000–20000/Kg, as seen in Indian markets alone. Therefore, this shift is seen in astaxanthin production, from a natural to a synthetic mode. Also, with rising concern for nature and its resources in the wild, overharvesting of natural sources of Astaxanthin like shrimp, algae, and probiotics has led to disruption in the ecosystem of the ocean. Also affecting the food chain and the food web. Looking into this problems, there have been various studies conducted where more natural plant-based products are researched upon to replace the oceanicts, such as Garlic, paprika (Yilmaz et al., 2013; Ebeneezar et al., 2020),

tomatoes (Maiti et al., 2017 ;Tiewsoh et al ., 2019) etc., which are well known and rich in pigments and can be exploited on large scale by increasing their production using upcoming environmentally friendly techniques like aquaponics, hydroponics, etc. Another con associated with high usage of Astaxanthin is its high cost in the market, which further increases the cost of the fish feed.

1.1.4 Regeneration

Regeneration in biology refers to the process of renewal, restoration, and tissue growth that enables organisms to recover from damage or disturbance. This process involves gene regulation, cell morphogenesis, and differentiation (Birbrair et al., 2013). It is seen in Plants and many of the animals, like Hydra, *Planaria*, fishes, salamanders , etc. As the complexity of body design increases, the regenerative capacity is seen to decrease in the organisms. Fishes show remarkable regeneration capabilities. There are three main types of regeneration patterns seen.

Epimorphic regeneration is a type of regeneration observed in higher-order species, where missing tissue is regenerated through cell proliferation at the injury site without affecting the structure of existing tissues. It involves forming a blastema, a mass of undifferentiated cells capable of giving rise to the regenerated tissues (Londono et al., 2018).

Morphallaxis is the regeneration by transformation, renewal, and rebirth of existing body tissues through the repatterning of existing tissues with little new growth, observed in lower animals (Gilbert, 2010).

Compensatory growth is a type of regenerative growth that occurs in several human organs after damage, removal, or when they cease to function. It can also be stimulated by increased functional demand in tissues and organs. Compensatory growth can involve increased cell size , increased cell division (Widmaier et al ., 2006).

Regeneration can also be classified into three types: (1) Physiological regeneration, which is tissue regeneration that takes place under normal conditions, i.e., not in response to injury. This is a

continuous process that supports the turnover of particular cells in a structure throughout the life of a given organism, maintaining the tissue homeostasis (e.g., the continuous replacement of cells in the epidermis and blood).

(2) Reparative regeneration, which refers to an organism's capacity to repair organs or tissues after a minor injury (e.g., healing of blood vessels and skin cells after a cut).

(3) Restorative regeneration (or "true regeneration"), which consists of the capacity to regrow a fully functional, scar-free structure after loss of that structure (e.g., regeneration of limbs, tail, external gills, and fins).

Fishes show Epimorphic regeneration, in amputated fins, tails, heart etc. (Yoshinari & Kawakami, 2011). Fish species with high regenerative ability include Zebrafish and certain teleost fish species such as the Medaka and the Senegal bichir. The African killifish has also been recently discovered as a novel vertebrate model that can regenerate its caudal fin and heart (Potts et al., 2021). Some ornamental fish, especially those from the Poecillidae family, show exceptional fin regeneration. Guppies are also known to show fin regeneration. Studies have shown regeneration of Guppy's, gonopodium, caudal fin etc. (Hopper, 1965). Ornamental fishes like Guppies are kept in pairs or schools. This sometimes cause injuries,to fin, scale, skin etc. This can cause

- Stress to the fish
- Fin Rot and further parasitic ailments like whirling diseases, Ichthyophonus etc.
- Death
- It may also lead to spread of diseases from one fish to the other
- And , ultimately decrease, the market value of the fish.

Usually the Guppies take 4-6 weeks for recovery from amputated fin , caused due to mechanical injury, a fungal infection may never recover,. During this period if the fish catches fungal infection, it can be detrimental (Fabian, 2021) .No specific medications are

currently known to directly promote fish fin regeneration. Various researches focuses on understanding the underlying molecular and cellular mechanisms of fin regeneration rather than pharmaceutical intervention (Kawakami, 2010). Many researchers are trying to address this issue using various plants products as natural source of medication (Mariappan et al., 2023). Carotenoids(Astaxanthin ,cantaxanthin) as a source of regenerating medication, almond leaves, and *Piper sarmentosum* are being researched in models like Zebrafish (Zainol-Abidin et al., 2020).Therfore, there is a necessity to study and explore the potency of various indigenous plants as source of feed additives in fishes (Das, 2016).

1.1.5 *Andrographis paniculata*

Andrographis paniculata is an indigenous plant of South and Southeast Asia. It belongs to the family Acanthaceae. It has quadrangular stem, reticulate leaf venation, raceme infloresence, and axile placentation. It has been used since ancient time is Ayurvedic medication as a source of treatment for Malaraia, Dysentry, Diabetes, wound healing, etc. It is known to have anti-pyretic, anti-inflammatory, anti-parasitic, and antioxidant properties. It has various phytochemicals like alkaloids, phenols, tannins, phlobatannins, hydrolysable tannins, flavonoids, terpenoids, and saponins (Kumar et al., 2021; Nagajothi et al., 2018). All these secondary metabolites are the reason behind its extraordinary and diversified medicinal properties. *Andrographis paniculata* is called as King of Bitters due to its bitter taste, which comes from a type of lactone present in the plant called andrographolide. The other two antioxidants leaving adrographoilde are Kalmeghin and Andrographin (Chauhan et al., 2014). *Andrographis paniculata* has been widely studied for its various phytochemicals present and its wound healing properties in rats (AI-Bayaty et al., 2012) and Planaria (Aref, 2023).

Though so many aspects regarding *Andrographis paniculata* has been studied, there is a huge gap in research regarding *Andrographis paniculata* as an indigenous medicinal herb for treatment in animals like fishes .Though so many studies are being currently conducted this field of study seems to be less explored.

1.2 AIM AND OBJECTIVES

To study the effect of *Andrographis paniculata* leaves extract on Colour Enhancement and Caudal fin Regeneration in Wild Guppies (*Poecilia reticulata*).

1) To evaluate the proximate nutrient contents of the *Andrographis paniculata* leaves.
2) To study the effect of *Andrographis paniculata* extract on colour enhancement in integument of *Poecilia reticulata* .
3) To estimate the effect of *Andrographis paniculata* extract on the caudal fin regeneration in *Poecilia reticulata.*

1.3 HYPOTHESES

The current study conducted where administered *Andrographis paniculata* extract via feed may bring about any colour enhancement in the *Poecilia reticulata a*lso , the administered feed may contribute in some way to reduce the stress in fish and increase the rate of regeneration in the fish.

1.4 SCOPE

The present study will involve proximate analysis of *Andrographis paniculata* extract to study its nutrient content, so it can be used as an additive in fish feed formulation, Further the formulated fish feed will be evaluated for its effect in inducing colour enhancement in integument of Guppy fish, and in increasing the rate of regeneration of caudal fin.

CHAPTER 2: LITERATURE REVIEW

2.1 Andrographis paniculata

Andrographis paniculata plant belonging to Acanthacea family , Is indigenous to Bangladesh, India, Nepal, Sri Lanka, and the Western Himalayas. It is an annual, herbaceous plant growing in plains, hills, seashores, roadsides etc. It is also a cultivable plant in the garden (Niranjana, 2010). It is an ethnobotanical medicine used in dysentery, malaria, fever, diabetes, etc (Akbar, 2011).

2.1.1 Antioxidant properties of *Andrographis paniculata*

Li et al. (2007) extracted secondary metabolites from the *Andrographis paniculata* in which two new flavonoids and one diterpenoid were isolated and their structure was determined using physicochemical and spectroscopic analysis. Andrographic acid was evaluated for cytotoxicity to KB cells.

Wahjuningrum et al. (2007) conducted a study where three herbal plants *A. paniculata, Psidium guajava, and Piper betle* were used as a cheaper source of medicine to treat *Aeromonas septicemia* in African catfish. Report shows combination of 1.0 gram *A. paniculata*, 0.75 gram *P. guajava* and 0.25 gram *P. betle* gave higher efficacy against *A. hydrophila* infection.

Rattanachaikunsopon and Phumkhachorn (2009) assessed six herbs, including *A.paniculata*, in treating *Oreochromis niloticus* infected with *Streptococcus agalactiae* .On Using swab paper disc assays *A. paniculata* showed largest inhibition zones,fish feed supplemented with either *A. paniculata* leaf powder or dried matter of *A. paniculata* aqueous extract reduced mortality of *S. agalactia*e infected Nile tilapia.

Chao and Lin (2010) in their review article reported various bioactive compounds from the *Andrographis paniculata* their extraction isolation and the various characteristics of each bioactive compounds.

Arslan and Ozcan (2010) compared sun, oven and microwave drying and proved via the experiments conducted how microwave drying prevents the colour degradation in food samples.

Song et al. (2013) carried out Qualitative and Quantitative Analysis of *Andrographis paniculata* by Rapid Resolution Liquid Chromatography and identified fifteen compounds, including flavonoids and diterpenoid lactones.

Chauhan et al. (2014) reported nutritional profiling of *Andrographis paniculata* comparing to other two plants suggesting less carbohydrates content compared to the other two plants.

Kshetrimayum et al. *(*2018), in their paper Quantitative Estimation of Total Chlorophyll and carotenoid content in *Oreopanax jalapenos,* reported the use of methanol or other alcohols as the best solvents for plant extraction.

Dai et al. (2019) provided an overview of the pharmacological activities of *Andrographis paniculata*, such as its beneficial effect on various ailments like fever, dysentery, cancer, diabetes, etc.

Sharma and Chauhan (2019) did a comparative study of Nutritional and Phytochemical Attributes of *Andrographis paniculata*, *Bryophyllum pinnatum* and *Clitoria ternatea* for nutraceutical applications.

Shi Y et al. (2020) mentioned that *Andrographis paniculata* has higher antioxidant capacity. It increases the SOD, CAT, activities, thus decreasing ROS.

Al Durrah et al. (2023) reported *A. paniculat*a extract as an antidepressant. The methanolic extract was seen to decrease the cortisol levels, and increase locomotion in zebrafish hence showing the antidepressant activity of the *A. paniculata* extract.

Aref (2023) reported that *A. paniculata* accelerated regeneration and minimized the stress induced by hydrogen peroxide in *Planaria* .

Ear et al. (2024) Reported that 0.6 g/kg of astaxanthin extract showed the highest weight gain, average daily growth, specific growth rate, feed efficiency and protein efficiency ratio, as well as the lowest feed conversion rate. Highest levels of RBCs and WBCs levels were found in 0.6 g/kg, it also showed lowest cumulative mortality, suggesting positive effects on hybrid catfish.

2.2 COLOUR ENHANCEMENT

Kaur and Shah (2017), through their experiment, showed the need for feed additives in feed as a source of pigmentation in ornamental fishes. It emphasized natural colouring pigments as an alternative to deteriorating synthetic pigments. Also, Das and Biswas (2016) reports the importance of natural plants as a source of fish feed in captive fisheries, owing to the adverse effects of synthetic pigments on the aquatic environment.

2.2.1 Pigmentation

Storebaken and No (1992) showed that carotenoid accumulation occurs more in integuments compared to other organs in fishes.

Torrisen (1989) primarily mentioned the set limit of the rate of pigment absorption in fish.

Paripatananont et al. (1999) showed 36–37 mg/kg astaxanthin as the optimal dosage to show colour enhancement in Goldfish. The four week study period showed no weight gain in fishes.

Harpaz and Podwicz (2007) Showed how 240mg /Kg of Oleoresin from Paprika pepper showed colouration in *Microgeophagus ramirezi*. The paper highlighted how the adult fish showed faster carotenoid accumulation (forty five days) compared to post larvae, which took seventy five days.

The paper by Tondiew et al. (2007) discusses the effects of *Morinda citrifolia and Andrographis paniculata* on pigmentation and phagocytosis in goldfish as an indicator to fish immune system wellbeing. It showed the combination of the two as an immunity booster for the fish.

Mukherjee et al. (2009) proved the significant effect of turmeric as a source of carotenoid, they showed pigmentation in fantail guppy at a concentration of 45mg/50g of fish feed.

Ramamoorthy et al. (2010) worked on *Amphiprion ocellari*s using Carrot, Marigold, Hibiscus and China rose. Carrot showed maximum colouration. It also mentions Astaxanthin is absorbed faster compared to lutein and Zeaxanthin.

Yesilayer et al. (2011) mentions the effect of various carotenoid sources on pigmentation in Goldfish, where Astaxathin, Cantaxanthin, and *Oleoresin paprika*, showed best colour results compared to *Gammarus sp*.

Boonyapakdee et al. (2015) conducted a four-week study on the efficacy of Natural and Synthetic astaxanthin. 50mg/Kg of Natural Astaxanthin showed better efficacy in colouration in Fancy carp compared to synthetic astaxanthin.

Safari and Atash (2015) suggested that for carotenoid accumulation in fish skin, the lipid content of the feed formulated must be high.

Maiti et al. (2017). Showed effect on colour enhancement in Koi carp using Beetroot, Carrot, and Tomato. Where Beetroot showed high carotenoid pigmentation in the fish skin followed by a mixture of the three products, they also explained how a decrease in the deposition of pigments in the skin is due to the limitation of the rate of absorption.

Dhananjaya et al. (2020) using annatto seed dyes as a source of colour enhancer, pigmentation in Goldfish fed with 250 mg/Kg of AD was studied. The fish showed the highest pigmentation, at day 60, total carotenoid content seemed to decrease.

Sawant et al. (2020) reported in their review article the need for colour enhancement in ornamental fishes and the various factors affecting colour enhancement in fishes.

Bisht et al. (2022) showed how spirulina powder had positive results in growth, survival, and pigmentation in Guppies.

Hien et al. (2022) reported the null effect of Astaxanthin, Canthaxanthin, and Xanthophylls on weight gain in Bighead catfish, however the fish fed with Xanthophylls showed the highest amount of muscle pigmentation at the end of the experiment. The pigment deposition was reported to be constant in all fishes after four weeks.

Huang et al. (2023) experimented to show the comparison between natural and synthetic astaxanthin in black tiger prawns, which proved Natural astaxanthin showed better results .

2.3 REGENERATION

2.3.1 Regeneration experiment in various model organisms

Kolluru et al. (2006) reported in his experiment that the carotenoid sources or the amount of feed received had no effect on tail regeneration in male guppies.

Chansue and Tangtrongpiros (2006) reported the optimal level of 0.03g/L of *Andrographis paniculata* and dried Indian almond leaves improved wound healing in fancy carp.

Da Cunha et al. (2015) Experimented with the effect of clove oil as anesthesia on male and female guppies. Male guppies subjected to 50mg/L of clove oil died after anesthesia, whereas no effect was seen on females. 5omg/L for juveniles also gave them too long induction of anesthesia.

Patel et al. (2019) Studied regeneration in *Poecilia latipinna* using a Vernier calliper. For twenty -five days. The first 5dpa showed the maximum rate of regeneration, followed by a decline in the rate. At 6dpa lepidotrichia starts bifurcating.

Zainol-Abidin et al. (2020) provided results from his study, in which *Piper sarmentosum* showed accelerated regeneration in Zebrafish and toxicity with concentrations above 60 μg/ml.

Uemoto et al. (2020) showed that longer fin rays in fish fin takes longer time to regenerate. Growth rate increases at 3-6dpa.

Mallawa et al. (2023) showed the importance of herbs in caudal fin regeneration in Siamese Fighting fish. How the presence of tannins prevented infection, also mentioning that herbs used in rearing water of fishes increased pigmentation.

2.4 CAROTENOID EXTRACTION AND ESTIMATION

Pavia et al. (1999) isolated chlorophyll and carotenoids using TLC, and column chromatography from the Spinach leaves.

Ambati et al. (2014), through their studies of the extraction of astaxanthin, proved acetone to be the best extraction solvent for astaxanthin.

CHAPTER 3: METHODOLOGY

Before carrying out experiments, various research papers were analyzed and read to find the standardized protocols. Research papers were searched via various search engines, like Google Scholar and Mendeley. The Sci-hub tool was used to access various unaccessed papers.

3.1 MATERIALS AND SAMPLE COLLECTION

The wild juvenile guppies (N=100, 0.322±94.6g) required for experimentation were purchased from ICAR-Old Goa. They were acclimatized in dechlorinated water for about two weeks prior to experimentation. All the fishes were maintained in same dimension tanks, (28.8 cm x 21.6cm x 13.9cm), and each study was conducted in the same time frame. The fishes were fed at the same hours daily. Post-feeding the fishes, observations were made for ten minutes to ensure whether all the fishes were feeding and the uneaten food was siphoned out. The tanks were aerated continuously, with partial water changes alternatively.

*Andrographis paniculata re*quired for the experimentation were grown in the garden, and only those plants were used for the experimentation. Once the plant was uprooted the leaves were plucked and microwave dried (Arslan & Ozcan, 2010). They were ground and stored in air-tight containers in refrigerator.

The apparatus and chemicals required for the experimentation were issued from the Zoology Department of the Goa University.

3.1.1 Model organisms and their classification.

Guppy (*Poecilia reticulata*)	**Chiretta (*Andrographis paniculata*)**
Kingdom : Animalia	Kingdom : Plantae
Phylum: Chordata (Notochord present)	Phylum: Magnoliophyta (Flowering plants)
Class: Actinopterygii (Ray-finned fishes)	Class: Magnoliopsida (Dicots)
Order: Cyprinodontiformes (unlobed Caudalfin)	Order: Lamiales (Capsular seeds)
Family: Poeciliidae (Live bearers)	Family: Acanthacea (No stipules)
Genus: *Poecilia* (Speckled coloured fishes)	Genus: *Andrographis (*waterwillows)
Species: *P. reticulata*	Species : *A. paniculata*

3.2 EXPERIMENTAL PROTOCOLS

3.2.1 Proximate analysis of *Andrographis paniculata*.

For conducting proximate analysis the grounded powder of *Andrographis paniculata* was used. The powder was homogenized using motor and pestle and extracted using menstruum. Ethanol was the menstruum used for polar compounds i.e Proteins and carbohydrates (Abubakar & Haque, 2020). Prior to testing *Andrographis paniculata* powder for proximate analysis , Standards were prepared using stock solution to gain a standard curve . For analysing proximate contents in AP, three set of replications were performed for each test, to gain a mean and reduce error. Further the concentrations and content analysis was estimated using standardized calculations.

a. Proteins

Proteins present in (AP) were estimated using Lowry's method (Lowry et al.,1951). BSA (Bovine Serum Albumin) stock was prepared using (5mg/20ml) in NaOH. Lowry's reagents were prepared (4% Na2C03, 2% CuSO4, 4% Na-K-Tartarate all mixed in ratio of (98:1:1). Folin's reagent in 1:1 ratio with distilled water. The plant extract (2g/40ml ethanol) was used as the sample, the experiment was repeated three times to obtain an average value and reduce error. The testubes were labelled B,1 ,2,3,4,5,S . The addition of the regents was done as shown in **(Table 3.2.1 Appendix I)**.Absorbance was recorded using a UV/Visible spectrophotometer **(Fig 3.2.1).** Protein concentration was calculated using the **y** equation on the graph, obtained using Standards.

Protein conc (mg/ml) = Optical density (UK sample) / y value from graph.

The concentration was estimated per gram of sample and converted into percentages**.**

b. Carbohydrates

The carbohydrates estimation in AP were done using Anthrone method (Ludwig & Goldberg, 1956) of Crude Carbohydrate analysis. The Reagents used were Anthrone (0.1g in 50 ml sulphuric acid), Glucose stock solution (0.010g in 50ml distilled water) and sample 1g/10ml ethanol). The additions were done as shown in the (**Table3.2.2 Appendix I**) .The absorbance was recorded using UV/Visible spectrophotometer **(Fig 3.2.2)**.Concentration was calculated using the **y** equation on the graph obtained using Standards.

Carbohydrate conc (mg/ml) = Optical density (UK sample) / y value from graph

The concentration was estimated for per gram of sample and converted into percentage.

c. Ash and Moisture

Ash and moisture content in AP were estimated using AOAC 936.03 (2000); AOAC 930.15 (2009) (**Fig 3.2.3**).The AP leaves were weighed (2g). Crucibles were pre-weighed (w), in which the samples were added and weighed again (W1). The crucibles were kept in an oven at 103°C for three hours. Once removed and cooled, they were weighed (W2). The moisture content was calculated using the following calculation.

Moisture content % = (W1-W2)/W1X100

For the ash content, similarly, the leaves were weighed (2g). After weighing the crucibles (W), the sample was added to the crucibles and weighed again (W1). The crucibles were kept in a muffle furnace at 520-550°C. The crucibles were removed the next day, and they were weighed along with ash (W2). Using the following calculation, the ash contents were measured.

Ash content % = (W3- W) / (W-W) X 100

d. Crude fats

Crude fats were analysed using AOAC 948.22(2000) modified method, in AP. A beaker was weighed (W), 2g of sample(S) was weighed into a beaker .The sample was dried in oven at 102°C for five hours. Petroleum ether was added to the beaker and the beaker was covered using aluminium foil and placed above water bath till it boils and all the extract is evaporated **(Fig 3.2.4)**.The beaker was cooled and weighed (W1). The crude fat content was calculated as follows

Crude fat % = (W1 - W) /S X100

e. Crude Fibres

Crude fibres in AP was estimated using AOAC 978.10 (2005) method. The diluted sulphuric acid (3.5ml/496.5 distilled water) was heated in a conical flask on a hot plate till it started

boiling **(Fig 3.2.5)**. The sample powder 2g(S) was added to the flask and heated for thirty minutes; the solution was filtered using Watmans filter paper, and continuous water washes were given till all the acid was washed and the residue became acid-free; after every wash using a pH paper, the residue was checked for neutrality. In another conical flask, NaOH (6.39g in 500ml distilled water) was heated till it boiled on a hot plate. The residue was added to the solution and boiled. Filtered it and again washed to remove all the basic solution till it turned neutral. The residue was collected in the crucible and placed in the oven for 110°C and left for overnight. The residue was weighed (a) and incinerated in a muffle furnace, and the ash content was weighed (b).

Crude fibre % = a-b/S X100

Based on the data from proximate analysis ,entire nutrient profile of the AP was obtained . Using this information and Guppy's nutrient requirements (Mohanta & Subramanian, 2011; Sales & Janssens , 2003) fish feed was formulated by partial replacement with AP extract. For formulating fish feed Pearson's Square Method was used (Wagner & Stanton, 2012). The formulated fish feed included 97.87g of wheat (30% Carbohydrate source and as binding agent) , Mackeral flesh 70.7g and Soyabean 29.26g as a source of protein(48%). Palm oil as source of fats (5.4ml; 5%) and pinch of vitamin mix. The fish feed prepared were of five types, Control **'C**' (containing no pigment source), Control positive **'C+'** (Containing astaxanthin 9mg/100g fish feed as a pigment source), Experimental I,II,III, **'EI', 'EII', EIII'** (containing AP extract 9mg, 45mg, 90mg per 100g of fish feed respectively) **(Fig 3.2.6).**

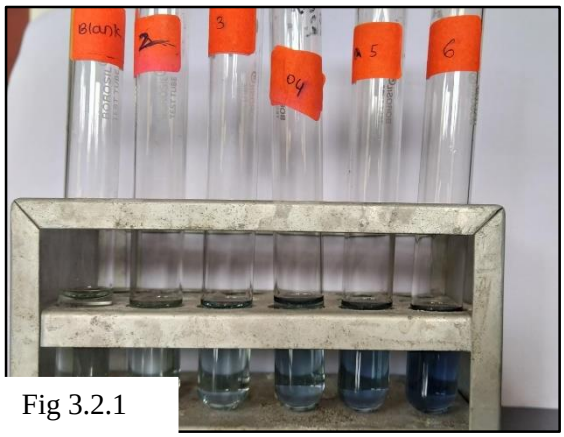


Fig 3.2.1

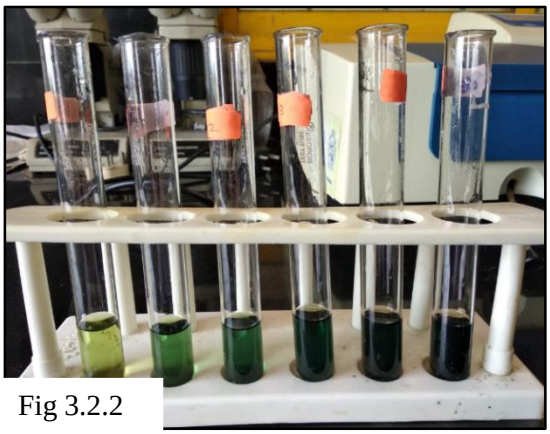


Fig 3.2.2

Fig 3.2.3

Fig 3.2.4

Fig 3.2.5

a

b

Fig 3.2 c d

Fig 3.2.1 The intense blue colour developed after all additions in Protein estimation using Lowry's method
Fig 3.2.2 The green colour developed, in standard testubes ,for estimation of carbohydrates using Anthrone method
Fig 3.2.3 Measuring crucibles with sample for calculation of ash and moisture
Fig 3.2.4 Estimation of Crude fats content using AOAC 948.22 method (modified).
Fig 3.2.5 Estimating crude fibres, sample being boiled in solution of Sulphuric acid on hot plate.
Fig 3.2.6 a) depicting Weighing of ingredient (soyabean powder), b) Dough made of mixing all ingredients, c) after drying fakes formed, d) *Andrographis paniculata* powder and Final fish feed prepared.

3.2.2 Protocol for Colour Enhancement in Guppy Integument.

The protocol followed for colour enhancement was derived from (Torrisen & Naevdal,1984). The experiment was carried out for a period of 20 days. And the set of readings were taken on every fifth day, to check for any colour enhancement in the integument of Guppies. Randomly three fishes were collected using net from the Control tank. To minimise the error ,for fishes picked from remaining tanks male:female ratio was maintained as per ones picked from Control tank. The fishes were euthanised using ice-chilling method, where fishes were placed on block of ice. The dead fishes were deskinned using sharp and sterile blade and the skin was collected in the petri plate. The skin sample was measured. The sample was transferred to acetone rinsed motor and pestle. To the sample was added 10 ml of acetone , and pinch of anhydrous Sodium sulphate to remove the water content from the sample. Adding, acetone , helps extract the carotenoids from the sample as carotenoids are extracted in non-polar solvents and carotenoids are best known to be extracted in acetone (Saini & Keum , 2018). The sample was homogenised , till all the colour was extracted. The coloured solution was stored in eppendorf tubes sealed with parafilm in dark at 4-5°C. The tubes were incubated for 24 hours, the following day samples were centrifuged at 5000rpm for five mins and the readings were taken using UV/Visible spectrophotometer (Fig 3.2.7).Prior to collecting the empirical data, λ max was obtained of Astaxanthin and coloured solution obtained from homogenised fish integument. The scan was run from 300- 800nm .And based on λ max obtained the wavelength to be set for running spectrophotometer was decided. Wavelength set were 476nm and 442 nm for Control, 476nm for control-positive and at, 442nm for all the Experiment I, II, III. The readings were noted and using E_1%- coefficient, concentration of the carotenoids in the fish skin were calculated. E_1%- coefficient used for estimation was 2500 (Torrisen &

Naevdal,1984). For data analysis Kruskal –Walis test was used, followed by post-hoc Dunn's test.

Calculations: 1) $E_1\%$ x MW = E_{molar} x 10

MW= Molecular weight of compound

$E_1\%$= Extinction coefficient

E_{molar}= Molar extinction coefficient

2) A= E_{molar} C l

A=Absorbance/OD

E_{molar}= Molar extinction coefficient

C= Concentration g/M

l=Path length (cm)

3.2.3Identifying pigment in fish integument

The fish skin sample, along with *Andrographis paniculata* extract were simultaneously loaded on TLC and , the spots obtained, were matched with eachother (**Fig 3.2.8).** For clarity, observation was done using UV torch light. The spot of *Andrographis paniculata* corresponding to fish sample spot was visualised, based on the experiment conducted further for identifying pigments in *Andrographis paniculata*, the pigment absorbed in integument was identified.

3.2.4 Protocol for Analysing Carotenoids in the *Andrographis paniculata*

Post colour enhancement the following experiment was conducted to determine the type of carotenoids absorbed in fish skin.

a.Extraction

Firstly the, AP leaves were washed and homogenized in three ml acetone and three ml methanol separately, till all coloured solution was extracted. Following the extraction the solution was centrifuged at 1000 rpm for two minutes. The supernatant was used as the sample to be used to spot in Chromatography. Also, to decide the suitable solvent for the chromatography of the carotenoids, various mixtures of solvents were chosen, based on the nature of the solvent and the carotenoids to be extracted. These various solvents were used for both the extracts, Acetone and methanol (Pavia et al., 1999). Depicted in (**Table 3.2.3**).

Table 3.2.3: Depicting the types of solvent mixtures tried as solvent phase in TLC and their formulation ratio

Sr.no	Solvent mixture	Ratios (5ml)
1.	Methanol	100%
2.	Methanol and Dichloromethane	1:1
3.	Methanol and Chloroform	1:1
4.	Petroleum ether	100%
5.	Petroleum ether and acetone	1:1
6.	Butanol	100%
7.	Dichloromethane	100%

Chromatography was carried out on both paper and thin layer chromatography for all these solvents. The two best solvents, were chosen amongst all for further experiment i.e., Methanol: Dichloromethane and Methanol: Chloroform, further they were analysed in various ratios to find the best among all combinations possible. Also, both these solvents were tested for both the extracts, i.e, Acetone and methanol, depicted in (**Table 3.2.4).**

Table 3.2.4: Depicting various ratios tried for the chosen two solvent phase for TLC

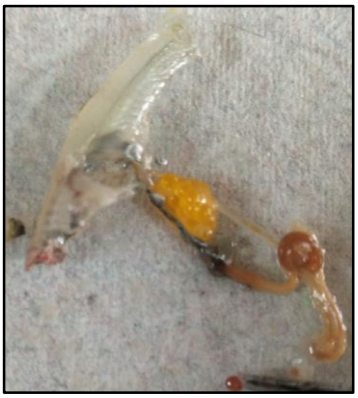
Sr.no	**Solvent mixtures**	**Ratios**
1.	**Methanol and Dichloromethane**	**1:1** **6:4** **7:3** **8:2** **9:1**
2.	**Methanol and Chloroform**	**1:1** **6:4** **7:3** **8:2** **9:1**

Among the following (90: 10) Met:DCM showed best spots and colour spots separation on the TLC plate **(Fig 3.2.9)**.Following these, the experiment was repeated to eliminate error. To confirm and extract the spots on TLC plate the Column chromatography was performed.

b. Estimation by Column Chromatography

The Chromatographic Column was washed with distilled water and acetone. The acetone extract *of* 10g *Andrgraphis paniculata* powder was prepared, strained, and collected in a beaker. The column apparatus was prepared as follows: The cotton was plugged at the bottom of the column apparatus to keep the silica loaded, trapped. Following cotton, almost half of the column was loaded with silica powder (60 x 120 mesh) in methanol solvent. The column was tapped with a rubber tube so all the silica settled down to form a uniform layer. The layer of solvent phase was almost always kept above the level of the silica gel layer. The silica gel layer is not supposed to run dry. Once the setup was complete , the sample was loaded from the top, followed by constant pouring of Methanol: DCM solvent. The solvent was added to the column to not let it run dry **(Fig 3.2.10).** The fractions separated were collected and stored. The fractions were collected using colour band separation and labelled **(Fig 3.2.11**). Each fraction was again spotted on TLC to make sure, the collected fractions were separate pigments. The fractions showing the same corresponding spots were mixed. Till the spots of all fractions showed different Retention factor, this procedure was continued. The final fractions were labelled accordingly and kept to dry till all the solvents evaporated. The powder obtained was weighed and dissolved in 10ml Acetone, and absorbance was recorded from 300- 800 nm for each fraction to record its λ max. Using the database, the fractions were identified from their following λ max in acetone as solvent (Pavia et al., 1999).

Fig 3.2.7



a b c

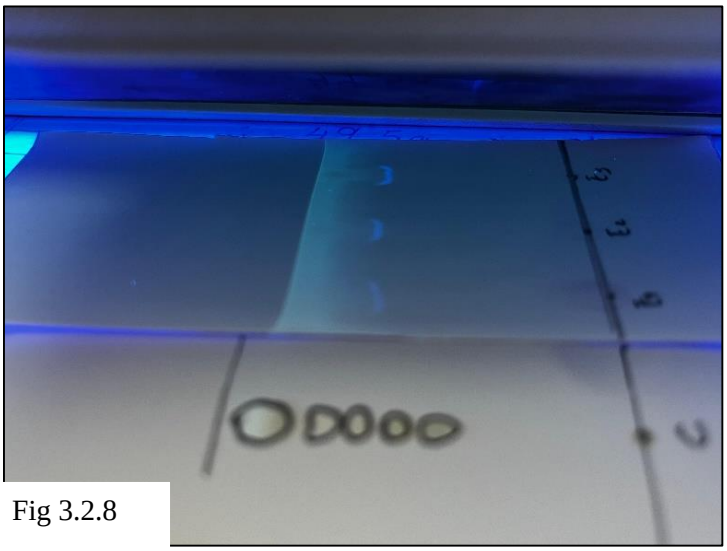


Fig 3.2.8

Fig 3.2.7: The three pictures , a- depicting the fish euthanized by ice –chilling. b – Deskinning the guppy integument. c- The homogenisation of the integument to extract the pigments.

Fig 3.2.8 : Integument samples loaded with *Andrographis paniculata* extract spot to match the spots and pigment . The pigment corresponding is the second spot (Xanthophyll).

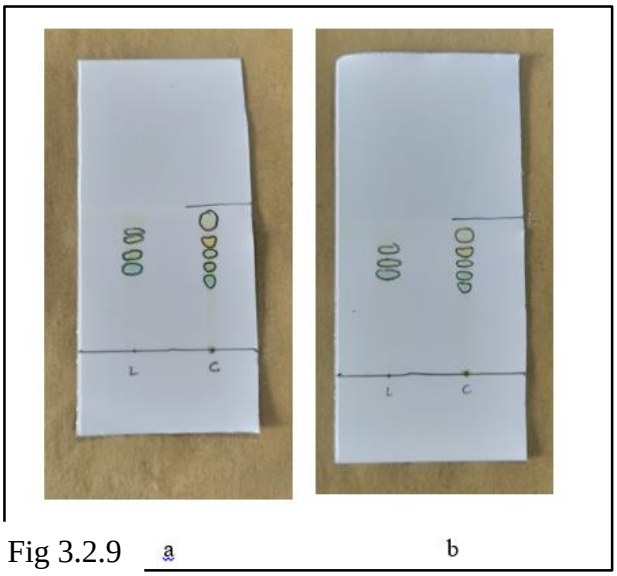


Fig 3.2.9

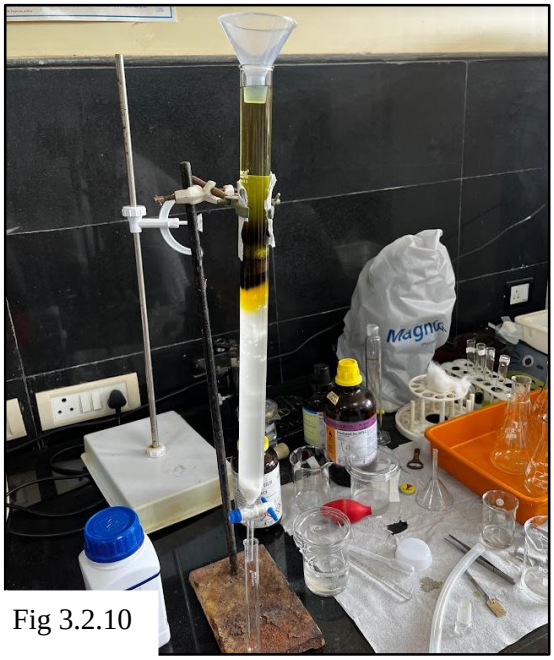


Fig 3.2.10

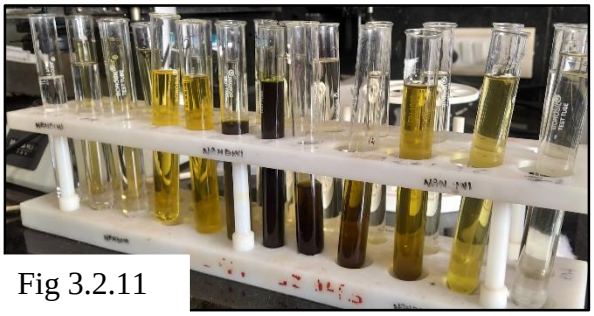


Fig 3.2.11

Fig 3.2.9: Depicting the TLC spotting on a Met: DCM (90:10) and Met: Chloroform (90:10). Met:DCM showing brighter spots and better separation

Fig 3.2.10: Column Chromatography set up, with separation of bands occurring, and fractions being collected

Fig 3.2.11: Fractions collected based on colour bands separation and each fraction was numbered

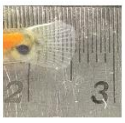
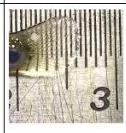
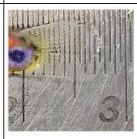
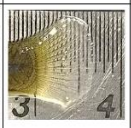
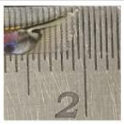
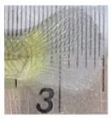
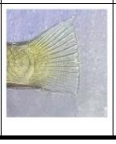
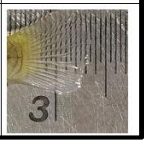
3.2.5 Protocol for Regeneration of Caudal fin.

The protocol for regeneration followed was according with the slight modification in procedures of (Zainol-Abidin et al., 2020).The experiment was carried out for a period of twenty one days. The readings were taken with a period of three days. Day 0 was the tail amputation day followed by day 3, 6,9,12,15,18,21. For anesthetising the fish, clove oil was used, the dosage was decided according to (Da Cunha et al., 2015).

Prior to the experiment, the set-up was arranged, which included a bowl of water with 2 drops of clove oil. Scale, Vernier calliper (6.1 inch WEN Digital Calliper, model-10761) (**Fig 3.2.12 Appendix II**). The pictures were taken using a Xiomi MI A3 (46 megapixel camera at 2.7X) (**Table 3.2.5).** During the experiment, another tank with continuous aeration was kept ready for the recovery of the fishes post-amputation.

The fish were collected using the net from the respective tanks and placed in a bowl containing clove oil; the time required for the fish to anesthetise completely were recorded. Each fish was collected on a Petri plate and placed above the scale. The caudal fin was computed using a sterile blade at a region proximal to lepidotrichia. The fish was released back to the tanks. The fishes were fed with formulated fish feed. Post-amputation readings were taken after every three days. The total length of the caudal fin amputated stump length, and growing length after every three days were noted. The growth per three days and growth rate were calculated. For data analysis, data was scanned through Shapiro-Wilk test for checking normal distribution of data, followed by RM-TWO –WAYANOVA and post hoc Tuckey's test and linear regression was used to check linearity between two factors.

Table 3.2.5 Tabular format depicting the growth of amputated fin for period of 21 day

Days →	Pre-amputation	Day 0	Day 3	Day 6	Day 9	Day 12	Day 15	Day 18	Day 21
C									
C+ (Astaxanthin 9mg/100g)									
EI(*A.paniculata* 9mg/100g)									
EII (*A.paniculata* - 45mg/100g)									

CHAPTER 4: ANALYSIS AND CONCLUSION

The data collected using the protocols mentioned in Chapter 3 were analyzed using various Statistics, Descriptive and Inferential. For analysis, two software applications were used: Graph-Pad Prism version 10.2.2 and Microsoft Excel 2013.

4.1 RESULTS

4.1.1 Proximate analysis of *Andrographis paniculata*

The proximate content of *Andrographis paniculata* has been reported in **(Table 4.1.1**). The plant's leaves are high in Crude Fibres 130mg/g and Ash content of 96.03mg/g. Crude proteins (CP) content was 3.953mg/g, Total carbohydrates (TC) 0.721mg/g, Crude fats (CF) 16.2mg/g, Ash content (AC) 96.03mg/g, Moisture content (MC) 38.93mg/g, Crude fibers (Cf) 130mg/g, which shows 0.3% CP, 0.0721% TC, 1.6%CF,9.6%AC, 3.89% MC and 13% Cf. All six analyses made were significantly varying by p=9.93E-11 **(Fig 4.1.1).**

Table 4.1.1 Depicting the Proximate analysis data of *Andrographis paniculata* leaves in mg/g of tissue

Nutrients	**Crude Proteins**	**Total Carbohydrates**	**Crude Fats**	**Crude Fibres**	**Ash**	**Moisture**
mg/g	**3.953**	**0.721**	**16.2**	**130**	**93.06**	**38.93**
%	**0.39**	**0.072**	**1.62**	**13**	**9.3**	**3.8**

4.1.2 Colour Enhancement in Fish Integument

The fish integument sample analysed was a green-coloured sample, showing increased colour intensity from C< C+ < EI < EII > EIII. EII showed the most intense colour in the solution. The concentrations of pigments deposited in the fish integument are depicted in the **(Figure 4.1.2).** The concentration of pigments in the sample seemed to decrease around after 15 days. Kruskal–Wali's analysis showed no significance between tanks for the effect of colour enhancement for 20 days. Acetone was a better solvent as a menstruum for

extracting pigments from *Andrographis paniculata* leaves than methanol. The solvent Methanol: DCM in a (90:10) ratio was the best solvent phase for TLC and Column Chromatography (pigments separation) of *Andrographis paniculata*, as it gave better and distinct spot separation. The λ max obtained for astaxanthin was between 472-476 nm, and the λ max obtained for the fish integument sample was 442nm, which corresponded to Fucoxanthin having λ max= 444nm, considering the type of pigment getting deposited in fish integument as fucoxanthin.

4.1.3 Caudal fin Regeneration

The data recorded were subjected to Normality distribution using the Shapiro–Wilk test. The regeneration data collected for all four tanks was normally distributed. For the caudal fin regeneration, the growth rate plotted on a line graph is depicted in (**Figure 4.1.3).** The growth rate was highest in fish fed with EII (45mg/100g) of *Andrographis paniculata* extract in fish feed. The fish fed with EII showed complete fin regeneration by day fifteen. Some fish fed with C+ diet (Astaxanthin 9mg/100g) showed complete regeneration after twenty one days. The growth rate was highest on day 6-9 days post amputation in fishes of Control tanks and treatment tanks, except of tank (EII), where growth rate was seen highest on day three post amputation (**Figure 4.1.4**). Also, the RM-TWO-WAY ANOVA showed insignificant differences between C, EI, and C+ and EII with respect to dosage **(Fig 4.1.5)** .Time period and Growth rate were tested for linear regression. Linear regression predicted the inverse relationship of Growth rate and time, Time period is significantly negatively linear with time period, in C+, EI and EII. However, there was no significance in the linearity between growth rate in C and time period, as depicted in (**Figure 4.1.6**).

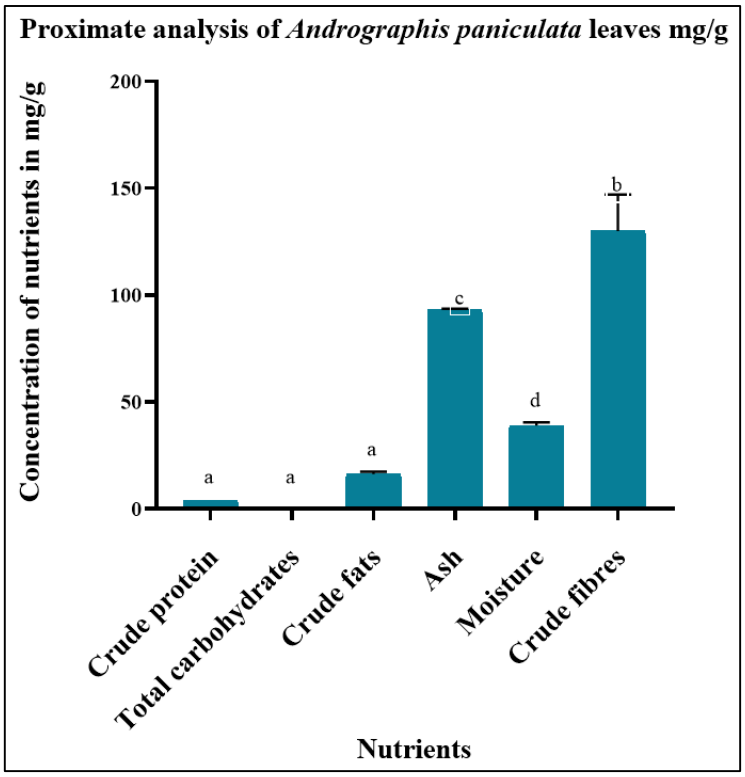


Fig 4.1.1: Depicting the mean concentration (Mean ±SD) of Nutrients in *Andrographis paniculata* leaves in mg/g, same letter no significance, different letters suggests significance ($p < 0.05$)

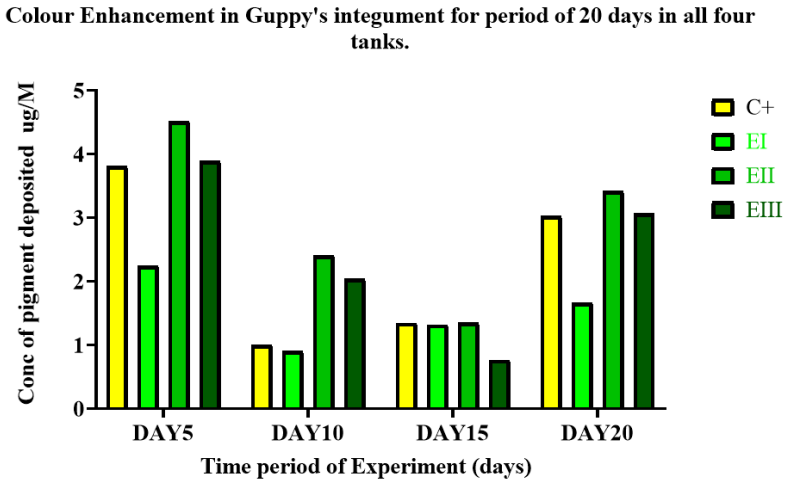


Fig 4.1.2: Depicting the concentrations of the pigment in Guppy's integument for a period of 20 days in all four tanks

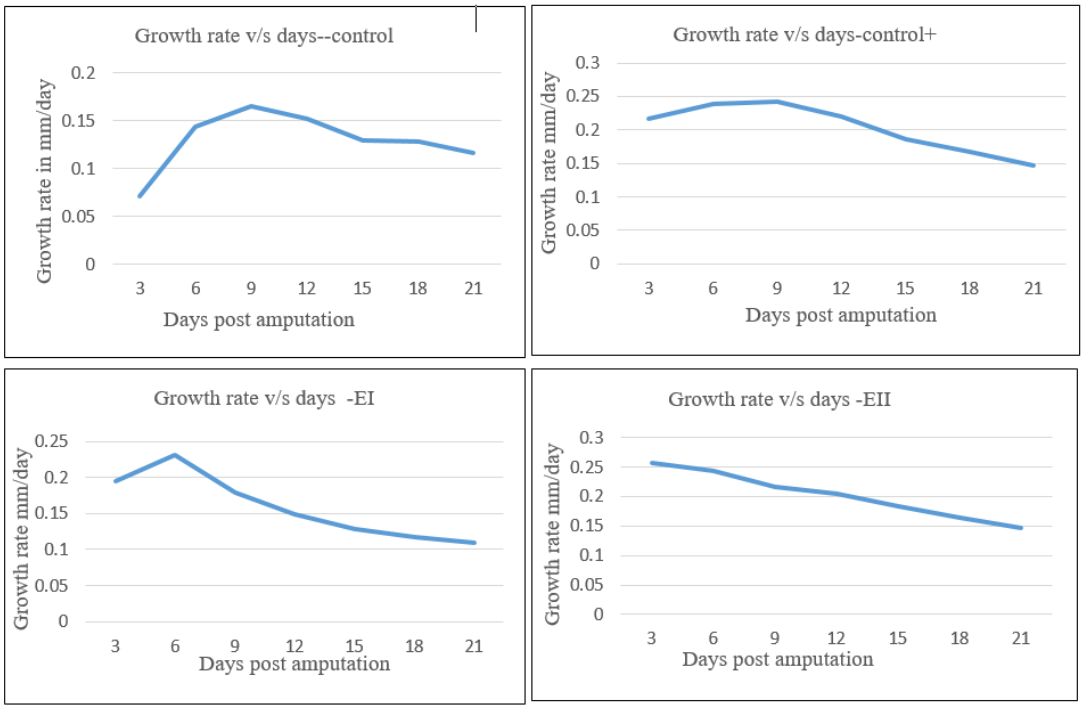


Fig 4.1.3: Depicting the growth rate trend over the period of 21 days in all four tanks, C, C+, EI and EII**.**

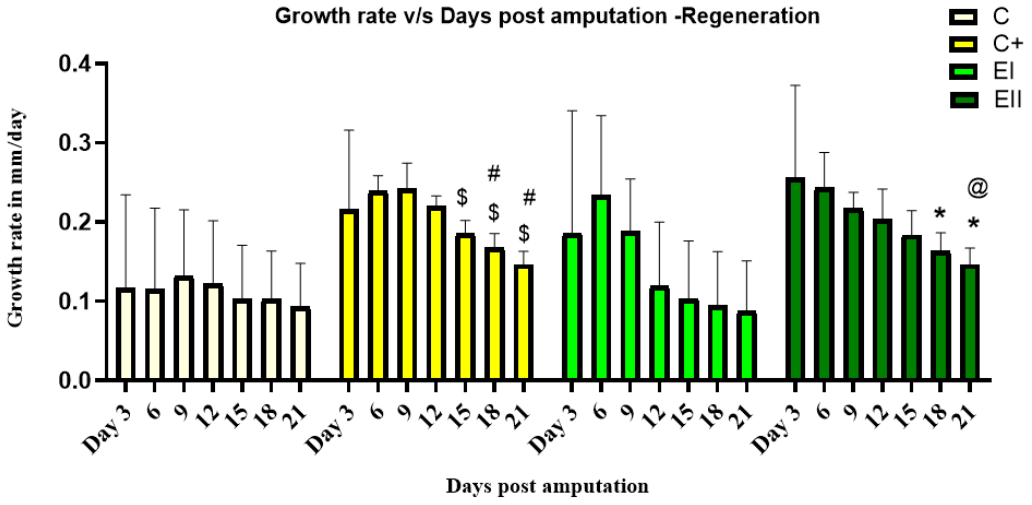


Figure 4.1.4: Depicting the growth rate in all the four tanks for overall period of 21 days (Mean ± SD) . $, $# shows significance differences between time periods in group C+ and *,*@ shows significance difference between time periods in group EII.

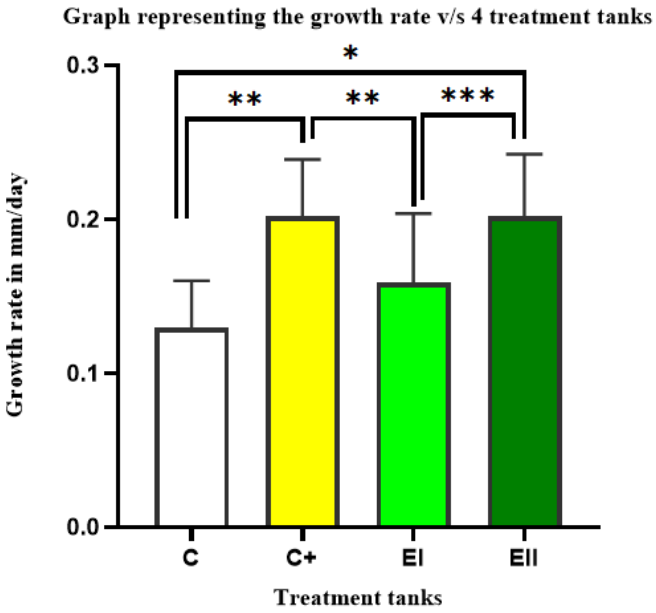


Figure 4.1.5: Depicting the significant differences between four tanks, with respect to growth rate (Mean ± SD). ***= 0.0005, **=0.002, * 0.01

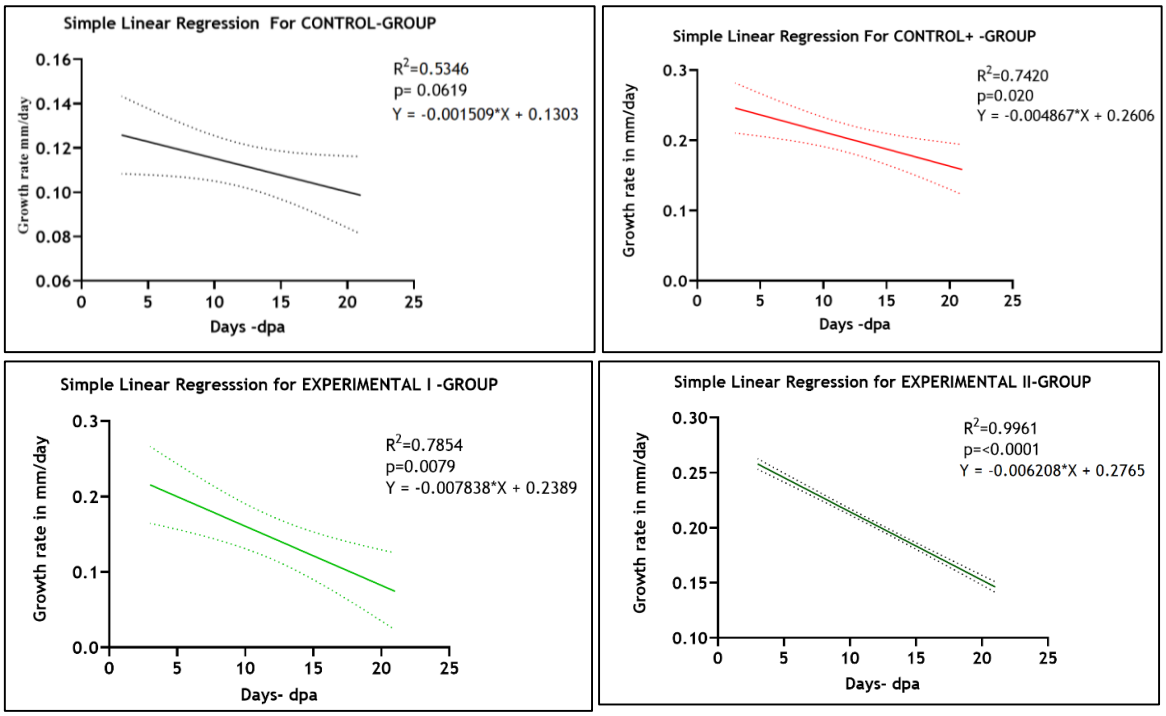


Figure 4.1.6: Linear Regression graphs for Regeneration of Caudal fin of Guppies , showing the linearity between Growth rate and time period, with significance value p=0.05

4.2 DISCUSSION

4.2.1 Proximate Analysis of *Andrographis paniculata*

The nutrient analysis was carried out on leaves of *Andrographis paniculata* , where nutrients like Crude proteins, Crude fats, Total Carbohydrates, Moisture, and Ash content were estimated. The current study proposes the plant is rich in Cf, followed by ash content. The readings differ from one reported by Sharma and Chauhan (2019), where CP was 10.3% and TC was 64.8%. Whereas the Cf reported are almost the same, they reported Cf= 12.7% per gram of leaves sample. Also, the results reported in a comparative study conducted by Chauhan et al. (2014) on the nutritional composition of three plants suggest that *Andrographis paniculata* consists of a high amount of MC, 73.02%, which contradicted our study. They reported fairly low protein and carbohydrate content; Cf in their study was 1.28%. Suggesting the data obtained in all three studies were differing from each other; this could be due to the geographical attributes. He and Nara (2007), explained how agro-ecology and agro-biodiversity enhance symbiosis and how this improves the uptake of nutrients, which improves or changes the nutrient contents of plants.

4.2.2 Colour Enhancement in Guppy Integument

Colour Enhancement in Guppy integument was studied by measuring the absorbance and calculating the concentration using the Extinction coefficient from the databases.

Therefore, the dependent variable, ie. The concentration of pigment and the two independent variables, time period and dosage of the pigment source, were analysed using the Kruskal- Walis test followed by post –hoc Dunn’s Test. The concentrations of pigment deposited in the integument of guppies in all four tanks were found insignificant for the dosage difference and time period $p>0.05$. Previously, no work has been reported on colour enhancement using *Andrographis paniculata*, nor has a study been reported on the pigment

content in the leaves of this plant. Also, Maiti et al. (2017) in her study observed decreases in pigment deposition as the days succeeded, mainly due to the limitation of the absorption rate of pigments, and there is a minimal amount of carotenoids a particular fish can absorb and retain. Torrisen et al. (1990) have studied limitations in the rate of absorption in a few fishes like Salmonoids, and further studies on many other fishes are lacking. No significance in pigment concentration in Guppies fed with Astaxanthin and Guppies fed with *Andrographis paniculata* (EI,EII,EIII*)* suggests that astaxanthin and AP(at all doses) had a similar effect on colour enhancement in the fish's colour physiology due to the retention factor of pigments Torrisen et al. (1990) , Also Safari and Atash (2015) suggests how adequate amount of lipid content in fish feed is important for pigmentation to occur. Acetone , helps extract the carotenoids from the sample as carotenoids are extracted in non-polar solvents and carotenoids are best known to be extracted in acetone reported by Saini and Keum (2018), this similar results were shown in current experiment as well. The probable reason for Methanol :DCM (90:10)being effective in separation could be its eluting strength, because of its polarity as mentioned by Somano et al. (2020), where they extracted this novel phytopigment Crocin from *Gardenia jasminoides* using methanol .The TLC spot of AP corresponding to spot of fish skin sample showed λ max =442. Xanthophylls are the carotenoids that show λ max range of (440-449nm). According to a study reported by Kita et al. (2015), Fucoxanthin is a xanthophyll with λ max at 444nm in acetone.

4.2.3 Caudal fin Regeneration

Regeneration in the caudal fins of guppies was studied by recording the growth and growth rate at interval of three days.The one dependent and the two independent variables were analysed for their contributing and simultaneous effect using RM TWO-WAY ANOVA

with alpha=0.05, followed by Tuckey's post-hoc test. Within each tank, there were significant growth rate changes seen for 21 days (p=0.0028). The growth rate was seen highest in fishes fed with EII (45mg of *Andrographis paniculata*), since three dpa. The growth rate between fishes fed with C diet and fishes fed with E1diet was insignificant, suggesting no particular effect of 9mg/100g dosage of *Andrographis paniculata* on regeneration. Fishes fed on Astaxanthin showed a faster growth rate than the fishes fed with C diet and fishes fed with EI ($p<0.05$). EII and C+ treatment showed positive effects in growth rate with respect to dosage. Still, there was faster growth in fish caudal fin regeneration in fishesfed with EII compared to fishes fed with C+ ,with respect to the time period (p=0.0031). Post hoc tests showed no significance concerning time period or dosage of plant extract between tanks, which could be the reason for the smaller sample size. The results seen are similar to as reported by Patel et al. (2019), in *Poecilia lattipinna* where growth rate post amputation was highest in first five dpa. In the current experiment, fishes fed with regular fish feed showed the highest growth rate on day 9dpa, whereas fishes fed with Astaxanthin and *Andrographis paniculata* (9mg/100g) showed the highest growth rate on six dpa. The growth rate was recorded highest in Fish fed with *Andrographis paniculata* (45mg/100g) on three dpa, which was 0.25mm/day. Uemoto et al. (2020), report that regeneration in zebrafish is highest on day 6 six dpa as the regeneration gene marker fa93e10 produced by mesenchymal cells shows increased activity at day six dpa. This means that in Fish fed with AP (45mg/100g), the gene expression was marked much earlier. Also Shi. Y et al., 2020 reported in their studies on *Monopterus albus* that an increase in AP dosage increases the antioxidant capacity, which increases SOD (Superoxide dismutase), CAT(Catalase) activities, thus decreasing the ROS (Reactive Oxygen Species) and as clearly seen in results from the current studies, where increasing the dosage five times, increased the regeneration in Guppy's caudal amputated fin. *Andrographis paniculata* is

known to have immune-stimulatory properties and various antioxidants as reported by Akbar (2011); the major antioxidant, namely andrographolide, is known to reduce oxidative stress in fish. Andrographolide regulates the Nrf2 transcription factor, which allevates the antioxidant enzyme system as reported by Mussard et al. (2019). *Andrographis paniculata* also shows DPPH scavenging activity was experimentally showed by Rafat et al. (2010) .As seen from the results of linear regreesion, of current study .Similar findings were also reported by Uemoto et al. (2020) where the growth rate of the caudal fin of zebrafish decreased with an increasing time period. Suggesting that antioxidants present in C+, EI AND EII has some faster effect on growth rate.

4.3 CONCLUSION

Andrographis paniculata is a medicinal plant indigenous to South and South-East Asia. It has various properties ranging from antioxidant, immune-stimulative, and anti-bacterial etc. From the current study *Andrographis paniculata proves* to be a potential medicinal plant for regeneration in wild Guppies at (45mg/100g). It is a potent antioxidant source with high regenerative inducible properties. It has not shown exemplary effect in Colour enhancement in wild Guppies; however, it is equally effective as Astaxanthin. According to the current study conducted it can be concluded that ,the dosage above 90mg/100g can be beneficial in colour enhancement, if the subsequent amount of lipid content in feed is increased, respective of the pigment retention factor of the model fish . Along with this, increasing the dosage period will also be beneficial, considering the various pigments (fractions collected) present in *Andrographis paniculata*. The current research demands a longer period of experimentation and a larger sample size for better analysis. Our results suggest that *Andrographis paniculata* may be as effective as astaxanthin in promoting colour enhancement and superior in accelerating the rate of fin regeneration. Thus, *Andrographis paniculata* can be suggested as a potent natural drug in Ornamental Fish Keeping, which is natural, environmentally friendly, and has no harmful effects as synthetic pigments available. It thus has the potency to replace Astaxanthin (synthetic or natural) as a source of pigmentation in ornamental fishes and also as a source of antioxidants for the regeneration of fins.

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Appendix I

Tables and Figures

Table 3.2.1: Depicting the the additions made, in protocol for Lowry's test

Test tube	BSA (ml)	Distiiled water (ml)	Conc (mg/ml)	Lowry's reagent (ml)	10 mins incubation at room temperature	Folin' s reagent (ml)	20 mins incubation at room temperature	OD 660nm
B	**0**	**1**	**0**	**5**		**0.5**		
1	**0.2**	**0.8**	**0.05**					
2	**0.4**	**0.6**	**0.10**					
3	**0.6**	**0.4**	**0.15**					
4	**0.8**	**0.2**	**0.2**					
5	**1**	**0**	**0.25**					
S	**-**	**-**	**?**					

Table 3.2.2: Depicting the additions done according to the protocol of Anthrone method

Test tube	Glucose stock (ml)	Distilled water (ml)	Conc (mg/ml	Anthrone (ml)	100°C incubation for 10 mins	OD 620 nm
B	**0**	**1**	**0**	**5**		
1	**0.2**	**0.8**	**0.04**			
2	**0.4**	**0.6**	**0.08**			
3	**0.6**	**0.4**	**0.12**			
4	**0.8**	**0.2**	**0.16**			
5	**1**	**0**	**0.2**			
S	-	-	**?**			

Fig 3.2.12 : Picture of the vernier calliper model used for measuring the growth in caudal fin