4

RESULTS AND DISCUSSION

The results and discussion of the study entitled as **"Physicochemical Characteristics, Safety of Under-Utilized Selected Natural Food Colourants and Development of Food Colour Sensor"** is discussed under the following headings:

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PHASE – I

4.1. Study on Natural and Synthetic Food Colourants

For more than three decades various studies carried out on the artificial food colors (AFCs) have proved to affect the health status of the children's behavior. Food and Drug Administration Food Advisory Committee convened to evaluate the current status of evidence regarding attention-deficit/hyperactivity disorder (ADHD) caused due to artificial food colours. Artificial food colors appear to be more of a public health problem than an ADHD problem, where it seems to affect children and have an aggregated additive or synergistic effect on most children who suffer from behavioral decrement. The edible dyes used in the food industries are not proved to improve the safety and quality of nutrition in foods, indicating the removal or replacement of currently used food colourants and their supplies for the betterment of booming food industries (*Zhou et al., 2022*).

An increased need for brightly coloured food has resulted in the undeniable incorporation of some questionable inorganic and organic chemistry being used in food products. A limited number of synthetic dyes are still used in foods today, but health concerns and the consumer driven demand for natural colorants has brought about a change in the way food is coloured. The proliferation of products with labels stated that they contain "No artificial colours" on supermarket shelves suggests that the future of azo dyes and their various derivatives is strictly limited. Nature produces an abundance of colours and many of these are extracted and used as natural food colorants; however, they are subject to limitations in the application and stability problems. Significant research by academia and industry into methods to stabilize and expand the application possibilities for the various approved natural food colorants is ongoing, but most developments that food colour manufacturers proclaim are enhanced vehicles for delivering established natural pigments into food products (*Tom Coultate et al., 2018*).

According to *Zhou* and *Tom (2022)*, the synthetic food colourants used in the foods have harmful effects on humans especially in children, however if the approved limitations are followed by the food manufacturers, there will be no harm in the consumption of the food colourants. But, as they are used in excess amount, eminent researchers have proved that they can be replaced with natural food colourants, which deliver the future for booming food producers.

4.1.1. Market Survey on Synthetic Food Colourants

A crucial ingredient to determine the consumers' preferences are food colourants. According to the investigator, the recent status of availability and usage of natural and synthetic food colourants in the Indian markets are growing tremendously, which also included global markets. Based on the case studies published on the effects of synthetic food colourants, rules and regulations were imposed by Food Safety and Standards Authority of India on both natural and synthetic food colourants. A significant fall back on the research emphasising on the impacts of synthetic food colourants identified and analysed through literature survey has showed a prominent scope and need for the appraisal of the prevailing issue, where an increased necessity of renewing policies on food colourants is essential need for the hour among the Indian population. Along with inappropriate labelling of natural food colourants, illegal usage of internationally banned food colourants, adulteration, mislabelling of permissible level of the synthetic food colourants and ignorant consumers have become a serious issue of the nation. Rules and regulations imposed must be comprehensively labelled on the products, along with renewed standards and policies to necessitate the consumers on responsive awareness on illegal use and adulteration of colourants to understand the use of natural food colourants that has become a predicted breakdown of Indian market (*Ressin et al., 2023*).

On the other hand, synthetic and artificial colours were studied, the commonly used artificial food coours are: Red 18 (ponceau 3R), Red 3 (Erythroxine), Red 40 (Allure Red), Green (Fast Green FCF), Yellow 5 (Tartrazine) and Yellow 6 (Sunset Yellow). Each colours are allotted with a specific chemical compound number and chemical class that highly describes these colours are unfit for human consumption. The percentage of distribution in confectionaries, beverages and permissible amount to be added in food were also taken in consideration to enhance the fact that the synthetic colours cause ill effects (*Ahmad et al., 2021*).

A market survey was conducted in 150 supermarkets and shops in Coimbatore city, Tamilnadu, India to know the customers' preference on synthetic food colorants present in the commercial market, along with the frequency of purchase and most commonly used brand of synthetic food colourants were considered.

Table – XI

Consumer Preference of the Synthetic Food Colourants from the Selected Shops

Brand Names Coded	No. of Shops Selling the Brands	Types of Colours Sold			
		Red	Green	Yellow	Blue
BK only	28	27	20	14	11
BU only	21	18	11	14	1
GC only	34	22	6	22	5
BK and BU	4	-	-	-	-
BU and GC	2	-	-	-	-
GC and BK	4	-	-	-	-
All three Brands BK, BU and GC	1	-	-	-	-
Others	56	28	16	20	6
TOTAL	**150**	**95**	**53**	**70**	**23**

From the market survey, the researcher had conducted the study on the highly used commercial brands available in the local supermarkets and shops. The brands preferred were BK, BU and GC which were sold in 28, 21 and 34 supermarkets respectively. Whereas four shops sold the brands like BK and BU, two shops sold the brands BU and GC, another four shops sold the brands like GC and BK. Only one supermarket sold all the three brands.

The market survey results showed that 28 supermarkets and shops that sold synthetic BK, 27 supermarkets sold synthetic red food colourant, 20 supermarketsand shops sold green synthetic food colourant, 14 supermarkets and shops sold yellow synthetic food colourant and 11 supermarkets and shops sold blue synthetic food colourant.

Out of 150 supermarkets and shops there were 21 supermarkets and shops that sold the brand BU. Out of21 supermarketsand shops, 18 supermarketsand shops sold synthetic red food colourant, 11 supermarkets and shops sold synthetic green food colourant, 14 supermarkets and shops sold synthetic yellow food colourant and one supermarket sold synthetic blue food colourants.

Whereas for the brand GC, out of 150 supermarkets and shops 34 supermarketsand shops sold this brand, whereas 22 supermarketsand shops sold synthetic red food colourant, six supermarkets and shops sold synthetic green food colourant, 22 supermarketsand shops sold synthetic yellow food colourant and five supermarketsand shops sold synthetic blue food colourant. Out of 150 supermarkets and shops, 94 supermarkets and shops sold the three major selling synthetic food colourants, in which only one supermarketand shops sold all the three brands. Meanwhile, 56 supermarkets and shops sold the brands other than BK, BU and GC.

The brand GC has become the highest selling synthetic food colourant among the other two brands, BK had got the second highest number of consumers and BU is the least common among the consumers. Based on the findings of market survey, three brands which were commonly purchased by the consumers were selected for further analysis to test the presence of heavy metals by Inductively Coupled Plasma Mass Spectrometry (ICP-MS) method.

4.1.2. Study on Selected Natural Food Colourants among the Selected Respondents

Using the formulated questionnaire market survey was conducted on the basis of knowledge related to the sources and usage of natural colourants among the selected 100 home makers. From the survey, the frequently used natural food colourings, their sources, uses and healthy benefits is collected and listed in table – XII.

Table – XII

Knowledge on Natural Food Colourants among the selected Home Makers (N = 100)

Common and Botanical Name of the Selected Plant Sources	Plant Parts Used	Awareness on Natural Food Colourants among Home Makers (%)	Colour Obtained	Colours used in Food Products	Health Benefits
Annatto *(Bixa orellana)*	Seeds	18	Orange Red	Colouring cheese and flavored milk	Antioxidant, antimicrobial and anticancer properties
Beet root *(Beta vulgaris)*	Root	100	Red, yellow and Bluish-red (depending on betanin content)	Yogurts, ice cream, ice bars, candy, noodles and pasta	Reduces the risk of cancer, cardiovascular, cerebrovascular diseases, liver and kidney damage
Carrot *(Daucus carota)*	Root	100	Orange yellow	Pro-vitamin A agent and colouring agent in dietary supplements	Promotes vision, aids in weight loss, skin health, improves immunity and brain health
Eucalyptus *(Eucalyptus grandis)*	Bark	23	Yellow Brown	Medicines – cough syrup, oil	Antioxidants, cold relief, treats dry skin, promotes relaxation and pain relief
Grapes *(Vitis vinifera)*	Fruit skin	72	Red to deep purple	Soft drinks, ice creams, yogurts, jams and confectioneries	Rich in antioxidant, antidiabetic, antiobesity and anti-inflammatory activities
Jamun fruit *(Syzygium cumini)*	Fruit	90	Pink to crimson black	Juice, squash, jam, jelly and toffee	Improves hemoglobin count, manages diabetes, improves gut health and acts as an immune booster
Marigold *(Calendula officinalis)*	Petals	35	Vibrant yellow, orange	Cheese and butter	Anti-inflammatory that promotes wound healing for eczema, sunburns, bruises and varicose veins
Madder *(Rubia cordifolia)*	Root	18	Brown Red	Medicine for kidney stones, menstrual disorders and urinary tract disorders	Treating jaundice, obstruction in spleen, palsy and bruises
Paprika *(Capsicum annuum)*	Fruit	100	Orange red	Meat products, confectionery, cheese, snacks, soups and salad	Rich in antioxidant properties, reduces the risk of cancer, heart diseases and improves immunity

Roselle *(Hibiscus sabdariffa)*	Petals	19	Dark Red	Wine, syrup, ice cream, pies, snacks, tarts and other desserts	Laxative effect, used in treating cracks in feet, sores and wounds
Sandal wood *(Santalum album)*	Bark, root	31	Yellow	Sandalwood oil used as flavoring agent	Lowers blood pressure, urinary tract infection, reduces common cold, liver and gallbladder problems
Tamarind *(Tamarindus indica)*	Pulp, Seeds	21	Brown, No Colour	Pulp is used for preparing jams, jelly and toffee	Rich source of antioxidants, has anticancer properties, improves heart health, liver protective and anti-diabetic effects
Tanner's cassia *(Senna auriculata)*	Flowers	16	Yellow	Candy, jam and tea powders	Anti-diabetic, relief from rheumatism, conjunctivitis, ulcer and constipation
Turmeric *(Curcuma longa)*	Root	100	Bright Yellow to deep orange	Pickles, seasoning, cheese, pies, candies, frozen desserts, beverages and snacks	Traditionally used against respiratory infections, arthritis, allergies, digestive disorders and liver disease

Table – XII represents the natural colouring agents that were commonly known by the selected home makers. The present study revealed that 78 percent of the home makers were familiar with all the natural colours except for annatto seeds (*Bixa orellana*) only 18 percent of the home makers were aware of annatto seeds come under natural food colourants. Likewise, the knowledge on eucalyptus bark (*Eucalyptus grandis*), madder root (*Rubia cordifolia*), roselle petals (*Hibiscus sabdariffa*), tamarind seeds (*Tamarindus indica*) and tanner's cassia petals (*Senna auriculata*) were accounting to only 23 percent, 18 percent, 19 percent, 21 percent and 16 percent respectively by the home makers.

In consideration towards the survey conducted, the natural food colourant sources on which the awareness and knowledge were below 25 percent were selected for further analysis among the all the other natural food colourants. The natural sources that were selected are: annatto seeds (*Bixa Orellana*), eucalyptus bark *(Eucalyptus grandis)*, madder root (*Rubia cordifolia*), roselle petals (*Hibiscus sabdariffa*), tamarind seeds (*Tamarindus indica*) and tanner's cassia petals (*Senna auriculata*) were selected for further analysis as the percentage of familiarity and knowledge about the natural food colouring substances were very low among the home makers than compared to the other natural food colourants. Also, only limited research was carried out in the above mentioned selected natural food colourants.

4.1.3. Knowledge Attitude and Practice regarding the Selected Natural Food Colourants among the Home Makers and Food Vendors

Survey was conducted among the selected home makers and food vendors of Coimbatore city to assess their knowledge, attitude and practice towards the usage of synthetic food colourants.

By adopting random sampling method, selected participants of home makers (N=100) and a group of food vendors (N=100) were selected, For the present study, selected home makers were between the age group of 25 – 45 years in Coimbatore city, they were selected to assess Knowledge, Atitude and Practice using the developed questionnaire.

The total percentage of the selected population were home makers, street food vendors, fast food vendors and restaurants with 48, 13, 28 and 11 percentage respectively. The populations' knowledge on rules, limitations and regulations imposed on the limitations of food colourants through Food Safety and Standards Authority of India (2012) were assessed with questions based on their knowledge. Their attitude and practice of cooking food with the food colourants were also assessed based on some basic questions in regard with the usage of food colourants.

Table – XIII

Educational Qualification of Selected Home Makers and Food Vendors

Educational Qualification	Selected Respondents (%)	
	Home Makers	Food Vendors
High School	30	32
Higher Secondary	31	32
Graduates	35	31
Illiterates	4	5

The literacy rate of the selected population was interviewed. Among the selected respondents of home makers, only four percentage of the population was illiterate, whereas 30 percentage of the respondents who completed their high school, 31 percentage of the respondents had completed their higher secondary and 35 percentage of the home makers were graduates. Likewise, among the respondents of food vendors 32 percentage of the vendors had completed their high school, 32 percentage had completed their higher secondary schooling and 31 percentage of the food vendors had obtained a degree and are graduates, whereas five percentage of the food vendors were illiterates, who are mostly drop outs from school.

Table – XIV

Knowledge Attitude and Practice of the selected Home makers and Food Vendors

Type of Respondents	Knowledge on SFC* (%)	Attitude on SFC* (%)	Practice on SFC* (%)
Home makers	37	25	47
Food Vendors	44	50	50
TOTAL	**81**	**75**	**97**

***** Synthetic Food Colourants

From the selected respondents, out of 48 percentage of home makers, 37 percent had good knowledge on synthetic food colourants, whereas only 25 percent of home makers had the attitude of accepting the regulations in using the synthetic food colourants in foods. All the selected home makers had the habit of using food colourants in their cooking.

Likewise, out of 52 percent of food vendors, 44 percentage had sound knowledge on synthetic food colourants, 50 percentage of them had the attitude to accept the regulations of FSSAI, whereas all thefood vendors had the practice of incorporating synthetic food colourants into their foods.

From this survey conducted among the home makers and food vendors in Coimbatore, its evident that, 81 percentage of the selected respondents had knowledge on food colourants, where 75 percentage of them had the tendency and good attitude towards the rules and regulations formulated by FSSAI. But, according to their style of preparation, target customers and commercial competition in the society.All the selected respondents had the practice of using synthetic food colourants in the food that they prepare on day-to-day basis.

Table – XV

Statistical Analysis on the Knowledge on Regulations of FSSAI and Usage of Synthetic Colourants in Food among the Selected Respondents

Type of Respondents	Knowledge on Regulations of FSSAI on SFC*			Usage of Synthetic Colourants		
	%	Chi sq	*p* value	%	Chi sq	*p* value
Home makers	47	2.11	0.54*	46	0.94	0.81^{NS}
Food Vendors	53			53		

^{NS} ***p value* is Not significant**

In terms of the percentage, 47 percentage of home makers had a good knowledge on regulations and limitations of synthetic food colourants imposed by FSSAI, but 46 percentage of the home makers population use food colourants to make their children feel that the prepared food appetizing enough to eat. Among the food vendors, 53 percentage of them had knowledge on the regulations of the FSSAI on synthetic food colourants, but all the food vendors had acknowledged the usage of synthetic food colourants to the food that they prepare for their customers.

Here, in the table – XV, which shows the knowledge and level of usage of synthetic food colourants among the home makers and food vendors, eventhough they have the knowledge on the harmful effects of synthetic food colourants, knowing the regulations of FSSAI and its limitations on synthetic food colourants, their usage of synthetic food colourants is high and also the significance value is more than 0.05, which shows that the selected respondents' knowledge is not significant to the usage or practice of using synthetic food colourants.

The food vendors had also reasoned out their usage of synthetic food colourants in the food they prepare, explaining that the food colourants are added to the food to attract either a specific target group of population or to have more customers than their competitors, especially to make the food look more appetizing, attractive and appealing to consume.

PHASE – II

4.2. Processing, Shelf Life Study, Microbial Assay and Testing of Primary Toxicity in the Selected Natural Food Colourants

To overcome the problem of characteristic changes in food and to ensure the good manufacturing practices and consumer safety and physical properties of the natural food colourants are analyzed (*Novais et al., 2022*). The following measures were taken to analyze the physical property of change in colour in the developed natural food colourants annatto seeds (*Bixa Orellana*), eucalyptus bark *(Eucalyptus grandis)*, madder root (*Rubia cordifolia*) and roselle petals (*Hibiscus sabdariffa*).

4.2.1. Measuring the Intensity of Colours of the selected Natural Food Colourants using Food Colour Reader

Food Colour Reader is used to detect the physical change taking place in the extracted natural food colorants. The raw ingredients of the natural food colourants were prepared in three different methods as powdered form, aqueous extraction method. In the powdered form no discolouring was found when it was placed in room temperature at 27^{0}C as well as refrigeration at 5^{0}C, whereas the aqueous extracts of annatto seeds (*Bixa Orellana*), eucalyptus bark *(Eucalyptus grandis)*, madder root (*Rubia cordifolia*) and roselle petals (*Hibiscus sabdariffa*) showed increase in colour intensity and discolouring of colours. The Food Colour Reader was used to monitor the colour change in aqueous extracts every fourth day of the week.

The natural colour of the extracts of annatto seeds (*Bixa orellana*), eucalyptus bark (*Eucalyptus grandis*) and madder root (*Rubia cordifolia*) were measured on every forth day to know the rate of discolouring. The food colour reader was calibrated using black and white cavity covers, placing it food colour reader's lens evenly on the cavity. Then the food samples were placed on a watch glass, with a plain white base and the colours were measured. Around 0.15 ml (that is 150 micro liters) of the extracts of annatto seeds (*Bixa orellana*), eucalyptus bark (*Eucalyptus grandis*) and madder root (*Rubia cordifolia*) were pipette out in the watch glass. The cavity of the lens in the food colour reader was placed over the liquid extracts and the reading was measured using L* as lightness, a* as either red or green and b* as either yellow or blue. The extracted colours were analyzed for discolouration using food colour reader and the change in colour was recorded and compared between aqueous food colour extract and powdered substances is noted in the below table.

Table – XVI

Computed Values of the Natural Food Colour Extracts Extracted by Aqueous Extraction Method on Every Fourth Day in a Week

No. of Days	Natural Food Colours extracted by Aqueous Extraction Method	L*	a*	b*	$\sqrt{(\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2}$	ΔE* ab
1	**Annatto Seeds** (*Bixa orellana*)	41.84	8.43	1.08	√ 1822.80	42.69
2		43.60	8.47	12.43	√ 2127.20	46.12
3		48.91	6.27	2.79	√ 2439.27	49.38
4		47.06	6.92	3.02	√ 2271.64	47.66
1	**Eucalyptus Bark** (*Eucalyptus grandis)*	37.55	5.48	3.71	√ 1453.79	38.13
2		39.62	3.80	6.16	√ 1622.12	40.27
3		45.11	8.30	5.34	√ 2129.31	45.35
4		46.26	4.41	7.70	√ 2218.71	47.10
1	**Madder Root** (*Rubia cordifolia*)	48.37	5.24	1.49	√ 2369.32	48.67
2		47.25	8.58	6.59	√ 2057.43	48.47
3		47.31	6.80	3.90	√ 2299.68	47.95
4		46.37	5.00	6.20	√ 2213.61	47.04
1	**Roselle Petals** (*Hibiscus sabdariffa*)	41.36	8.21	2.17	√ 1782.74	42.22
2		38.98	1.34	0.68	√ 1521.69	39.00
3		27.88	15.90	6.25	√ 1069. 16	32.69
4		21.58	24.26	4.60	√ 1075.39	32.79

L* represents lightness

a* represents either red or green

b* represents either yellow or blue

In *Table-XVI,* Delta-E is a standard measurement that qualifies the difference between the two colours that appear, where the lower Delta-E figures indicate greater accuracy, while the high Delta-E levels indicate a significant mismatch. The intensity of colour was measured by the food colour reader and the values were calculated using the formula for Delta-E. The CIE LAB colour was measured using Food Colour Reader to note the hue difference. For eucalyptus bark (*Eucalyptus grandis)* and annatto seeds (*Bixa orellana*) extract colour increased from 38.13 to 47.10 hue and 42.69 to 47.66 hue respectively. The madder root (*Rubia cordifolia*) extract's colour changed from 48.67 to 47.04 hue. As for the extracts obtained from rosella petals (*Hibiscus sabdariffa*) the colour had decreased hues from 42.22 to 32.79 hue. The table also shows that, if the a* value is positive in nature, then it represents the colour red, if it is negative values represent green colour. Likewise, if b* value falls in positive side it is yellow in nature and in negative, it represents blue in nature. As all the values are positive, Researcher had concluded that the extracts' colours are either red, yellow, orange red or shades of yellow and red. The colour values also proved that the boiled extracts had shown discolouration for every two days with less stability, whereas the powdered substances did not show any change in colour for testing the physical characteristics.

4.2.2. Testing pH for the Selected Natural Food Colours Extracted using Aqueous Extraction Method

The pH of the extracts of annatto seeds (*Bixa Orellana*), eucalyptus bark *(Eucalyptus grandis)*, madder root (*Rubia cordifolia*) and roselle petals (*Hibiscus sabdariffa*) extracted by aqueous extraction method was measured using pH meter. The pH values were tabulated along with the quantity of the extracts that was obtained after boiling the raw compounds with distilled water at 60^{0}C for 60 minutes and strained using Whatman filter paper.

Table – XVII

Testing pH for the Selected Natural Food Colours Extracted using Aqueous Extraction Method

Natural Food Colours extracted by Aqueous Extraction Method	Raw Form (g)	Addition of Distilled Water (ml)	Extract Yield (ml)	pH (mol/L)
Annatto seeds (*Bixa orellana*)	25	250	230	6.33
Eucalyptus bark (*Eucalyptus grandis)*	25	250	160	4.57
Madder root (*Rubia cordifolia*)	25	250	170	5.69
Roselle petals (*Hibiscus sabdariffa*)	25	250	180	3.43

From *table-XVII*, it was evident to note that the pH values of the extracts from madder root (*Rubia cordifolia*), eucalyptus bark (*Eucalyptus grandis*), annatto seeds (*Bixa orellana*) and roselle petals (*Hibiscus sabdariffa*) in comparison with the normal pH values where pH is less than 7 indicates acidity and greater than 7 indicates a base. The range of pH is commonly considered to extend from 0 to 14. A pH value of 7.0 is neutral, because pure water has a pH value of exactly 7.0. Values less than 7.0 are considered acidic, while those greater than 7.0 are considered basic or alkaline (*William, 2016*).

The selected natural food colourants were extracted through reflux extraction method, the compounds were analyzed for pH ranges to analysis acidic in nature, where eucalyptus bark (*Eucalyptus grandis)* and roselle petals (*Hibiscus sabdariffa*) extracts were measured to be 4.57 mol/L and 3.43 mol/L of base with an yield of 160 ml and 180 ml from 250 ml. Whereas madder root (*Rubia cordifolia*) extract was slightly acidic with 5.69 mol/L with an yield 0f 170 ml and annatto seeds (*Bixa orellana*) extract had very mild acidity of 6.33 mol/L with an yield of 230 ml.

According to *Mohammad Shafuret al.*, (*2020*), the pH in food is to know the stability and biochemical reactions taking place between the molecules in a food, where, if the food’s pH is less than or equal to 3.0, then the food is considered ten times acidic as that of the foods with the pH of greater than or equal to 4.0 mol. The study on the pH of the natural food colourants had ranged between 3.4 and 6.33 mol/L, stating the fact that all the extracted colours can be considered as edible.

The processed aqueous extracts of annatto seeds (*Bixa Orellana*), eucalyptus bark *(Eucalyptus grandis)*, madder root (*Rubia cordifolia*) and roselle petals (*Hibiscus sabdariffa*) were observed for change in colour and it showed leeching off of colours. So, the intensity in change of colours were monitored every fourth day and the pH of the extracts of annatto seeds (*Bixa Orellana*), eucalyptus bark *(Eucalyptus grandis)*, madder root (*Rubia cordifolia*) and roselle petals (*Hibiscus sabdariffa*) were also monitored.

4.2.3. Shelf Life Study of the Extracted Natural Food Colourants at Different Temperatures

The shelf life of foods is affected by several aspects, mainly chemical and microbial events, resulting in a considerable decline in consumer's acceptance. There is an increasing interest to substitute synthetic preservatives with the plant based bioactive ingredients which are safe and natural. However, full implementation of this replacement is postponed by some challenges associated with bioactive ingredients, including their low chemical stability, off-flavor, low solubility and short term effectiveness (*Hamed et al., 2021*).

The aqueous extracts, when stored in sterile glass bottles showed quick contamination. On 10^{th} day, the natural food colourants extracts stored at room temperature started forming creamy layers and on 15^{th} day, the creamy layers became darker in colour forming a slimy layer on the surface of the extracts. Same way on 15^{th} day, the extracts placed in the refrigerator (5°C), formed a slimy thick layer at the bottom of the bottle and the colour of the extract was too dark than the original colour. The slimy layers formed were confirmed to be microbial growth as it began to grow thicker and larger day-by-day. To analyse the slimy layers, microbial assay was carried out.

4.2.4. Microbial Analysis for the Selected Natural Food Colourants

To identify the presence of microorganisms in the natural food colour extract following assay was carried out.

4.2.4.1. Fungal Assay by Rose Bengal Chloramphenicol Agar Medium

Fungal colonies were identified after inoculating into a sterilized petric plates using standard pour plate culture with Rose Bengal Chloramphenicol Agar Medium. The plates were kept at room temperature (27°C) for the formation of colonies. From *table*, the number of colonies formed on the third day was counted and recorded.

Table – XVIII

Identification of Fungal Colonies Count at Different Temperature in the Selected Natural Food Colourants

Natural Food Colour Extract	No. of Colonies Identified	
	Room Temperature (27°C)	Refrigerator (5°C)
Annattoseeds (*Bixa orellana*)	22	5
Eucalyptus bark (*Eucalyptus grandis*)	25	8
Madder root (*Rubia cordifolia*)	4	2
Roselle petals (*Hibiscus sabdariffa*)	26	17

The selected natural food colourants extracts in the room temperature at 27°C showed extra fungal growth than that of the extracts that had been refrigerated at 5°C. Annatto seeds (*Bixa orellana*), eucalyptus bark (*Eucalyptus grandis*), madder root (*Rubia cordifolia*) and roselle petals (*Hibiscus sabdariffa*) extracts placed at the room temperature had shown bigger fungal colonies formation 22, 25, 4 and 26 respectivelythan that of the refrigerated extracts 5, 8, 2 and 17 respectively.

Table – XIX

Colony Forming Unit for Fungal Growth in Aqueous Extraction of the selected Natural Food Colourants

Natural Food Colour Extract	*Colony Forming Unit (CFU)*	
	Room Temperature (27°C)	Refrigerator (5°C)
Annatto seeds (*Bixa orellana*)	2	8.8
Eucalyptus bark (*Eucalyptus grandis*)	10	3.2
Madder root (*Rubia cordifolia*)	1.6	0.8
Roselle petals (*Hibiscus sabdariffa*)	10.4	6.8

Table shows the fungal colony forming unit calculated with the mentioned formula, where the dilution factor was six and the volume of the specimen is 15 ml. Thus, the CFU per ml of the annatto seeds (*Bixa orellana*), eucalyptus bark (*Eucalyptus grandis*), madder root (*Rubia cordifolia*) and roselle petals (*Hibiscus sabdariffa*) were 2, 10, 1.6 and 10.4 CFU count of microorganisms noted at room temperature (27°C) and 8.8, 3.2, 0.8 and 6.8 CFU count of microorganisms were observed at refrigerated (5°C) temperature.

4.2.4.2. Bacterial Assay by Nutrient Agar Medium

For detecting the presence of bacteria, Nutrient agar medium is prepared and culture was inoculated in sterilized petric plates, using standard pour plate method was followed. For the period of 24 hour growth of bacterial colonies were identified. In *table*, the bacterial colonies formed was counted and recorded.

Table – XX

Identification of Bacterial Colonies Count at Different Temperature in the selected Natural Food Colourants

Natural Food Colour Extract	No. of Colonies Identified			
	Room temperature (27°C)		Refrigerator (5°C)	
	Bigger Colonies	Total Colonies	Bigger Colonies	Total Colonies
Annatto seeds (*Bixa orellana*)	28	72	15	34
Eucalyptus bark (*Eucalyptus grandis*)	6	79	10	62
Madder root (*Rubiacordifolia*)	69	86	23	39
Roselle petals (*Hibiscus sabdariffa*)	38	91	23	39

The natural food colour extracts at the room temperature 27°C had shown more bacterial growth than that of the extracts that had been refrigerated at 5°C. Madder root (*Rubia cordifolia*) had 69 big bacterial colonies from the total of 86 colonies; eucalyptus bark (*Eucalyptus grandis*) had six bacterial colonies from total of 79 colonies. In annatto seeds (*Bixa orellana*) extract's petric plate, there were a total of 72 bacterial colonies out of which 28 bacterial colonies were bigger colonies whereas in roselle petals' (*Hibiscus sabdariffa*) petric plate, out of 91 colonies 38 bacterial colonies was bigger in size. Colonies found in the extracts placed in the room temperature had shown bigger colonies formation than that of the refrigerated extracts 39, 62, 34 and 39 respectively.

Table – XXI

Colony Forming Unit for Bacterial Growth in Aqueous Extraction of the selected Natural Food Colourants

Natural Food Colour Extract	*Colony Forming Unit (CFU)*	
	Room Temperature (27°C)	Refrigerator (5°C)
Annatto seeds (*Bixa orellana*)	28.8	13.6
Eucalyptus bark (*Eucalyptus grandis*)	31.6	24.8
Madder root (*Rubia cordifolia*)	34.4	15.6
Roselle petals (*Hibiscus sabdariffa*)	36.4	15.6

As for bacterial colony forming units was calculated with the dilution factor is six millilitre and the volume of specimen is 15 ml. CFU is calculated using the total colonies count. Thus, the CFU per ml of madder root (*Rubia cordifolia*) is 34.4 CFU at room temperature (27°C) and 15.6 CFU at refrigerated (5°C) temperature. Likewise for eucalyptus bark (*Eucalyptus grandis*), annatto seeds (*Bixa orellana*) and roselle petals (*Hibiscus sabdariffa*), it was noted that 31.6, 28.8 and 36.4 CFU at room temperature (27°C) and 24.8, 13.6 and 15.6 CFU at refrigerated (5°C) temperature respectively.

From the below *table,* the fungal colonies formed in the petric plates were physically identified, in consideration with their colour, physical appearance and growth rate in the particular time (72 hours) using Crystal-Violet Gram Staining Method.

Table – XXII

Physical Characteristics of Identified Fungi in the selected Natural Food Colourants

Natural Food Colour Extract	Identified Fungi	Physical Characteristics
Annatto seeds (*Bixa orellana*)	*Rhizopus*	White gray layer
	Aspergillus fumigatus	Greenish black spongy layer
	Trichoderma	Pale green root like layer
	Pencillium	Olive green spots
Eucalyptus bark (*Eucalyptus grandis*)	*Aspergillus niger*	Green strands of hairy cotton clusters
	Mucor species	White hairy layer with spores
	Curvularia species	Shiny velvet-black fluffy growth
Madder root (*Rubia cordifolia*)	*Flavus*	Yellow green spongy spores
	Rhizopus stolonifer	White fluffy layer
Roselle petals (*Hibiscus sabdariffa*)	*Aspergillus niger*	Green strands of hairy cotton clusters
	Flavus	Yellow green spongy spores
	Pencillium	Olive green spots

From the plate-4.2.4.2, through the physical characteristics of the fungal group the species were identified manually. White gray layer was *Rhizopus*, greenish black spongy layer was *Aspergillus fumigatus*, pale green root like layer was found to be *Trichoderma* and olive green spots were found to be *Pencilium*. All these four species were found in the extracts of annatto seeds (*Bixa orellana*) (*Plate 4.2.4.2 a).*

Whereas in eucalyptus bark extracts (*Eucalyptus grandis*) showed three colonies were identified with green strands of hairy cotton cluster was formed, it was identified to be *Aspergillus niger*, white hairy layer with spores was a species of mucor and shiny velvet-black fluffy growth was *Curvularia* species (*Plate 4.2.4.2 b).*

Table shows that two different fungal colonies were observed in madder root (*Rubia cordifolia*) extract. The fungal colonies of *Flavus* which was yellow white spongy spores and *Rhizopus stolonifer* which was a white fluffy layer were observed in *Plate 4.2.4.2 c*.

In rosella petals extract (*Hibiscus sabdariffa*), green strands of hairy cotton clusters known as *Aspergillus niger*, yellow green spongy spores known as flavus and olive green spots known as *Pencilium* was identified in *Plate 4.2.4.2 d*.

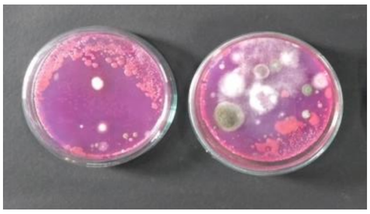


Plate – 4.2.4.2 (a)
Annatto seeds (Bixa orellana)

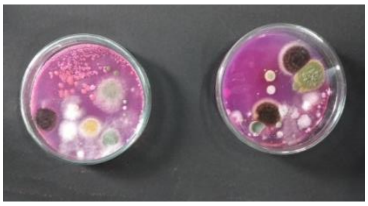


Plate – 4.2.4.2 (b)
Eucalyptus bark (Eucalyptus grandis)

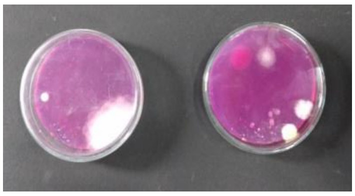


Plate – 4.2.4.2 (c)
Madder root (Rubia cordifolia)

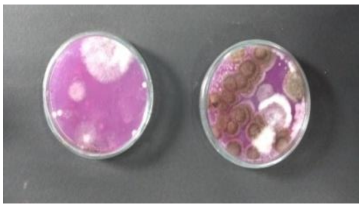


Plate – 4.2.4.2 (d)
Roselle petals (Hibiscus sabdariffa)

Plate –4.2.4.2 (a, b, c, d)– Fungal Identification in Inoculated Petric Plates

Table – XXIII

Physical Characteristics of Identified Bacteria in the selected Natural Food Colourants

Natural Food Colour Extract	Identified Bacteria	Characteristics of Bacteria
Annatto seeds (*Bixa orellana*)	***Bacillus species***	**Gram positive:** rough, opaque, fuzzy half-white circular colonies with uneven edges
Eucalyptus bark (*Eucalyptus grandis*)	***Staphylococcus species***	**Gram positive:** round, smooth, golden-white colonies with glisten-rough edges
Madder root (*Rubia cordifolia*)	***Bacillus species***	**Gram positive:** rough, opaque, fuzzy half-white circular colonies with uneven edges
Roselle petals (*Hibiscus sabdariffa*)	***Staphylococcus species***	**Gram positive:** round, smooth, golden-white colonies with glisten-rough edges

From the *table*, the bacterial identification of the extracts was identified through Crystal-Violet Gram Staining Method, through gram positive staining. The major bacterial colonies found are: madder root (*Rubia cordifolia*) showed the presence of *Bacillus* species, eucalyptus bark (*Eucalyptus grandis*) showed the presence of *Staphylococcus* species, annatto seeds (*Bixa orellana*) showed the presence of *Bacillus* species and roselle petals (*Hibiscus sabdariffa*) showed the presence of *Staphylococcus* species.

The study on microorganisms had proved that bacterial and fungal interactions have always been a hindrance in improvising human health activities and now also in natural food extracts. Roselle petals (*Hibiscus sabdariffa*) extract had always shown the highest contamination rate in comparison with the other extracts in room temperature with 10.4 CFU/ml in fungal assay and 36.4 CFU/ml in bacterial assay. Eucalyptus bark (*Eucalyptus grandis*) come next in place in the rate of highest contamination with 10 CFU/ml in fungal assay and 24.8 CFU/ml in bacterial assay in refrigeration. The results proved that the aqueous extracts from the natural substances are easily contaminated in a short duration. Further preservation techniques have to be equipped in preserving these natural extracts for a longer shelf-life. Microbial assay carried out for the selected natural food colourants in aqueous extracts showed that the culturing of bacteria and fungi has practically confirmed the presence of both bacteria and fungi in any food substances at different temperature.

4.2.5. Analysis for Primary Toxicity Level in the selected Natural Food Colourants using Brine Shrimp Assay

The probability of toxicity of chemical compounds means that all new compounds should be regarded as toxic until their safety is confirmed. Food additives sometimes destroy vitamins in food (adding caramel to a food is found to cause a deficiency of Vitamin B6), are used to make bad quality food look good and can cause allergy in many people like diarrhea, skin irritation, stomach disorders, vomiting or an increase in the body hear. Also, it may destroy the nutritional value of food. Several food colouring have been banned due to their tendency to cause cancers and tissue injuries. Tartrazine as a food additive has been proven to cause many different side effects and allergic responses in individuals. These may comprise migraines, nervousness, asthma attacks, hazy vision, eczema, other skin rashes and thyroid cancer (*Kamal et al., 2018*).

4.2.5.1. Primary Toxicity Assay using Brine Shrimp

The results of the brine shrimp assay for annatto seeds (*Bixa orellana*), eucalyptus bark (*Eucalyptus grandis*), madder root (*Rubia cordifolia*) and roselle petals (*Hibiscus sabdariffa)* of both powdered substances and aqueous extractions is tabulated.

Table – XXIV

Brine Shrimp Lethality Assay of the Selected Natural Food Colourants in Powdered Form

Natural Food Colour Extract	Concentration (µg/ml)	Mortality of Brine shrimp (no. of shrimps dead/hour)					
		1	2	4	6	24	% Mortality (at 24 hour)
Annatto seeds (*Bixa orellana*)	**100**	0	0	0	0	1	**3**
	250	0	1	1	1	2	**7**
	500	0	1	2	2	2	**7**
	1000	0	1	1	1	2	**7**
	1500	0	1	1	1	2	**7**
Eucalyptus bark (*Eucalyptus grandis)*	**100**	0	0	0	0	2	**7**
	250	0	0	0	0	2	**7**
	500	0	0	0	0	2	**7**
	1000	0	0	1	1	2	**7**
	1500	0	0	2	3	4	**13**
Madder root (*Rubia cordifolia*)	**100**	0	0	0	0	1	**3**
	250	0	0	0	1	2	**7**
	500	0	0	0	1	7	**23**
	1000	0	0	1	1	10	**30**
	1500	0	0	1	3	12	**40**
Roselle petals (*Hibiscus sabdariffa*)	**100**	0	0	0	0	2	**6**
	250	0	1	1	2	4	**13**
	500	0	1	1	3	7	**23**
	1000	0	1	1	4	10	**30**
	1500	1	1	5	12	23	**80**
Control $K_2Cr_2O_7$	**1(mg/ml)**	**30**	**-**	**-**	**-**	**-**	**100**
Blank	**Saline water**	0	0	0	0	0	**0**

In table-XXIV, the lethality of the brine shrimp for the powdered forms were expressed and revealed that annatto seeds (*Bixa orellana*) powder showed the lowest rate of mortality of seven percent shrimp death even after 24 hour. Whereas in madder root (*Rubia cordifolia*), the percentage of mortality at 24 hour showed that 40 percent in which nearly 12 shrimp were found dead at the highest concentration of solution of 1500 µl. In eucalyptus bark (*Eucalyptus grandis*) powder solution, the highest percent in rate of mortality was found in 1500µl the highest concentration of solution with 13 percent, which can also be expressed as four shrimp were found dead. In comparison to all the powdered samples of the natural food colourants, roselle petals (*Hibiscus sabdariffa)* had shown 80 percentage mortality percent of the brine shrimp. On the whole, the preliminary toxicity testing of powdered samples of annatto seeds (*Bixa orellana*), eucalyptus bark (*Eucalyptus grandis*), madder root (*Rubia cordifolia*) and roselle petals (*Hibiscus sabdariffa)* had shown maximum of 40 to 80 percentage of mortality.

Table – XXV

Brine Shrimp Lethality Assay of the selected Natural Food Colourants in Aqueous Extraction

Natural Food Colour Extract	Concentration (µg/ml)	Mortality of Brine shrimp (no. of shrimps dead/hour)					
		1	2	4	6	24	% Mortality (at 24 hour)
Annatto seeds (*Bixa orellana*)	**100**	0	0	0	0	11	**36**
	250	0	0	0	0	13	**43**
	500	0	0	0	1	13	**43**
	1000	0	0	0	2	16	**53**
	1500	0	0	0	2	16	**53**
Eucalyptus bark (*Eucalyptus grandis)*	**100**	0	0	0	1	23	**76**
	250	0	0	0	2	24	**80**
	500	0	0	0	1	25	**83**
	1000	0	0	7	20	29	**96**
	1500	0	0	22	27	30	**100**
Madder root (*Rubia cordifolia*)	**100**	0	0	0	1	21	**70**
	250	0	0	4	4	24	**80**
	500	0	0	1	1	28	**93**
	1000	0	0	1	1	30	**100**
	1500	0	0	0	2	30	**100**
Roselle petals (*Hibiscus sabdariffa*)	**100**	0	25	29	30	30	**100**
	250	0	27	30	30	30	**100**
	500	0	30	30	30	30	**100**
	1000	0	30	30	30	30	**100**
	1500	0	30	30	30	30	**100**
Control $K_2Cr_2O_7$	**1(mg/ml)**	**30**	**-**	**-**	**-**	**-**	**100**
Blank	**Saline water**	0	0	0	0	0	**0**

The mortality rate in percentage of 30 shrimp in each solution of natural food colourants had been shown in the above table. In the blank solution there was no shrimp found dead. But in the study sample extracts from madder root (*Rubia cordifolia*) and eucalyptus barks (*Eucalyptus grandis)***,** the mortality rate was noticed. In the annatto seeds (*Bixa orellana*) extract minimum mortality rate of 53 percentage (that is 16 shrimps) were found dead at the rate of highest concentration of 1500 µl.

Extract of madder root (*Rubia cordifolia*) had shown the minimum lethality of 70 percent in the concentration of 100 µl, whereas 100 percent lethality was found in 1000 µl and 1500 µl. Twenty one shrimps were found dead and in the eucalyptus bark (*Eucalyptus grandis*) extract also had the minimum mortality percentage of 76 percentage at its lowest concentration level. But other than annatto seeds (*Bixa orellana*) extract, all the other extracts in their highest concentration level of

1500 µl, nearly two-third of the shrimps were found dead. Table – XXIV revealed that the brine shrimp lethality assay carried out for powdered substances show less mortality rate and for further analysis, the powdered form of natural food colourants was used for further analysis.

Whereas, in **roselle petals (*Hibiscus sabdariffa*) all the shrimp were found dead at different concentration, had proved to be toxic in nature so it was ruled out from the study for further analysis.**

Statistical Analysis for the Rate of Mortality Observed in the Brine Shrimp Assay

To prove the rate of mortality observed in the brine shrimp assay, the statistical analysis of the sample, especially the lethality of the brine shrimp between the aqueous extraction and the powdered substance solution is calculated using one way ANOVA along with t-test.

Table – XXVI

Rate of Mortality between Powdered Form and AqueousExtract of Natural Food Colourants in Brine Shrimp Assay

Type of Natural Food Colourants	Rate of Mortality (within 24hour)	Factor (F)	Significance (sig.)
Powdered Form	11.7 ± 10.7	1.81	0.20^{NS}
Aqueous Extraction	73.7 ± 22.8		

^{NS} **Not Significant**

From the *table*, the calculated t-test value as the factor value is represented as f and the value obtained was 1.81, whereas the significant value between the powdered and aqueous extraction of natural food colourants is 0.20. As the factor value and significant value is greater than 0.05, it was statistically proved that there is no significant difference in the mortality rate of the death of shrimp in powdered substances and aqueous solution.

PHASE – III

4.3. Analysis for Chemical, Characterization properties and Secondary Toxicity Testing of the Selected Natural Food Colourants

There is increasing interest in the development of chemically stable food colorants from natural sources that may help replace synthetic dyes. Anthocyanins are natural flavonoid water-soluble pigments that provide red, violet and blue hues to different organs and tissues of many plant species, serving various roles in the plant, including attraction of insects and animals for pollination and seed dispersal and protection against biotic and abiotic stresses. In addition, these pigments have potent antioxidant and anti inflammatory properties and their consumption has proven beneficial effects on human health, with regards to reducing the risk of cardiovascular diseases and some cancer types, improving glucose regulation and aiding in the prevention neurological disorders (*Maria et al., 2022*). For the process of extraction, the natural sources that produce relevant colours to that of the synthetic colours were selected and the results of the analysis was recorded for interpretation.

4.3.1. Analyzing the Antioxidants in the Selected Natural Food Colourants

Antioxidant Assay: Antioxidants have also been replaced in the encapsulation studies used for the preservation and stabilization of food components. Antioxidants are groups of compounds that neutralize free radicals and Reactive Oxygen Species (ROS) in the cell (*Abuajah et al. 2015*). Antioxidant activity in food and beverages has become one of the most interesting features in the science community. These antioxidants provide protection against damage caused by free radicals played important roles in the development of many chronic disease including cardio vascular diseases, aging, heart disease, anemia, cancer, inflammation (*Cuma Zehiroglu et al, 2019*). The results of antioxidant assay of samples, DPPH assay method was used with ascorbic acid as standard for antioxidant assay.

The natural food colour extracts were extracted to replace synthetic colours in the food that are consumed on daily basis. These natural extracts and the powdered substances were tested for antioxidants and phytochemical metabolites. In the results of antioxidant assay of selected natural food colour samples, using ascorbic acid as standard is shown in below table.

Table – XXVII

Concentration Vs Inhibition by DPPH Assay in Different Forms of Natural Food Colourants

Different Concentration (µl)	Inhibition (%) of Natural Food Colourants					
	Annatto seeds *(Bixa orellana)*		Eucalyptus bark (*Eucalyptus grandis)*		Madder root (*Rubia cordifolia*)	
	Powdered Form	Aqueous Extracts	Powdered Form	Aqueous Extracts	Powdered Form	Aqueous Extracts
10	**82.79**	57.39	**67.21**	**97.39**	**67.21**	60.00
50	67.21	63.48	62.30	94.78	65.57	73.91
150	64.75	76.52	59.84	87.83	59.84	**92.17**
250	63.93	87.83	59.84	87.83	57.38	84.35
350	59.84	90.43	59.02	85.22	57.38	80.87
500	54.92	**92.17**	56.56	77.39	54.10	78.26
750	54.92	-	54.10	68.70	50.82	-

According to *Renan et al., (2011*), all the extracts from annatto seeds (*Bixa orellana*) had scavenging property even when tested at the lowest µg/ml range, where the extracts of annatto seeds with the highest antioxidant capacity is present in the lowest IC_{50} values. In accord with the same, hexane and phenolic extracts from annatto seeds had shown low free radical scavenging activity in consideration with the antioxidant and colourant properties, as the solvents with medium polarity must be used to extract functional properties from it *(Claudia et al., 2008)*, whereas in food products, addition of annatto seeds powder had shown good antioxidant activity and presence of phytochemicals (*Stoica et al., 2023*). In this experimental study, the annatto seeds (*B. orellana*), the percentage of inhibition in aqueous extracts had been increased as that of the standard ascorbic solution from 57.39 percentage to 92.17 percentage with increase in concentration from 10µl to 500µl respectively, whereas in the powdered substances the rate of inhibition had decreased from 82.79 percentage to 54.92 percentage.

Finely powdered and aqueous extract of eucalyptus bark is proved had a good source of phytonutrients and antioxidant properties. In the DPPH assay, IC_{50} value had been observed to be 77.20 µg/ml, producing suitable dye as food colourant with no toxicity and aflatoxins the study praline with Naseer's (*et al., 2019*) studies. Different extracts of *E. globulus* that is eucalyptus bark and the isolated fractions, exhibited different antioxidant activity. This is due to the fact that they contained different amounts of flavonoid and phenolic compounds as per their ability to solubilize these compounds; the high scavenging property of *E. globulus* may be attributed to hydroxyl groups existing in the phenolic compounds. All the samples exhibited different extent of antioxidant activity (AOA) and showed higher potency when compared with BHT in scavenging action of DPPH free radical (*Srivastava et al., 2012*), whereas in abovetable eucalyptus barks (*E grandis*) extract the percentage of inhibition was decreased from 97.39 percentage to 68.70 percentage with increase in concentration (10 µl, 750 µl respectively). In eucalyptus barks powder there was decrease in inhibition percentage from 67.21 percentage to 54.10 percentage.

In the aqueous extracts of madder root treated with DPPH antioxidant assay, the antioxidant activity was not hindered and had the suitable antioxidant properties (*Verma et al., 2016*). According to the present study, the rate of inhibition in madder root (*R. cordifolia*) had gradually been increasing from 60 percentage to 92.17 percentage till 150 µl and then it had reduced from 250 µl with 84.35 percentage to 78.26 percentage at 500 µl . Ravikiran's (*et al., 2022*) study had proved that the root extracts had the presence of higher amounts of antioxidants over the stem and leaf extracts and its potential was determined also by the DPPH assay, whereas in the powdered substances of the madder root, the percent of inhibition hadreduced from 67.21 percentage to 50.82 percentage.

4.3.2. Analyzing the Phytonutrients in the Natural Food Colourants

Phytochemical Screening: Phytochemicals are widely present in food and have been confirmed to be bioactive, thereby contributing to human health. However, some phytochemicals are sensitive to light owing to their structures and may suffer from photo degradation, especially when sensitizers exist, resulting in sensory quality change, nutrient loss in food and even the formation of toxic compounds. The photo oxidation of phytochemicals occurs through three different mechanisms: by directly absorbing luminous energy, with triplet excited state sensitizers through electron transfer or proton transfer (type I photo oxidation) and with singlet oxygen produced by O_2 (type II photo oxidation). On the basis of these mechanisms, adequate antioxidants can be added to quench the triple-excited state sensitizers or singlet oxygen to protect against the photo-oxidation of phytochemicals in food (*Baiyi Lu et al, 2017*).

Phytochemical screening was carried out to detect the presence of metabolites essential to increase the acceptability rate of natural food colourants in the food, enabling the food colourants to be applicable even in common foods.

Table – XXVIII

Screening for Phytochemicals in Powdered Form and Aqueous Extracts of the Selected Natural Food Colourants

Metabolites	Tests Performed	Annatto seeds (*Bixa orellana*)		Eucalyptus bark (*Eucalyptus grandis)*		Madder root (*Rubia cordifolia*)	
		Powdered Form	**Aqueous Extracts**	**Powdered Form**	**Aqueous Extracts**	**Powdered Form**	**Aqueous Extracts**
Alkaloids	+Mayer's reagent	-	-	-	-	-	-
	+Dragendorff's reagent	-	-	-	+	-	+
Flavonoids	Alkaline test	+	+	+	+	-	+
	$+H_2SO_4$	+	+	+	+	+	+
	+lead acetate	+	-	+	+	-	+
	Shinoda test	-	-	-	+	-	-
Sterols (Libermann test)	$+CHCl_3$+Acetic anhydride+Conc. H_2SO_4	+	+	+	+	+	+
Terpenoids (Libermann test)	$+CHCl_3$+Acetic anhydride+Conc. H_2SO_4	-	-	-	-	-	-
Anthraquinone (Borntrager's test)	$+FeCl_3$+Conc.HCl+diethyl ether+Ammonia	-	-	+	+	-	-
Anthocyanin	HCl Test	-	-	-	-	-	-
Proteins	+2% Ninhydrin reagent	-	-	-	-	-	-
	+2% $CuSo_4$+95% ethanol+KOH pellet	-	-	-	-	-	-
	+Conc. HNO_3	-	-	+	-	+	-
Phenolic Compound	+5% neutral $FeCl_3$	-	-	-	+	+	-
	Gelatin test	-	-	-	+	+	-
	+Ellagic acid test	-	-	-	+	-	-
Quinones	Conc. HCl	-	-	-	-	-	-

	Alcoholic KOH	-	-	-	-	-	-
Carbohydrates	Molisch's test	+	+	+	+	+	+
	Fehling's test	+	+	+	+	+	-
Tannin	Braymer's test	-	-	+	+	-	-
	+Gelatin test	-	-	+	+	-	-
	10% NaOH test	-	-	+	-	-	-
Saponins	Shaken with water	-	+	-	+	+	+
Cardiac glycosides	+Baljet reagent	+	+	-	+	-	-
	Bromine water test	-	-	-	-	-	-
	Keller-killai test	-	-	-	+	-	-
Glycoside's test	Borntrager's test	-	-	-	-	+	+
	Aq. NaOH test	+	+	-	-	-	-
Lignin	+Gallic acid	-	-	-	-	-	-
Coumarins	+10% NaOH+$CHCl_3$	-	-	-	-	-	-
Volatile oils	Fluorescence test	-	-	-	-	-	-

Presence of the metabolites are indicated as '+'

Absence of the metabolites are indicated as '-'.

Among the three extracts of annatto seeds (*Bixa orellana*), eucalyptus barks (*Eucalyptus grandis*) and madder root (*Rubia cordifolia*), the extracts of madder root (*Rubia cordifolia*) and eucalyptus bark (*Eucalyptus grandis*) extracted by aqueous extraction had shown the presence of alkaloids through Dragendorff's reagent test, whereas in annatto seeds (*Bixa orellana*) alkaloids were absent. The alkaloids present in the powdered forms have its own therapeutic values by acting as anti-inflammatory, antimicrobial, anticancerative, antibacterial agent and many more properties.

The phytochemical analysis in Indian madder root (*Rubia cordifolia*) had proved to have clinical importance where the antioxidant and free radical scavenging activities have impressive effects on cancers and tumors (*Devi et al., 2014*). All the extracts and the powdered form had shown the presence of flavonoids. The test for sterols shows that the extracts as well as the powdered substances had shown the presence of sterols. In the powdered form all the selected three natural food colourants annatto seeds (*Bixa orellana*), eucalyptus barks (*Eucalyptus grandis*) and madder root (*Rubia cordifolia*) show the presence of sterols. The absence of terpenoids had been found in all the six variants. In anthraquinone test, only eucalyptus barks (*Eucalyptus grandis*) extracts and the powder have shown the presence with the formation of reddish orange colour. The other extracts like madder root (*Rubia cordifolia*) and annatto seeds (*Bixa orellana*) had shown the absence of anthraquinone where no colouration was observed.

All the three selected natural food colourants extracts have indicated the absence of protein other than madder root and eucalyptus bark powders. Eucalyptus bark (*Eucalyptus grandis*) extracts and the madder root powder have indicated the presence of phenolic compounds. But annatto seeds (*Bixa orellana*) indicated the absence of phenolic compounds in all the three tests of +5 percent in neutral $FeCl_3$, gelatin test and ellagic acid test. The variants have indicated the absence of quinones through both the tests, where yellow and red precipitates were not formed.

The test for carbohydrates showed the presence of carbohydrates in all the three variants of natural food colourants, whereas tannin was present only in eucalyptus barks (*Eucalyptus grandis)* showed its presence. As for madder root (*Rubia cordifolia*) and annatto seeds (*Bixa orellana*), absence of tannin was observed. Annatto seeds (*Bixa orellana*), eucalyptus barks (*Eucalyptus grandis*) and madder root (*Rubia cordifolia*) hadshown the presence of saponins, where according to *Marta Oleszek et al., (2019)* saponins are highly related to antitumor, anti-inflammatory, antiviral, antifungal and antiparasitic activities. Whereas the other two extracts showed the absence of cardiac glycosides, where cardiac glycosides are responsible for controlling blood pressure, myocardiac ischemia and infraction (*Benito Soto Blanco, 2022*).

Only eucalyptus bark (*Eucalyptus grandis*) had shown the presence of cardiac glycosides, whereas the other two extracts showed the absence of cardiac glycosides and glycosides in either one of the tests. For the detection of lignin, coumarins and volatile oils, the extracts and the powders have showed their absence without any reaction.

Test for the phytochemicals shows the presence of 17 different metabolites. The madder root' (*Rubia cordifolia*) aqueous extracts had the presence of six metabolites and in powdered substances, there were seven metabolites out of 17 metabolites. While, testing the eucalyptus bark(*Eucalyptus grandis*), there were nine and six metabolites, out of 17 metabolites in aqueous extracts and powdered substances respectively. As for annatto seeds, the aqueous extracts and powdered substances had an equal count of six metabolites each out of 17 metabolites.

Testing the Level of Significance by Paired Comparison Test for Free Radical Analysis

The difference between the aqueous extracts and the powdered substances difference in the anti-free radical analysis has been statistical tested.

Table – XXIX

Level of Significant Difference in Free Radical Activity of Natural Food Colourants

Natural Food Colourants in different forms		**Mean of the Free Radical Scavenging Activity (M ± SD)**	**t test**
Annatto seeds *(Bixa orellana)*	Powdered Form	64.05 ± 09.54	**0.83****
	Aqueous Extracts	66.83 ± 32.40	
Eucalyptus bark*(Eucalyptus grandis)*	Powdered Form	59.83 ± 04.17	**0.00***
	Aqueous Extracts	85.59 ± 09.88	
Madder root *(Rubia cordifolia)*	Powdered Form	58.90 ± 05.87	**0.48****
	Aqueous Extracts	67.08 ± 31.19	

*Significant **Not significant

From the *table-XXIX*, the mean, standard deviation and the significance value of the aqueous extracts and the powdered substances in their inhibition percent of antioxidant activity were recorded. The overall mean in the powdered form of annatto seeds (*Bixa orellana*), eucalyptus bark (*Eucalyptus grandis*) and madder root (*Rubia cordifolia*) were 64.05±09.54, 59.83 ± 04.17 and 58.90 ± 29.28 respectively. Whereas in the aqueous extracts of madder root (*Rubia cordifolia*), eucalyptus bark (*Eucalyptus grandis*) and annatto seeds (*Bixa orellana*) were 67.08 ± 31.19, 85.59 ± 09.88 and 66.83 ± 32.40 respectively.

As for the level of significance value, the values of madder root (*R. cordifolia*) and annatto seeds (*B. orellana*) were 0.48 and 0.83, which showed that it was insignificant and there was no difference in the inhibition percentage of antioxidant activity. But in eucalyptus barks (*E. grandis*) there was a statistically significant difference in antioxidant activity between aqueous extracts and powdered substances.

As the level of significance in the paired comparison t test is less than 0.05, then it had been proved that there is significant difference in the radical scavenging activity between the aqueous extracts and the powdered substances.

This study on phytochemicals have scientifically proved that the antioxidants activity in the natural food colourants have an effect over increase in the concentration of substances. The presence of metabolites in both the aqueous extracts and the powdered substances has proved that both have significant health benefits, which can be used as alternative for synthetic food colourants. Also, in the emerging phase of various diseases, the selected natural food colourants have proved to act as biomarkers, especially for the enhancement of mental health.

4.3.2 Analyzing for Secondary Toxicity Level in the selected Natural Food Colourants and Synthetic Food Colourants.

Inductively Coupled Plasma Mass Spectrometer couples use of an ICP with MS for elemental analysis in madder root (*Rubia cordifolia*), eucalyptus bark (*Eucalyptus grandis*) and annatto seeds (*Bixa orellana*) along with the synthetic food colourants by generation of ions. This method is carried out to detect the presence of toxic heavy metals that are harmful for consumption.

4.3.2.1 Analysis of Inductively Coupled Plasma Mass Spectrometer in the selected Natural Food Colourants

Inductively Coupled Plasma Mass Spectrometry (ICP-MS) appears clearly that ICP-MS is at present one of the most useful techniques in the field of food classification. The potential of strontium isotope ratio and of multi element profiling is proved by the large number of studies on different foodstuffs, from the most simple production chains like fruit and vegetable products to the most complex ones like meat and fishes (*Aceto, 2016*).

The heavy metals analysed to check the toxicity of the selected natural food colourants are represented as follows: Lithium (Li), Berylium (Be), Boron (B), Aluminium (Al), Phosphorus (P), Titanium (Ti), Vanadium (V), Chromium (Cr), Manganese (Mn), Iron (fe), Cobalt (Co), Nickel (Ni), Copper (Cu), Zinc (Zn), Arsenic (As), Molybdenum (Mo), Silver (Ag), Cadmium (Ca), Tin (Sn), Antimony (Sb), Cesium (Cs), Mercury (Hg) and Lead (Pb) is shown in the table - .

Table – XXX

Heavy Metals Analysis in the Selected Natural Food Colourants

Heavy Metals Elements	**Annatto seeds** (*Bixa orellana*) **(µg)**	**Eucalyptus bark** (*Eucalyptus grandis*) **(µg)**	**Madder root** (*Rubia cordifolia*) **(µg)**
Lithium	Nil	0.06046	1.02786
Berylium	0.00978	0.00000	Nil
Boron	12.70282	16.05903	17.18369
Aluminium	16.07565	131.94823	376.40988
Phosphorous	1476.31634	1749.23633	1023.76806
Titanium	5.96367	123.90094	41.51459
Vanadium	0.07031	0.68988	0.95588
Chromium	0.25484	1.73141	5.48116
Manganese	21.31282	812.37734	30.70771
Iron	52.61333	184.71820	412.34194
Cobalt	0.23656	0.38276	0.21510
Nickel	9.53278	7.13936	1.25535
Copper	7.42697	5.75965	19.50650
Zinc	14.69122	17.04302	20.02754
Arsenic	0.00711	0.02292	0.12016
Molybdenum	0.34667	0.15631	0.30099
Silver	Nil	Nil	Nil
Cadmium	0.01074	0.02449	0.05162
Tin	0.12110	0.13752	0.45545
Antimony	Nil	0.00293	0.01406
Cesium	0.00748	0.01040	0.06730
Mercury	0.17127	0.16796	0.15526
Lead	0.57672	0.45234	3.28560

The table, represents the presence of metallic substances present in the powdered natural food colouarants annatto seeds (*Bixa orellana*), eucalyptus bark (*Eucalyptus grandis*) and madder root (*Rubia cordifolia*). Lead, cadmium, chromium and mercury are considered the primary toxins that can affect human health.

In annatto seeds (*Bixa orellana*) and eucalyptus bark (*Eucalyptus grandis*) the level of lead 0.57672 µg, 0.45234 µg, cadmium 0.01074 µg,0.02449 µg, chromium 0.25484 µg and 1.73141 µg respectively. But the level of mercury in eucalyptus bark (*Eucalyptus grandis*) and annatto seeds (*Bixa orellana*) is 0.16796 µg and 0.17127 µg. The other toxic metals were higher in concentration in the powdered particles of madder root (*Rubia cordifolia*) than in eucalyptus bark (*Eucalyptus grandis*) and annatto seeds (*Bixa orellana*). Whereas madder root was considered more toxic than the other two natural food colourants as it has high amount of lead 3.28560 µg, cadmium 0.05162 µg and chromium 5.48116 µg. Thus, **madder root (*Rubia cordifolia*) are considered toxic and was ruled out for further analysis**.

4.3.2.2 Analysis of Inductively Coupled Plasma Mass Spectrometer in the selected Synthetic Food Colourants

Apart from the natural food colourants, the most commonly used synthetic food colourants were also analysed for toxic heavy metals. From the survey conducted in the supermarkets and shops selling the synthetic food colourants, the popular brands were tested for heavy metals, to utilize the brand in preparation of standard selected recipes for the further analysis. The list of heavy metals present in the popular brands of BK, BU and GC are listed below in the table.

Table – XXXI

Heavy Metals Analysis in the Selected Synthetic Food Colourants

Heavy Metals Elements	Common Synthetic Food Colourants Brands sold in the Market		
	BK (µg)	BU (µg)	GC (µg)
Lithium	0.016	0.005	0.0635
Berylium	Nil	0.007	Nil
Boron	Nil	Nil	0.5227
Aluminium	1.736	Nil	2.2715
Phosphorous	46.697	5.911	13.0863
Titanium	0.609	Nil	1.4395
Vanadium	0.114	Nil	0.0235
Chromium	0.095	0.009	0.2799
Manganese	0.280	Nil	0.7825
Iron	10.865	Nil	9.5050
Cobalt	0.003	Nil	0.0061
Nickel	0.105	0.036	0.0987
Copper	Nil	Nil	0.0244
Zinc	Nil	Nil	0.0627
Arsenic	0.031	0.011	0.0078
Molybdenum	Nil	Nil	Nil
Silver	Nil	Nil	0.0046
Cadmium	Nil	Nil	0.0011

Tin	0.013	Nil	Nil
Antimony	Nil	Nil	Nil
Cesium	0.001	Nil	0.0025
Mercury	0.383	Nil	0.0846
Lead	0.038	0.033	0.6707
Lithium	0.008	Nil	0.0244

From the findings of market survey, the researcher had come to know that the three brands mostly commonly used are BK, BU and GC. Thus, the three brands were subjected to heavy metal analysis to comparatively know their rate of toxicity. The above table, represents the presence of earthen metallic substances present in the commercially available synthetic food colourants that are more commonly used by consumers of Coimbatore city.

As lead, cadmium, chromium and mercury were considered the primary toxins that can affect human health, the commonly used GC brand had been detected of the metals with 0.0244 µg, 0.0011 µg, 0.2799 µg and 0.6707µg respectively. Whereas, the other metals in GC brand is lithium of 0.0635 µg, boron of 0.5227 µg, aluminium of 2.2715 µg, phosphorous of 13.0863 µg, titanium of 1.4395 µg, vanadium of 0.0235 µg, chromium of 0.2799 µg, manganese of 0.7825 µg, iron of 9.5050 µg, cobalt of 0.0061 µg, nickel of 0.987 µg, copper of 0.0244 µg, zinc of 0.0627 µg, arsenic of 0.0078 µg, silver of 0.0046 µg, cesium of 0.0025 µg and barium of 0.0846 µg are present.

Even though the brand BU is least sold and consumed by the customers, the heavy metals toxicity had proved that the brand BU has the least concentration of chromium with 0.009 µg, lead with 0.33 µg, also the other two heavy metals cadmium and mercury are not present. Thus, the synthetic food colourant Bush is advisable for usage than the brands BK and GC.

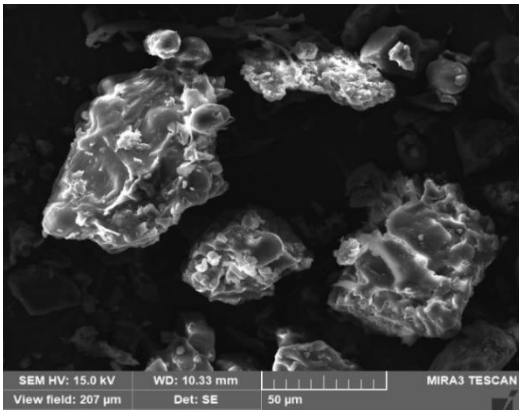
To test the organoleptic evaluation of the incorporated selected recipes, synthetic food colourant brand BU was used in standard recipes.

4.3.3 Analysis for Characterization of the selected Natural Food Colourants

4.3.3.1 Analyzing the Microstructure of the selected Natural Food Colourants by Field Emission Scanning Electron Microscope (FE-SEM)

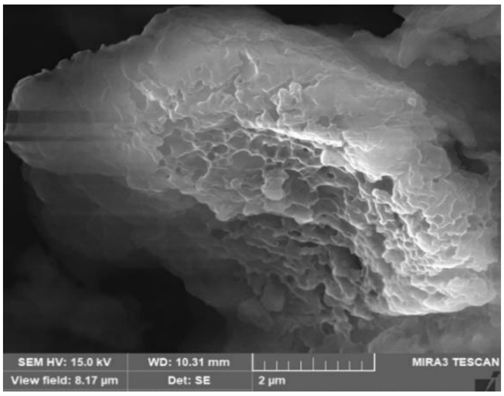
Field Emission Scanning Electron Microscope (FE-SEM) is an advanced technology used in capturing the microstructure image of any material. FE-SEM is conducted with high voltage of 10,000-20,000 kV and with high vacuum, as gas molecules intersects the electron beams which emit secondary and backscattered electrons used for capturing the image of the material. Before exposing the natural food colourants, annatto seeds (*Bixa orellana*) and eucalyptus bark (*Eucalyptus grandis*) to the beam of electrons or x-rays, coating the material in vacuum with evaporated carbon is must and this process is known as sputtering. Particles morphology of the samples were investigated using Nova 200 Nano Lab field emission scanning electron microscope (FE-SEM) (*Ali Reza Jahangiri et al., 2021*).

In this study, in the field of food science the technique FESEM needs to be adapted to understand food structure, since the processing conditions that convert biological raw materials into food result in structural and textural changes and affect the properties and materials of the food. The technique serves as a promising and reliable method for microstructure analysis particularly in novel formulations, innovations with particular properties, food textures and also detection of defects in foods (*Vasudha et al., 2019*).



(a)

View Field	207 µm
Particle Size	50 µm
Magnification View	1.00 kx

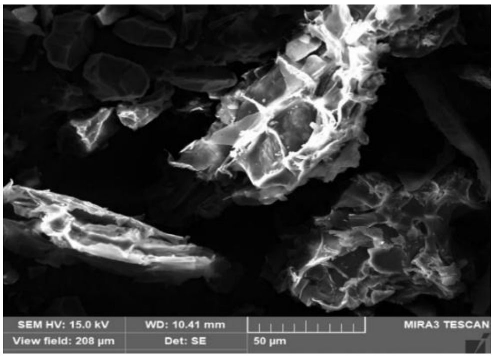


(b)

View Field	8.17 µm
Particle Size	2 µm
Magnification View	25.4 kx

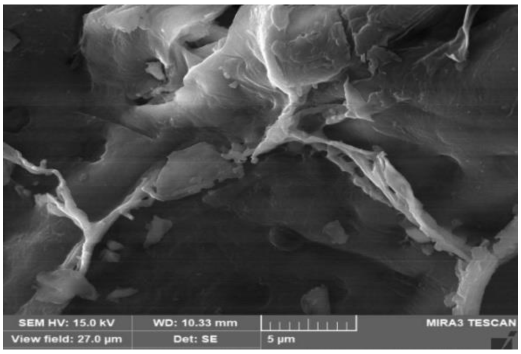
Plate – 4.3.3.1 (a) (b)

Micro structure of Field Emission Electron Microscopic view of Annatto Seeds Powder (*Bixa orellana*)



(a)

View Field	208 µm
Particle Size	50 µm
Magnification View	999 kx



(b)

View Field	27.0 µm
Particle Size	5 µm
Magnification View	7.69 kx

Plate – 4.3.3.1 (a) (b)

Micro structure of Field Emission Electron Microscopic view of Eucalyptus Bark Powder (*Eucalyptus grandis*)

The above *plates* are the imaging of the powdered particles viewed under MIRA3 TESCAN Scanning Emission Microscope that magnifies the physical structure of annatto seeds (*Bixa orellana*) and eucalyptus bark (*Eucalyptus grandis*). In the physical structural view of annatto seeds (*Bixa orellana*) powder plate (a), the view field at 207 µm, the magnification view is at 1.00 kx which had exhibited the particle size to be 50 µm. Likewise, the closer view field at 8.17 µm of 25.4 kx magnification view the particle size is seen to be 2 µm.

The plate of eucalyptus bark (*Eucalyptus grandis*) shows the view field at 208 µm and 27 µm, the particles are being magnified at 999 kx and 7.69 kx. The particle size at 999kx is 50 µm, whereas the particle size at 7.69 µm is 5 µm. According to *Sandra (et al., 2024)*, in the eucalyptus bark (*Eucalyptus grandis*) powder, the needle like structure exhibits the presence of residue of cell lignin particles that are crystalline cellulose embedded in the form of lignin matrix responsible for the rigidity of the bark substance.

4.3.3.2 Analyzing the Chemical Composition by X-Ray Diffraction (XRD) inthe Selected Natural Food Colourants

To generate X-rays, a source of electrons, an accelerating mode with the electrons at high speeds and a target material to receive the impact of the electrons to interact with them is needed. Crystalline solids consist of regular arrays of atoms, ion or molecules with inter atomic spacing on the order at 100 pm or 1 Å. The wavelength of the incident light has to be on the same order as the spacing of the atoms. In powder diffraction, the powder of annatto seeds (*Bixa orellana*) and eucalyptus bark (*Eucalyptus grandis*) composed of many small and finely ground crystals, known as crystallites. These crystallites are (assumed to be) randomly oriented to one another. If the powder is placed in the path of a monochromatic X-ray beam, diffraction will occur from the planes in those crystallites that are oriented at the correct angle to fulfil the Bragg condition (*Hayat Khan et al., 2020*).

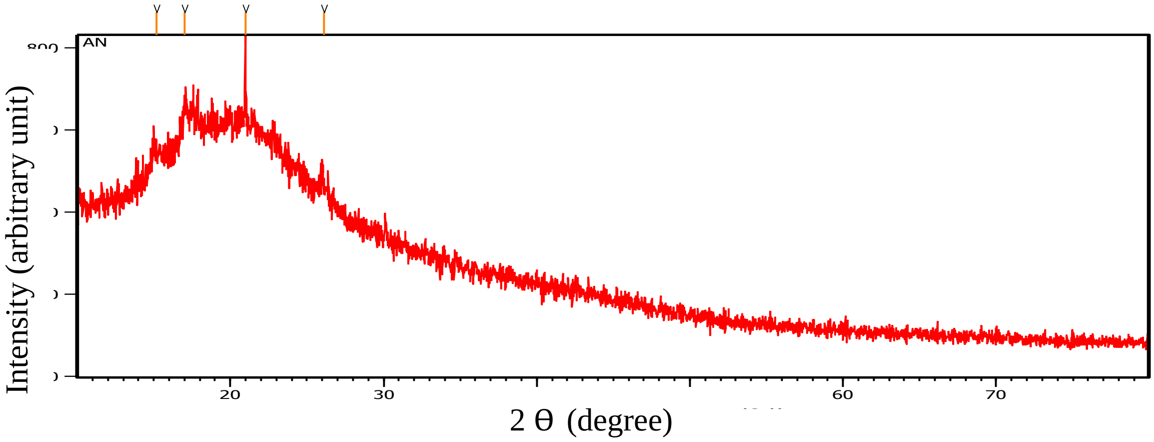


Figure - 4.3.3.2 (a): XRD Crystalline Nature of Annatto Seeds Powder (*Bixa orellana*)

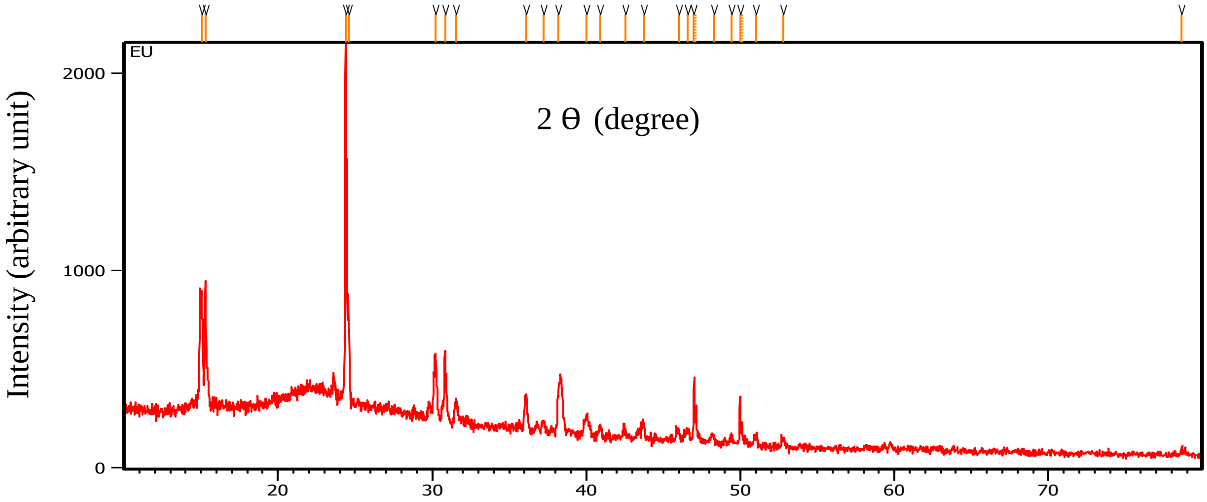


Figure - 4.3.3.2 (b): XRD Crystalline Nature of Eucalyptus Barks Powder (*Eucalyptus grandis*)

From the XRD Charts, the higher peak intensity of the annatto seeds powder have shown that there are higher number of atoms in the crystal proving it to be amorphous is nature, whereas the highest peak in the eucalyptus bark powder indicated that the higher degree of crystallinity is due to the large crystals of particles present in it, proving it to be crystalline in nature.

Also in the powdered form of eucalyptus bark (*Eucalyptus grandis*), the peaks in the graph of XRD crystalline nature of eucalyptus bark powder(*Eucalyptus grandis*) had fallen at 24^0C of two theta value showing the crystallinity index. It represents the presence of more number of heavy metals present in the powdered substances. Thus, Inductively Coupled Plasma Mass Spectrometer (ICPMS) for heavy metal analysis was carried out. At the highest exposure to thermal treatment, the substance can become amorphized of the fibril surface by further degrading the celluloytic enzymes that peels the fibrils. The further drop in the peak is due to decrease in the crystallite width of application of temperature (*Sandra et al., 2024*).

4.3.3.3 Thermogravimetric Analysis (TGA) for the Selected Natural Food Colourants

Thermal gravimetric analysis or thermogravimetric analysis (TGA) is a method used to measure as a function of increasing temperature-with constant heating rate, or as a function of time-with constant temperature and/or constant mass loss. Thermogravimetric analysis, the sample is heated in a given environment at controlled rate. The change in the weight of the substance is recorded as a function of temperature or time. The temperature is increased at a constant rate for a known initial weight of the substance and changes in weights are recorded as a function of temperature at different time interval (*Gamzenur Ozsin et al., 2017*).

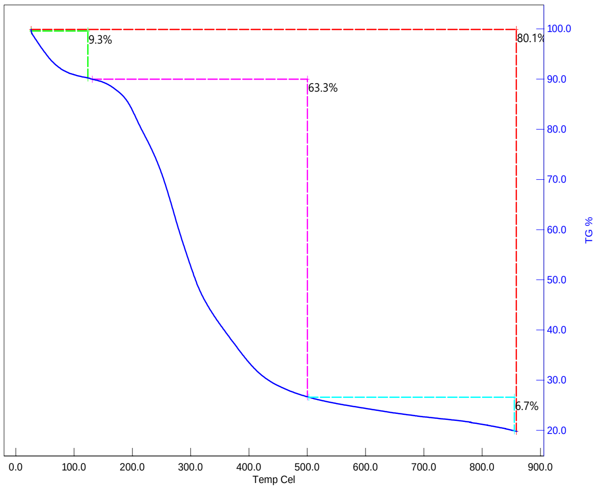


Figure – 4.3.3.3 (a): Thermo Gravimetric Analysis (TGA) of Annatto Seeds Powder (*Bixa orellana*)

The blue curve in the graph indicates the thermal stability of the annatto seeds (*Bixa orellana*) powder. According to *Handayani et al., (2021)* and *Satyanarayana et al., (2003),* pioneer researcher in annatto seeds had explained that the seed coat of annatto seeds are made up of cis-bixin, a soluble compound which dissolves in organic polar solvents gives orange colour to it. When the temperature is increased the stereoisomers of cis-isomers gets converted into trans-isomers, stable red colour compound that are mostly polar carotenoids. Thus, from the graph the slight drop in the curve at 100°C for 93 percentage is due to loss of water in the substance. The loss of actual mass of the powder has shown the conversion of cis to trans-isomers that has begun at 200°C and has dropped till 500°C with loss of mass to 63.3 percentage Thestability of the substances at 67 percentage for the highest thermal gravimetric analysis. When there is increase in temperature and also increase in loss of mass. As most forms of cooking is done within 60-200°C, the annatto seeds powder tested for thermal gravitivity has been physically proved to be stable to be used as natural food colourant.

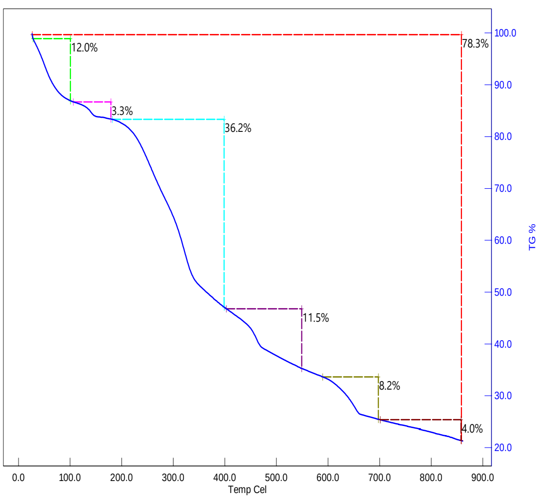


Figure – 4.3.3.3 (b): Thermo Gravimetric Analysis (TGA) of Eucalyptus Bark Powder (*Eucalyptus grandis*)

The blue curve in the graph indicates the thermal stability of the eucalyptus bark (*Eucalyptus grandis*) powder. The drop in the curve at 100°C for 12 percentage is due to loss of absorbed water in the substance, where the maximum weight loss must be due to the presence of calcium. The loss of actual mass of the powder has began at 200°C and has dropped till 400°C with loss of mass to 36.2 percentage, where according to *Trouve (et al., 2023),* calcium oxalate monohydrate looses its water along with thermal decomposition of lignin and cellulose with release of degradation products summing up to 11.5 percentage. After 500^{0}C the loss contributes to the charring, that degrades the densification of the bark where the thermal degradation of the inorganic salts take place. At 600-700^{0}C, the thermal degradation of calcium oxalate decarbonizes to form calcium carbonate (*Sandra et al., 2024*). But the eucalyptus bark powder, tested for thermal gravitivity effect have shown a slight loss of mass, when there is increase in temperature for every 100^{0}C.

4.3.3.4 Testing the Photosensitivity of the Selected Natural Food Colourants

The photodegradation reactions involved were found to be photo-oxidation, isomerisation, hydrogen transfer, hydrogen abstraction and photolysis, with a great variety of photodegradation products. No other approaches than standard methods have appeared to be used for the analysis of photodegradation studies, with UV as standard detection methods. For a better understanding of the nature of the photodegradation species is gained together with strategies to properly analyse the substances. Nonetheless, due to the complex composition of food products and the influence of other factors than light, more extensive research on this topic still needs to be performed (*Joshka Verduin et al., 2020).* The powdered samples of annatto seeds (*Bixa orellana*) and eucalyptus bark (*Eucalyptus grandis*) were exposed to LED light of 200 watts (maximum exposure) and the samples were used for photometric analysis. Due to turbidity in the solution, the results were not reliable and the graphical curves were also very unstable. But the decrease in the level of concentration was monitored using UV spectrophotometer with 215 nm.

Table – XXXII
Photosensitive Degradation of Selected Natural Food Colourant Solutions

Natural Food Colourants	Number of Hours exposed to LED Light	Change in Concentration of the Natural Food Colourants (μg/ml)
Annatto seeds (*Bixa orellana*)	**Zero**	**0.60**
	Two	0.46
	Four	0.45
	Eight	0.36
	12	0.32
	18	0.29
	24	0.28
Eucalyptus bark	**Zero**	**0.61**

(*Eucalyptus grandis*)	Two	0.47
	Four	0.46
	Eight	0.37
	12	0.33
	18	0.30
	24	0.29

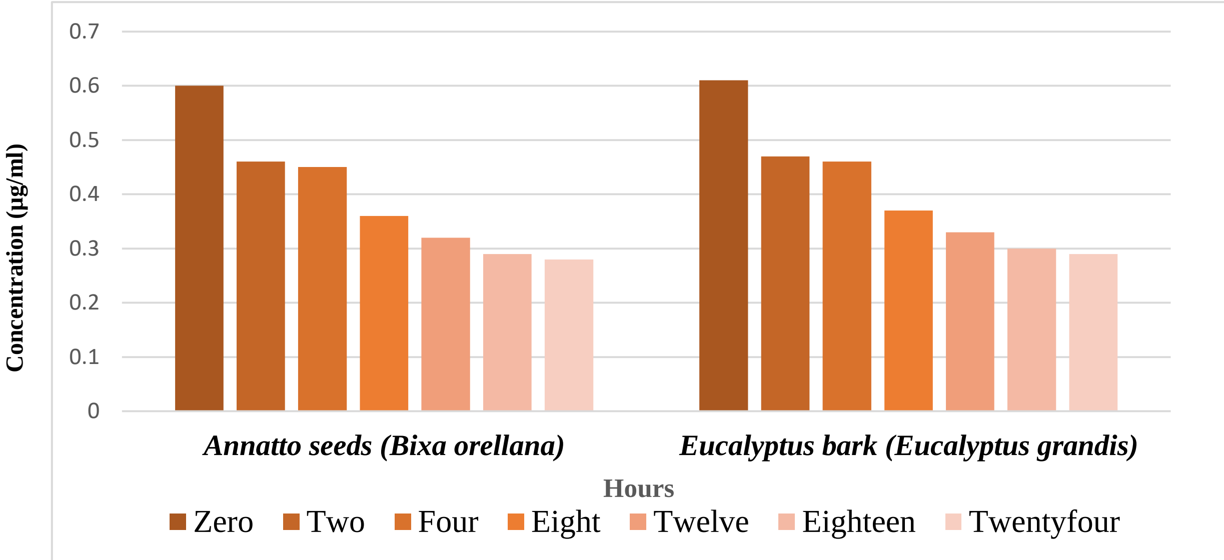


Figure - 4.3.3.4

Level of Concentration of the Selected Natural Food Colourants

From the table-XXXII, the level of concentration of degradation due to photosensitivity was measured using the standard curve measurement by spectrophotometer. The concentration level of annatto seeds (*Bixa orellana*) and eucalyptus bark (*Eucalyptus grandis*) were measured in zero hours, then gradually increased the time of exposure to the monochromatic light from zero hour to two, four, eight, twelve, eighteen and twenty-four hours respectively.

From zero hour to twenty-four hours, the degradation in concentration was 0.32 µg loss of strength in the concentration of annatto seeds (*Bixa orellana*), whereas the same level of degradation was measured as 0.32 µg of loss of concentration due to degradation is found in eucalyptus bark (*Eucalyptus grandis*).

Photosensitivity test has proved that when the selected natural food colourants annatto seeds (*Bixa orellana*) and eucalyptus bark (*Eucalyptus grandis*) powder are stored in transparents glass bottles, they are directly exposed to the light in the environment. Thus, there is degeneration of colours when exposed to light for a longer period of time. Proper storage and type of package to store the natural food colourants without losing the intensity or change in colour.

PHASE – IV

4.4. *In vivo* Study

The mechanisms of toxicant actions are multifactorial but many toxic effects converge on the generation of oxidative stress and chronic inflammation resulting in cell death, aging and degenerative diseases. Integration of food toxicology data obtained throughout biochemical and cell-based *in vitro*, animal *in vivo* and human clinical settings has enabled the establishment of alternative, highly predictable *in silico* models (*Alexander Gosslau, 2016*).

4.4.1. Testing the Toxicity of the Selected Natural Food Colourants in Wistar Albino Rats

Mice and rats are considered as primary substituents for monitoring the bodily functions of all the organs as that of human beings. Thus, the *in vivo* study was adopted to study the metabolic changes that is taking place in the organism. The appropriate level of the selected natural food colourants eucalyptus bark (*Eucalyptus grandis*) and annatto seeds (*Bixa orellana*) to be added in the foods will be verified using animal study that will remain prognostic for human consumption. The *in vivo* model study was demonstrated to prove that the selected natural food colourants have various health benefits in enhancing the quality of food. It was also conducted to analyze the biological effects of natural food colourants in animals through histopathological examination by enabling the colourants for further utilization. The permissible level of natural food colourants to be added in foods for human consumption will be determined after the in vivo study. The effects of developed natural food colourants will be tested biologically in model Wistar Albino rats to analyze the effects when consumed more frequently.

4.4.1.1 Pilot Study to Optimize the Level of Natural Food Colourants Administered to Wistar Albino Rats

The test groups for both the powdered forms of eucalyptus barks (*Eucalyptus grandis)* and annatto seeds (*Bixa orellana*) were named as test rat – 1 and test rat – 2. The Wistar Albino rats' level of water to be given was calculated with the body weight of the rats during the purchase. The calculation of aqueous feeding of natural food colourants are as follows for eucalyptus barks (*Eucalyptus grandis)* where the rat was named as Test rat – 1 (TR-1) and it weighed around 376g. The rat for annatto seeds (*Bixa orellana*) was named as Test rat – 2 (TR-2) and it weighed around 373 g.

Table – XXXIII

Experimentation Plan for Toxicity Testing

PARTICULARS	**NO. OF ANIMALS**	**FOOD AND DOSAGE GIVEN DURING EXPERIMENTATION**	**PERIOD OF TIME** (days)	**TYPE OF FEED**
Control Group	2	Commercial Feed (25 g) + Normal Drinking water (ml)	56	Oral
Experimental Group – 1	5	Commercial Feed (25 g) + Normal Drinking water (ml) + Specific Dosage of Food Colourants Eucalyptus bark (*Eucalyptus grandis)* (calculated according to the weight of the rat)	56	Oral
Experimental Group – 2	5	Commercial Feed (25 g) + Drinking water (ml) + Specific dosage of Food Colourants Annatto seeds (*Bixa orellana*) (calculated according to the weight of the rat)	56	Oral

From the scheduled feed plan, the wistar albino rats were weighed every week and checked for physical changes that indicates the weight gain or loss. 25 g of commercial pellets were fed everyday to the rats, dividing it for twice a day. Likewise, level of water consumption and intake of feed was also checked every day and total consumption was monitored every week.

Table – XXXIV

Oral feeding of Water Fed with Selected Natural Food Colourants

Natural Food Colourants	**Name of the Rat**	**Calculation of Level of Water to be fed**	**Determined Level of Water (ml)**
Eucalyptus bark (*Eucalyptus grandis)*	Test rat – 1 (TR1)	376 × 2 ÷ 100 = 7.5	**7.5**
Annatto seeds (*Bixa orellana*)	Test rat – 2 (TR2)	373 × 2 ÷ 100 = 7.46	**7.5**

According to the *table,* the determined level of water in which the natural food colourants were to be optimized was 7.5 ml for both the test groups of rats which was orally administered to the in vivo models with powdered forms of eucalyptus barks (*Eucalyptus grandis)* and annatto seeds (*Bixa orellana*).

The level of water determined according to the body weight was 7.5 ml, in which the natural food colourants annatto seeds and eucalyptus bark powder were diluted and this extract was given for five hours per day. Rest of the time the rats were exposed to normal drinking water along with the feed. To verify if the animals were under certain abrupt meal pattern due to the intake of natural food colourants, their normal drinking water consumption level was also monitored frequently.

Table – XXXV

Pilot Study on Optimization of the Eucalyptus bark (*Eucalyptus grandis)* Orally Fed to the Wistar Albino Rats

No. of Weeks	Water (ml)	Ppm	Calculation of ppm*	ppm of Natural Food Colourants mixed in Water (mg)
1	7.5	2	7.5 × 2 ÷ 1000	0.01
	7.5	12	7.5 × 12 ÷ 1000	0.08
2	7.5	14	7.5 × 14 ÷ 1000	0.10
3	7.5	28	7.5 × 28 ÷ 1000	0.20
4	7.5	41	7.5 × 41 ÷ 1000	0.30
5	7.5	54	7.5 × 54 ÷ 1000	0.40
6	7.5	67	7.5 × 67 ÷ 1000	0.50
7	7.5	80	7.5 × 80 ÷ 1000	0.60
8	7.5	94	7.5 × 94 ÷ 1000	0.70

*The water level administered to the rats multiplied by the parts per million (ppm) to be tested in 1000 ml of water, that is one liter of water *(Ramachandran et al., 2012).*

For eucalyptus bark (*Eucalyptus grandis*) in the first week two different ppm of two and 12 ppm of fed were diluted and orally fed to the rat with accounted to 0.01 mg and 0.08 mg of powdered eucalyptus bark sample. Wash out period for two days were given and the rat was fed with one milli gram of the natural food colourant. The rats used for pilot study drank the water completely without any wastage that was administered with the calculated amount of natural food colourants in it. Every five days in a week, the wistar albino rats were exposed to oral feeding, whereas the other two days the rats were left with normal drinking water, as washout period.

The acceptability rate was high among the rat and so every five days in a week and with two days of wash out period, the quantity natural food colourant added to the oral fed was increased by one milligram, which was diluted in water and administered to the wistar albino rat. On exposure to 7 mg of natural food colourant, the test rat 1 showed resistance to drinking water and left over of food pellets were large in quantity. Thus, three days of wash out period was given and 6 mg of eucalyptus bark powder in 7.5 ml of water was continuously fed to know the level of acceptability. As 6 mg, that is 94 ppm in 7.5 ml of water was acceptable, the level was optimized for eucalyptus bark, this can also be represented as 6000 µg/kg of body weight of a wistar albino rat.

Table – XXXVI
Pilot Study on Optimization of the Annatto seeds (*Bixa orellana*) Orally Fed to the Wistar Albino Rats

No. of Weeks	Water (ml)	Ppm	Calculation of ppm*	ppm of Natural Food Colourants mixed in Water (mg)
1	7.5	2	7.5 × 2 ÷ 1000	0.01
	7.5	12	7.5 × 12 ÷ 1000	0.08
2	7.5	14	7.5 × 14 ÷ 1000	0.10
3	7.5	28	7.5 × 28 ÷ 1000	0.20
4	7.5	41	7.5 × 41 ÷ 1000	0.30
5	7.5	54	7.5 × 54 ÷ 1000	0.40
6	7.5	54	7.5 × 54 ÷ 1000	0.40
7	7.5	50	7.5 × 50 ÷ 1000	0.37
8	7.5	47	7.5 × 47 ÷ 1000	0.35

*The water level administered to the rats multiplied by the parts per million (ppm) to be tested in 1000 ml of water, that is one liter of water *(Ramachandra et al., 2012)*.

From the *tables,* it was interpreted that 7.5 ml of water mixed with the natural food colourant from week one to week seven, where the ppm was increased every week and the maximum threshold level of the rats were analysed and the amount of the feed (ppm) to be fed to the wistar albino rats were optimized by the researcher.

As for the annatto seeds (*Bixa orellana*) powder, the same procedure was carried out, on the first week 0.01 mg and 0.08 mg, that is two ppm and 12 ppm were administered to the rat in 7.5 ml of water. The acceptability rate stopped in the fifth week, where 54 ppm that is 4 mg of annatto seeds powder extract was not consumed by the rat and so it was again administered during the sixth week as well to test if the rat is able to consume it, after exposing it continuously to 4 mg of annatto seeds powder. But the rat showed an aggressive behaviour of resistance to touch, inability to consume its food and inability to drink water. Thus, the dosage was reduced to 0.37 mg and 0.35 mg for the next two week with two days of wash out period, where again the same resistant reactions were found in the rat and so the optimization level of annatto seeds powder extracts to be fed orally with water was determined to be 3 mg in 7.5 ml of water, this can also be represented as 3000mg/kg of body weight of a rat. The further study was elaborated to the experimentation study of feeding the rats with natural food colourants of powdered forms.

4.4.1.2. Experimentation for Toxicity of the selected Natural Food Colourants in the Wistar Albino Rats

The animals were divided into two groups with five animals in each experimental group and two animals in control group. Group – 1 is the experimental group of eucalyptus barks (*Eucalyptus grandis)* and Group – 2 is the experimental group of annatto seeds (*Bixa orellana*), the Wistar Albino Rats was fed for 56 days.

Table – XXXVII

Change in Weight of the Wistar Albino Rats

No. of Weeks	Weight of the Wistar Albino Rats (g)											
	Control Group		Experimental Group – 1 Eucalyptus bark					Experimental Group – 2 Annatto seeds				
	CR_1	CR_2	$EuER*_1$	$EuER_2$	$EuER_3$	$EuER_4$	$EuER_5$	$AnER^{\wedge}_1$	$AnER_2$	$AnER_3$	$AnER_4$	$AnER_5$
Week 1	265	271	378	325	302	331	281	356	326	304	331	384
Week 2	265	281	349	313	302	325	284	361	319	284	338	386
Week 3	276	309	380	352	330	336	305	372	312	284	358	399
Week 4	273	301	387	344	338	332	314	369	316	289	352	400
Week 5	271	312	402	355	350	334	327	377	316	286	345	406
Week 6	286	325	407	368	352	337	337	383	318	276	363	415
Week 7	283	297	403	357	349	323	339	383	310	271	360	412
Week 8	282	298	407	374	354	338	340	388	314	265	364	422

*Eucalyptus bark Experimental Rat^Annatto seeds Experimental Rat

From *table-XXXVII,* the weekly weight gain or loss of the Wistar Albino rats was monitored regularly has been listed that were fed with eucalyptus bark (*Eucalyptus grandis)* and annatto seeds (*Bixa orellana*).

The length of the rats remained the same throughout the experimentation study and the overall average length of the rats ranged between 20 centimeters to 21 centimeters throughout the period of study.

Table – XXXVIII

Body Mass Index (BMI) of the Wistar Albino Rats Monitored during the Experimental Period

Wistar Albino Rats	Before		After		t – value	Significance (Sig.)
	Weight (g)	BMI	Weight (g)	BMI		
Control Group	268	0.61	290	0.65	-9.00	0.70^{NS}
Experimental Group – 1 Eucalyptus bark (*Eucalyptus grandis)*	323	0.72	362	0.81	-2.332	0.80^{NS}
Experimental Group – 2 Annatto seeds (*Bixa orellana*)	340	0.76	350	0.79	-0.757	0.49^{NS}

^{NS} Not Significant BMI = Body Weight (g)÷Square of the nose-to-anus length (cm)

According to Novelli (*et al.,* 2007), the Body Mass Index for normal Wistar albino rats range between the values of 0.45 to 0.68 g/cm^2, likewise when the reference range of BMI had increased, then the level of obesity also increased, classifying the weight gain as slight, moderate and severe obesity.

From the *table-XXXVIII,* the BMI value of all the three groups of rats were calculated and the weight gain had shown slight increase in the body mass of 0.7 g/cm^3 in control group and 0.78 g/cm^3 in experimental groups, respectively, whereas the actual BMI of the rats have to be 0.68 g/cm^3. This shows that the rats are slightly overweighed when compared to the range of BMI in Wistar albino rats.

The BMI of the wistar albino rats before and after the experimentation was calculated by measuring its weight. The paired sample t-test was applied to find the statistical difference before and after experimentation. The p value was greater than 0.05, which signifies that there is no significant difference before and experimentation, proving it to be that there has been no change in the weight of the body mass due to constant consumption of water or either due to the natural food colourants.

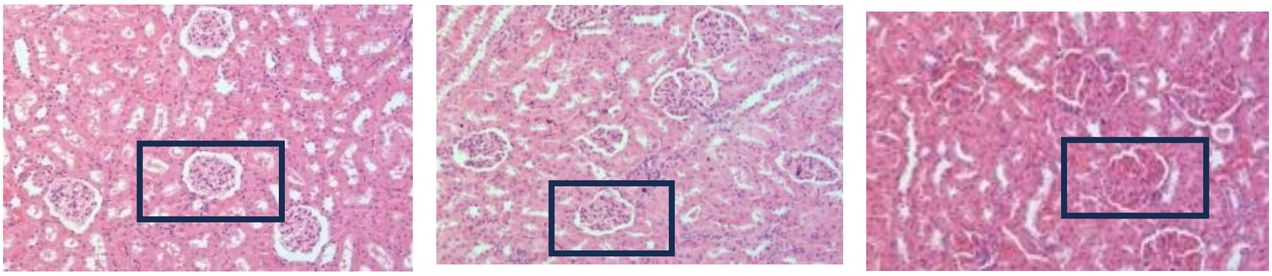
After 56 days, the animals were euthanasia for histopathology study, to identify the effects of the natural food colourant in both the experimental groups. All the animals were harvested and the organs (the heart, liver, kidney, intestine, stomach and spleen) were collected in solution of formaldehyde with water in the ratio of 1:10.

4.4.2 Histopathological Analysis

The microscopic examination of the various organs of the rats treated with eucalyptus bark (*Eucalyptus grandis)* and annatto seeds (*Bixa orellana*) powder extracts was monitored under 10x microscope, the cells and their morphological structure is illustrated below.

4.4.2.1 Renal Histology of the Wistar Albino Rats Administered with the selected Natural Food Colourants

According to *Ahmad Fauzi et al., (2020),* kidney is the only able organ that can regenerate cell damage rate upto 50 percentage, restoring the physiological functions of a being. This function of kidney has led to various investigations with various substances that have potential effect to cause nephrotoxicity in the living being. The organs are stained with hematoxylin eosin staining solution to get a better view of the cells.



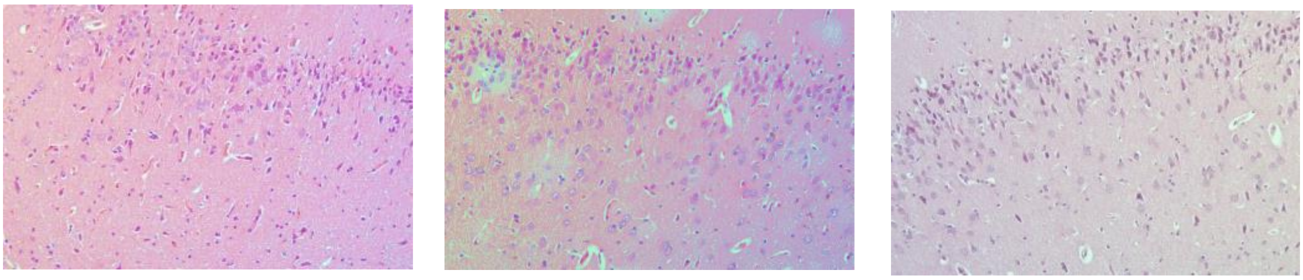
(a) **Control Group** **(b)** **Experimental Rat of Eucalyptus bark** **(c)** **Experimental Rat of Annatto seeds**

Plate – 4.4.2.1: Renal Histology of Bowman's Capsule in Wistar Albino Rats fed with Selected Natural Food Colourants

To analyse the renal histology of the wistar albino rats of control group and the experimental groups, 10x magnifying view is used. To get a view of the kidney cells plate (a) is the histology of control rats, plate (b) and (c) are the histology of wistar albino rats that had consumed the powder extracts of eucalyptus bark (*Eucalyptus grandis)* and annatto seeds (*Bixa orellana*).

Ahmad Fauzi et al., (2020) explains that in the untreated control group spherical glomerulus in the kidney cells are composed of endothelical cell capillaries with bowman's capsules round in shape, where the proximal contortus tubules with cuboid cellular microvilli and distal contortus tubules with round cell, no microvilli are found. Likewise, in the kidney cells of the experimental rat fed with eucalyptus barks (*Eucalyptus grandis)* and annatto seeds (*Bixa orellana*) had round shaped glomerulus are normal and solid in structure, where proximal tubules with cuboid round cells and villis are visible. **This indicates that is no nephrotoxicity taken place in the selected wistar albino rats fed with selected natural food colours.**

4.4.2.2 Cerebral Histology from the Brain of the Wistar Albino Rats Administered with the selected Natural Food Colourants



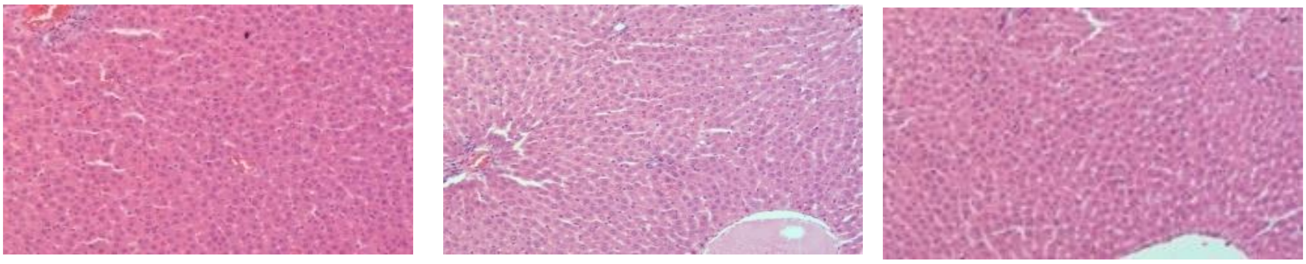
(a) **(b)** **(c)**

Control Group **Experimental Rat of Eucalyptus bark** **Experimental Rat of Annatto seeds**

Plate – 4.4.2.2 : Cerebral Histology of Brain in Wistar Albino Rats fed with selected Natural Food Colourants

According to *Sameh Farouk et al., (2021),* the cerebral section of the control group had displayed a normal arrangement of cerebral layer with clear outer fluid mater marked with neither congestion nor hemorrhage in the blood vessels of the cortical tissue. The cerebral histology of both Experimental group of rats I and II fed with eucalyptus barks and annatto seeds extracts had also displayed normal cerebral layer with outer fluid marked with no congestion and hemorrhage in the blood vessels and tissues, **which proves that there has been no neurotoxicity taken place in the experimental groups of wistar albino rats fed with selected natural food colours.**

4.4.2.3 Liver Histology of the Wistar Albino Rats Administered with Selected Natural Food Colourants



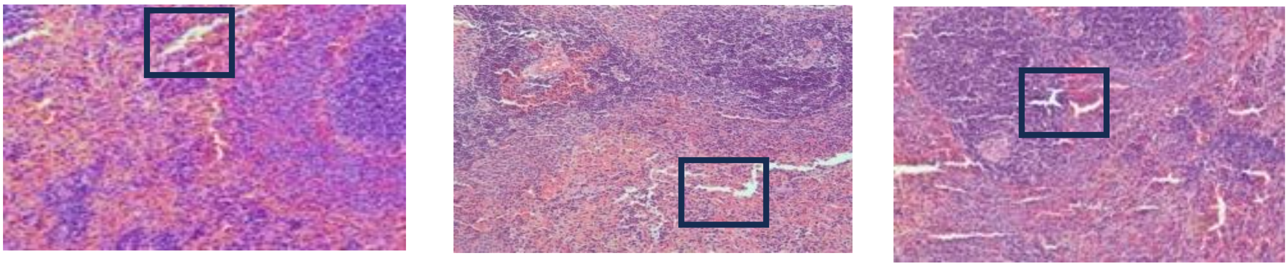
(a) (b) (c)

Control Group **Experimental Rat of Eucalyptus bark** **Experimental Rat of Annatto seeds**

Plate – 4.4.2.3 : Liver Histology of Wistar Albino Rats Fed with Selected Natural Food Colourants

According to *Monica Silva et al., (2022),* the results for liver histology had not shown any degenerated hepatic inflammation and necrosis. The analysed liver of the control group (Plate (a)), had exhibited the typical structure of tissues, normal physiology with hepatocyte plaques separated by sinusoids, with well defined nuclei of hepatocytes and observable Kupffer cells. Likewise, in the liver histology of eucalyptus barks (*Eucalyptus grandis)* and annatto seeds (*Bixa orellana*) experimental rats, **normal physiology with well defined nuclei of hepatocytes were observed proving it to have no hepatotoxicity in the experimental wistar albino rats fed with selected natural food colours.**

4.4.2.4 Histological Architecture of Spleen in the Wistar Albino Rats Administered with the selected Natural Food Colourants



(a) (b) (c)

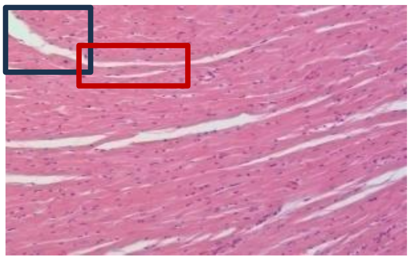
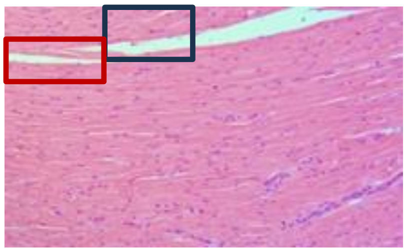
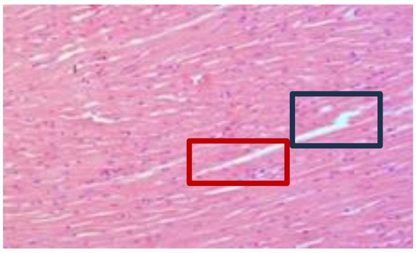
Control Group **Experimental Rat of Eucalyptus bark** **Experimental Rat of Annatto seeds**

Plate – 4.4.2.4: Histological Architecture of Spleen in Wistar Albino Rats Fed with Selected Natural Food Colourants

According to *El-Hak et al., (2018),* the architecture of the white pulps in the spleen should exhibit normal round scattered follicles with a one sided arterioles called central arterioles, where the cells are arranged around the arteriole and classified as thymus dependant zone, germinal center, follicular zone and marginal zone. These zones were observed in the organs of the control group

(Plate (a)), experimental groups of eucalyptus barks (*Eucalyptus grandis)* **(Plate (b)) and annatto seeds (*Bixa orellana*) (Plate (c)) proving to have found no inflammation in the organ fed with selected natural food colours.**

4.4.2.5 Histology in Association with Cardiovascular Tissues of Wistar Albino Rats fed with selected Natural Food Colourants

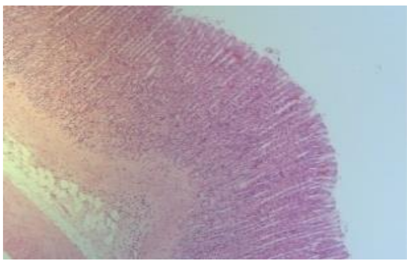
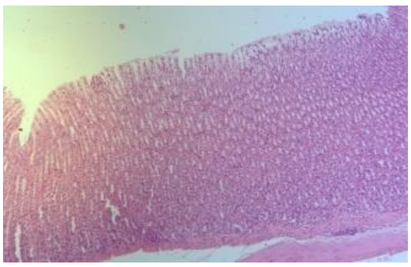
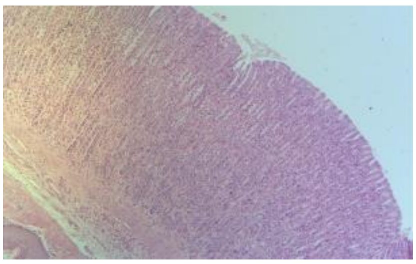
(a) **(b)** **(c)**

Control Group **Experimental Rat of Eucalyptus bark** **Experimental Rat of Annatto seeds**

Plate – 4.4.2.5 : Histology in Association with Cardiovascular Tissues of Wistar Albino Rats Fed with Selected Natural Food Colourants

With regard to *Ogundare et al., (2022)*, the control group (Plate – 4.4.2.5) (a)) had shown normal cellular and architectural integrity that did not deform and disrupt the heart's cyto structure, where the cardiac tissue section has exhibited distinct myocardiac fibers (black box) and nuclei (red box). In consideration with the control group the experimental group of rats fed with eucalyptus barks (*Eucalyptus grandis)* (Plate (4.4.2.5) (b)) and annatto seeds (*Bixa orellana*) (Plate (4.4.2.5) (c)) had also shown disrupted myocardiac fibers (black box) and nuclei (red box). **This proves that there has been no cardiac toxicity found in the experimental group of rats fed with selected natural food colours.**

4.4.2.6.Gastric Histology of Wistar Albino Rats Administered with Selected Natural Food Colourants

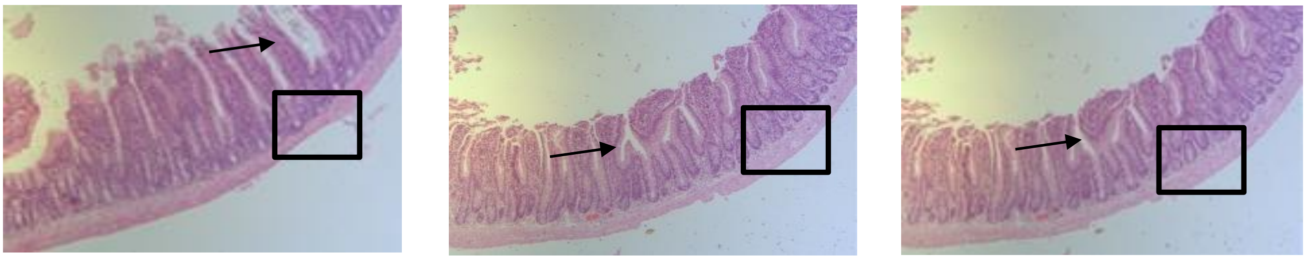
(a) **(b)** **(c)**

Control Group **Experimental Rat of Eucalyptus bark** **Experimental Rat of Annatto seeds**

Plate – 4.4.2.6 : Gastric Histology of Wistar Albino Rats Fed with Selected Natural Food Colourants

The gastric histology of effect of synthetic food colourant conducted by *Erhan (2024)* had proved that the stomach tissue had normal histological appearance with regular contours. The cytoplasm and nucleus were stained with normal hematoxylin eosin that exhibited no gaps or hemorrhage between the epithelial cells with mucosal degeneration. The experimental group of rats with eucalyptus barks (*Eucalyptus grandis)* (Plate (4.4.2) (vi) (b)) and annatto seeds (*Bixa orellana*) (Plate (4.4.2) (vi) (c)) had also shown regular contours with no gaps or hemorrhage between the epithelial cells with mucosal degeneration. **This proves that there is no toxicity in the natural food colourants that affects the stomach mucosal layer of the rats fed with selected natural food colours.**

4.4.2.7. Intestinal Histology of Wistar Albino Rats Administered with Selected Natural Food Colourants



(a) **Control Group** **(b)** **Experimental Rat of Eucalyptus bark** **(c)** **Experimental Rat of Annatto seeds**

Plate – 4.4.2.7 : Intestinal Histology of TM layer and Villi of Wistar Albino Rats Fed with Selected Natural Food Colourants

According to *Erhan (2024)*, the contours of the Tunica Muscularis (TM) layer (black box) in all the three groups of control group (Plate (4.4.2) (vii) (a)), experimental group of rats with eucalyptus barks (*Eucalyptus grandis)* (Plate (4.4.2) (vii) (b)) and annatto seeds (*Bixa orellana*) (Plate (4.4.2) (vii) (c)) had normal histological structure, where the crypts and villi (black arrow) maintained a structural integrity with no degenerations such as tears or necrotic area bleeding. **There were no ruptures, impairments or hemorrhage in the villi. This had also proved that there is no intestinal hemorrhage and the intestinal walls of TM are with no ruptures, exhibiting that the natural food colourants are non toxic to intestines.**

From the histology of Wistar Albino rats, the organs of the rats that consumed eucalyptus bark (*Eucalyptus grandis)* and annatto seeds (*Bixa orellana*) powder extracts did not show any colour or morphological changes when compared with the control group rats' organs.

In addition, the microscopic examination revealed that none of the organs from the treated Wistar Albino rats had shown any alteration in cell structure or any unfavourable effects under the light microscope using multiple magnification powers.

No pathologies were recorded in the histological sections of the vital organs (brain, liver, kidney, heart, stomach, intestine and spleen) of the control group. Equally, there was no change in the creatinine after administration of eucalyptus bark (*Eucalyptus grandis*) and annatto seeds (*Bixa orellana*) powder extracts when compared to the control group. Any alteration found in the level of creatinine show that there has been damage in the functioning of nephrons. The observations in the histopathology study of the kidney tissue have been recorded no changes. Thus, this study proves that eucalyptus bark (*Eucalyptus grandis*) and annatto seeds (*Bixa orellana*) nontoxic in nature and can be used as natural food colourant.

4.4.2.1 Analysis for Haematology and Blood Parameters for the Wistar Albino Rats fed with Selected Natural Food Colourants

The haematology and biochemical parameters are tested to identify the permissible level of natural food colourants to be added to food for human consumption, as animals and especially rats are miniature models for humans. In consideration with the reference interval given by *Vigneshwar et al., (2021),* the hematological and the biochemical range is included in the table.

Table – XXXIX
Hematological Analytes of Wistar Albino Rats fed with Natural Food Colourants

Hematological Parameters	Estimated Mean Value			
	Control Group	Experimental Group – I Eucalyptus bark	Experimental Group – II Annatto seeds	Reference Range* (Male Rats)
Hemoglobin (g/dl)	14.53	14.64	15.9	**11.80-16.20**
Red Blood Cells (10^6/mm^3)	7.56	7.66	8.35	**6.10-8.50**
Total White Blood Cells (10^3/mm^3)	4.93	4.70	4.2	**3.70-5.80**

Reference Range by *Vigneshwar et al., (2021)

The mean hematology analytes of the wistar albino rats were measured for the control group, experimental groups of eucalyptus bark (*Eucalyptus grandis*) and annatto seeds (*Bixa orellana*) which shows that the values of hemoglobin are 14.53 g/dl, 14.64 g/dl and 15.9 g/dl respectively, which falls under the range of 11.80-16.20 g/dl.

Likewise, the range of RBCs and WBCs of the wistar albino rats are 6.10 -8.50 ($10^6/mm^3$). Here, the control group and the experimental groups I and II RBCsmeasured are 7.56, 7.66, 8.35 ($10^6/mm^3$) and 4.93, 4.70, 4.2 ($10^3/mm^3$) respectively, which falls under the normal range of hematological values by *Vigneshwar et al., (2021)*.

The hematological range of White Blood Cells (WBCs) are 3.70-5.80 ($10^3/mm^3$). The control group and the experimental group I, WBCs of eucalyptus bark (*eucalyptus grandis*) experimental rats are measured as 4.93 and 4.70 ($10^3/mm^3$), respectively. For the experimental group II of annatto seeds (*Bixa orellana*) are measured as 4.2 ($10^3/mm^3$), which comes between the reference range indicated by *Vigneshwar et al., (2021)*.

Table – XL

Biochemical Analytes of Wistar Albino Rats fed with selected Natural Food Colourants

Biochemical Parameters	**Estimated Mean Value**			
	Control Group	**Experimental Group – 1 Eucalyptus Bark** (*Eucalyptus grandis*)	**Experimental Group – 2 Annatto Seeds** (*Bixa orellana*)	**Reference Range* (Male Rats)**
SGOT (U/L)	27.17	26.93	27	**24.13-67.45**
SGPT (U/L)	20.18	23	25	**18.7-26**
ALP (U/L)	72.45	72.56	72.45	**55.45-98.10**
Glucose (g/dL)	146	146	145	**90.75-180.25**
Cholesterol (g/dL)	92	92	90	**60.52-100.20**
Total Protein (g/dL)	6.5	6.53	6.5	**5.10-7.60**
Albumin (g/dL)	14.2	13.6	13.5	**12-20**
Creatinine (mg/dL)	0.40	0.63	0.60	**0.30-0.60**
Sodium (mg/dL)	137.3	141.24	147.9	**130.92-148.76**
Potassium (mg/dL)	4.1	4.1	6.5	**4.10-7.80**

*Reference Range as denoted by *Vigneshwar et al., (2021)*

Table represents the biochemical parameter of the rats to find if there has been any alteration in the renal functioning that was influenced by the powder mixed in water. Liver and kidney analysis is very important as the toxicity evaluation of the plant substance will be noted through the tissues, which is responsible for the survival of the organism.

The mean biochemical parameters of the control rats and the experimental group of rats were measured and comparison is done with the average range of biochemical parameters. The SGOT level in the control rats are 27.17 U/L, whereas for the eucalyptus bark (*Eucalyptus grandis*) and annatto seeds (*Bixa orellana*) it is 26.93 and 27 U/L respectively which measured under the range of

24.13 to 67.45 U/L. The SGPT level in the control rats are 20.18 U/L, whereas for the eucalyptus bark (*Eucalyptus grandis*) and annatto seeds (*Bixa orellana*) shows 23 and 25 U/L respectively which measured under the range of 18.7 to 26 U/L.

Likewise, the ALP value of the control rats are 72.45 U/L which is similar with experimental group of rats I and II fed with eucalyptus bark (*Eucalyptus grandis*) and annatto seeds (*Bixa orellana*) respectively, was found to be 72.56 and 72.45 U/L respectively, which ranges within the reference range(55.45 to 98.10 U/L).

The reference glucose ranges between 90.75 to 180.25 g/dL. From the reference range of glucose, the glucose level of control rats and the experimental group rats are normal which is measured to be 146 g/dL for both the control group and the experimental group – I, whereas experimental group – II ranges in 145 g/dL.

The cholesterol level ranges between 60.52 to 100.20 g/dL which is the reference range, also the control group and the experimental group – I and II ranges as 92, 92 and 90 g/dL. The total protein content of the wistar albino rats' reference value ranges between 5.10 to 7.60 g/dL and the control group and the experimental group – I and II ranges as 6.5, 6.53 and 6.5 g/dL.

The other biochemical parameters measured were albumin, creatinine, sodium and potassium. As for the reference value of albumin ranges from 12 to 20 g/dL, the measured range of control group is 14.2 g/dL, the experimental group of eucalyptus bark (*Eucalyptus grandis*) is 13.6 g/dL and the annatto seeds (*Bixa orellana*) is 13.5 g/dL. The measured range falls between the reference range of albumin. The reference range for creatinine is 0.30 to 0.60 mg/dL, likewise the control group of rats along with the experimental group 1 and 2 measures to be 0.40, 0.63 and 0.60 mg/dL respectively.

As for the reference values of sodium and potassium ranges from 130.92 to 148.76 mg/dL and 4.10 to 7.80 mg/dL, the measured values of sodium and potassium in the control group is 137.3 and 4.1 mg/dL. The experimental group of rats measured that were fed with eucalyptus bark (*Eucalyptus grandis*) and annatto seeds (*Bixa orellana*) consisted of 141.24 and 4.1 mg/dL of sodium along with 147.9 and 6.5 mg/dL of potassium respectively.

These measured mean values of the hematological and biochemical parameters have their ranges between the reference range, which indicates that there are no biochemical changes that has been taken place in the experimental group of rats of eucalyptus bark (*Eucalyptus grandis*) and annatto seeds (*Bixa orellana*) that were continuous fed for 56 days of sub chronic period.

Serum concentration of total protein and albumin in the experimental group I and II as well as in the control group confirms that the substance has not damaged the hepatocellular or secretory functions of the liver. Renal dysfunction is assessed through the concurrent values of creatinine values and their normal control group value reflects the normal renal functioning in the rats. The proper functioning of kidney is again proved through the standard values of sodium, potassium and chloride. **Thus, the hematology and the biochemical parameter values have proved that the eucalyptus bark (*Eucalyptus grandis*) and annatto seeds (*Bixa orellana*) substance did not affect the hepatocytic functions of the wistar albino rats.**

Conversion of Animal Study Dosage to Human Equivalent Dosage (HED)

HED (mg/kg) = Animal Dosage (mg/kg) × Animal Km ÷ Human Km
Km calculation: Km = Average Body Weight ÷ Body Surface Area

HED for Consumption of Annatto seeds (*Bixa Orellana*)

i) Animal Km = Annatto Body Weight ÷ Body Surface Area = 0.345 ÷ 0.025 = 13.8
Human Km = 60 ÷ 1.8 = 33.33

ii) HED Calculation: HED = 0.3 × 13.8 ÷ 33.33 = 0.3 × 0.41 = 0.123

iii) Total Dosage Calculation:
Total Dosage for 60 kg human = 0.123 mg/kg × 60 kg = 7.38 mg (i.e., 7 mg)

Total Dosage of Annatto seeds (*Bixa Orellana*) for 60 kg human = 7 mg(per day)

HED for Consumption of Eucalyptus bark (*Eucalyptus grandis*)

i) Animal Km = Annatto Body Weight ÷ Body Surface Area = 0.344 ÷ 0.025 = 13.76
Human Km = 60 ÷ 1.8 = 33.33

ii) HED Calculation: HED = 0.6 × 13.76 ÷ 33.33 = 0.6 × 0.41 = 0.246

iii) Total Dosage Calculation:
Total Dosage for 60 kg human = 0.246 mg/kg × 60 kg = 14.76 mg (i.e., 15 mg)

Total Dosage of Eucalyptus bark (*Eucalyptus grandis*) for 60 kg human = 15 mg (per day)

Table – XLI

Consolidated Overall Profile of the Wistar Albino Rats during the Experimentation Study

Profile of the Rats	**Control Group - I**		**Experimental Group – II Eucalyptus Bark** (*Eucalyptus grandis*)		**Annatto Seeds** (*Bixa orellana*)	
	Before	**After**	**Before**	**After**	**Before**	**After**
Type of Group	CR	CR	EuER	EuER	AnER	AnER
Sex	Male	Male	Male	Male	Male	Male
Age (months)	8-12 months	8-12 months	8-12 months	8-12 months	8-12 months	8-12 months
Weight (g)	268 g	290 g	323 g	362 g	340 g	350 g
Water Consumption(ml)	20-25 ml from 120 ml of water	20-25 ml from 120 ml of water	20-25 ml from 120 ml of water	10 ml + 8 ml of Sample	20-25 ml from 120 ml of water	10 ml + 8 ml of Sample
Food Consumption(g)	25 g	25 g	25 g	25 g	25 g	25 g
PHYSICAL STATUS						
Body Parts	**Appearance**					
Fur/Skin	Normal White	Normal White	Normal White	Normal White	Normal White	Normal White
Eyes	Normal Red	Normal Red	Normal Red	Normal Red	Normal Red	Normal Red
Ears	Normal Pink	Normal Pink	Normal Pink	Normal Pink	Normal Pink	Normal Pink
Nose and Mouth	Normal Pink	Normal Pink	Normal Pink	Normal Pink	Normal Pink	Normal Pink
Limbs and Nails	Normal	Normal	Normal	Normal	Normal	Normal
Tail	Normal Pink	Normal Pink	Normal Pink	Normal Pink	Normal Pink	Normal Pink
MENTAL STATUS						
Active	✓	✓	✓	✓	✓	✓
Sluggish	X	X	X	X	X	X
Aggressive	X	X	X	X	X	X
Irritated	X	X	X	X	X	✓
Sleepy	X	X	X	X	X	X

In accordance with the table, it has been proved that the wistar albino rats maintained under the condition of 22±2ºC had been in the same mental health and activeness, apart from the weight gain, as the purchase rats were adult male rats, the weight gain was an unavoidable circumstance during the experimentation for 56 days.

PHASE – V

4.5. Nutrient and Organoleptic Analysis of the Selected Recipes Incorporated with Natural Food Colourants

Analyzing the nutrients in the selected natural food colourants and formulating the traditional recipes incorporating with annatto seeds (*Bixa orellana*) andeucalyptus bark (*Eucalyptus grandis*) substance as natural food colourants have paved way for recipe evaluation.

4.5.1. Nutrient Analysis of SelectedNatural Food Colourants

The powdered samples of 100g of annatto seeds (*Bixa orellana*) andeucalyptus bark (*Eucalyptus grandis*) substance was analyzed for nutrients using the standard procedure of *AOAC (2021)* method.

Table – XLII

Amount of Nutrients in Selected Natural Food Colourants

NUTRIENTS	Natural Food Colourants	
	Annatto seeds (*Bixa orellana*)	**Eucalyptus bark** (*Eucalyptus grandis*)
Moisture	9.99	10.55
pH	5.23	4.12
Calorific Value (kcal)	351.33	345.59
Protein (g)	5.92	2.13
Fat (g)	2.13	0.39
Carbohydrates (g)	77.12	83.39
Fiber (g)	16.84	15.14
Vitamin A (IU)	175.13	75.51
β Carotene (mg)	105076.39	45308.27
Lycopene (mg)	28.09	34.69
Sodium (μg)	7.42	1.66
Potassium (μg)	609.28	91.03
ANTI NUTRIENTS		
Total Phenols (mg)	202.24	5534.34
Tannins (mg)	183.34	5808.24
Phytic acid (mg)	51.75	18.19

The nutrients suchas protein, fat, fiber, carbohydrates and calorific values was analyzed along with the micronutrients like vitamin A, beta carotene and lycopene were also analyzed. Minerals namely sodium and potassium, along with antinutrients like total phenols, tannins, phytic acid, moisture and pH were present in the natural food colourants of annatto seeds (*Bixa orellana*) andeucalyptus bark (*Eucalyptus grandis*) were analyzed to exhibit its nutritional effect on foods.

The protein content in the annatto seeds (*Bixa orellana*) andeucalyptus bark (*Eucalyptus grandis*) powders were 5.92 g and 2.13 g, respectively, whereas fat content was of 5.92 g and 2.13g, carbohydrates was 77.12 g and 83.39 g and crude fiber was 16.84 g and 15.14 g, thus provides the calorific value was 351.33 kcal and 345.59 kcal per 100g of each selected natural food colourants respectively.

The micronutrients analysed in annatto seeds (*Bixa orellana*) andeucalyptus bark (*Eucalyptus grandis*) powders were Vitamin A with 175.13 and 75.51 IU, respectively, beta carotene of 105076.39 mg and 45308.27 mg and lycopene of 28.09 and 34.69 mg respectively.

The minerals analysed for sodium was 7.42 µg and 1.66 µg, and potassium was 609.28 and 91.03 µg, respectively in annatto seeds (*Bixa orellana*) andeucalyptus bark (*Eucalyptus grandis*) powders, respectively.

The moisture content and the pH value were checked, where in annatto seeds (*Bixa orellana*) powder was 9.99 percent with a pH of 5.23 which is slightly acidic in nature. As for the powder of eucalyptus bark (*Eucalyptus grandis*), the moisture content was 10.55 percent and the pH is 4.12, which is also acidic in nature. With all the nutrients, minerals and moisture content, the natural food colourants from annatto seeds (*Bixa orellana*) andeucalyptus bark (*Eucalyptus grandis*) powders are considered suitable with sufficient amount of nutrients which will enhance the food in which the natural food colourants are being added.

The antinutrients found in annatto seeds (*Bixa orellana*) powder, the total phenols was 202.24 mg, tanins was 183.34 mg and phytic acid was 51.75 mg. Likewise, in the powdered substance of eucalyptus bark (*Eucalyptus grandis*), the content of total phenols was 5534.34 mg, tannins was 5808.24 mg and the phytic acid content was 18.19 mg.

The level of tannin present in 100 g of eucalyptus bark was measured as 5808.24 mg, whereas for the optimized level of eucalyptus bark is only 6 mg which gives tannins of 0.005 mg. Thus, proving the eucalyptus bark will be consider with in permissible level.

Important properties like antiseptics, anticarcinogenic and anti-inflammatory of tannins have been recorded as viable substance for pharmaceutical and nutraceutical industries. Because of certain recent concerns related to synthetic compounds used in human health and food industries, which leave high adverse effects on human health and environment, tannins offer an alternative chemical to recent emerging situtations (*Akhlash P Singh et al., 2020*).

4.5.2 Incorporation of the selected Natural Food Colourants in the selected Recipes and Measuring the Intensity of Colours in Incorporated Selected Recipes

The selected recipes prepared and incorporated with the natural food colourants are traditional sweets such as kesari, motichoor ladoo and jalebi, snacks such as chilli gobi, chilli chicken, tutti frutti, aloo bajji and aloo bonda and fruit preserves such as apple jam and jelly.

Using the standard procedure by Modern Cookery (*Thangam Philips., 2010*), these recipes were prepared and their intensity in colour was also recorded. In the standard preparation of all the recipes the one among popular brand with less presence of toxins and most commonly (from the market survey) used synthetic food colourant, Bush was used to note the colour difference between the natural and the synthetic food colourants.

From the table given, the Delta-E is a standard measurement that qualifies the difference between the two colours that appear, where the lower Delta-E figures indicate greater accuracy, while the high Delta-E levels indicate a significant mismatch. The colour intensity analysed by the food colour reader has been recorded and the values were calculated using the formula for Delta-E. The CIE LAB colour was measured using Food Colour Reader to note the hue difference.

All the recipes were prepared with one standard commercial synthetic food colourant. The optimized level of natural food colourant to be consumed that was experimentally calculated using *in vivo* study was used in preparing the different variations of the recipes.

Variation – I to variation – III were prepared using the highest concentrations of annatto seeds (*Bixa orellana*) powder of 1mg, 2 mg and 3 mg respectively, whereas from the next set of variation – I to III, highest concentrations of eucalyptus bark (*Eucalyptus grandis*) powder were used with 4 mg, 5 mg and 6 mg respectively. And also the least concentration of eucalyptus bark (*Eucalyptus grandis*) powder were not used as they did not produce sufficient colour to the selected recipes.

Table – XLIII

Intensity of Food Colour of Selected Traditional Sweets Incorporated with Synthetic and Natural Food Colourants

Selected SweetRecipes	Type of Colourant	Variations	Amount of Colourants used	L*	a*	b*	Result (ΔE* ab)
Kesari	**BU(SFC**)**	**Standard**	**1 mg (pinch)**	**21.54**	**10.83**	**16.47**	**29.19**
	Annatto seeds powder (*Bixa orellana*)	Variation – I	1 mg	17.82	7.87	16.24	22.28
		Variation – II	2 mg	19.66	5.94	14.47	25.12
		Variation – III	3mg	17.78	8.09	18.98	27.23
	Eucalyptus bark powder (*Eucalyptus grandis*)	Variation – I	4 mg	22.09	2.77	4.66	22.74
		Variation – II	5 mg	23.78	1.28	4.54	24.24
		Variation – III	6 mg	24.35	0.71	4.04	24.69
Motichoor Ladoo	**BU (SFC**)**	**Standard**	**1 mg (pinch)**	**13.31**	**13.99**	**10.59**	**22.02**
	Annatto seeds powder (*Bixa orellana*)	Variation – I	1 mg	17.80	4.16	13.17	22.52
		Variation – II	2 mg	17.05	5.11	13.89	22.57
		Variation – III	3mg	20.79	4.34	15.95	26.56
	Eucalyptus bark powder (*Eucalyptus grandis*)	Variation – I	4 mg	13.23	4.55	8.96	16.61
		Variation – II	5 mg	18.65	1.79	9.12	20.83
		Variation – III	6 mg	19.68	3.84	6.76	21.16
Jalebi	**BU (SFC**)**	**Standard**	**1 mg (pinch)**	**49.53**	**5.76**	**9.71**	**50.8**
	Annatto seeds powder (*Bixa orellana*)	Variation – I	1 mg	50.10	6.03	7.72	51.04
		Variation – II	2 mg	50.73	5.87	7.88	51.69
		Variation – III	3mg	51.04	5.44	7.77	51.91
	Eucalyptus bark powder (*Eucalyptus grandis*)	Variation – I	4 mg	50.31	5.44	7.51	51.15
		Variation – II	5 mg	50.39	5.60	7.36	51.23
		Variation – III	6 mg	50.74	6.01	8.01	51.71

**Synthetic Food Colourant
a*represents red or green colour
L* lightness of the product
b* represents yellow or blue colour

According to the lightness, saturation hue of a* for either red or green colour and b* for either yellow or blue colour were calculated using the food colour reader for the traditional sweet recipes of keasri, motichoor ladoo and jalebi.

Standardized recipes were prepared using synthetic food colourant of BU brand. To the other variations from I to III, the annatto seeds (*Bixa orellana*) powder eucalyptus bark (*Eucalyptus grandis*) powder were used. The standard kesari was prepared with synthetic food colourants BU, had 29.19 hue, whereas the recipe incorporated with annatto seeds (*Bixa orellana*) powder variation – I had 22.28 hue, variation – II with 25.12 hue and variation – III with 27.23 hue. Likewise, the variations of eucalyptus bark (*Eucalyptus grandis*) had 22.74, 24.24 and 24.69 hue respectively. In comparison to the standard recipe prepared with Bush, thevariation – III recipe incorporated with annatto seeds (*Bixa orellana*) powder had bright hue of 27.23, when compared with eucalyptus bark (*Eucalyptus grandis*) of hue 24.69.

The standard motichoor ladoo prepared with BU as synthetic food colourant had 22.02 hue. The standardized recipes prepared with variations using annatto seeds (*Bixa orellana*) powder had increase in hue range of 22.52, 22.57 and 26.56 hue, respectively. Likewise, for eucalyptus bark (*Eucalyptus grandis*) variations, the hues ranged from 16.61, 20.83 and 21.16 respectively. In comparison to the standard recipe, the variations – I and II of annatto seeds (*Bixa orellana*) with 22.57 and 26.56 and variation – III of eucalyptus bark (*Eucalyptus grandis*) 21.16 hue had their hues equalent to each other.

Jalebi, an all time famous traditional recipe was also prepared using synthetic food colourant BU and variations – I to III for both annatto seed (*Bixa orellana*) and eucalyptus bark (*Eucalyptus grandis*) powder as added food colourants. The hue of the standard recipe was 50.8 hue, wheras for the variations of annatto seeds (*Bixa orellana*) from I, II and III, it was 51.04, 51.69 and 51.91 respectively. As for the eucalyptus bark (*Eucalyptus grandis*) variations, the hues were 51.15, 51.23 and 51.71 respectively, which had increased slightly when compared to the standard recipe prepared using synthetic food colourant.

Table – XLIV

Intensity of Food Colour of selected Snacks Incorporated with Synthetic and Natural Food Colourants

Selected snack Recipes	Type of Colourants	Variations	Amount of Colourants used	L*	a*	b*	Result (ΔE* ab)
Chilli Gobi	**BU (SFC**)**	**Standard**	**1 mg (pinch)**	**16.42**	**14.30**	**15.12**	**26.50**
	Annatto seeds powder (*Bixa orellana*)	Variation – I	1 mg	9.30	8.22	8.69	15.15
		Variation – II	2 mg	11.59	12.74	9.02	19.44
		Variation – III	3mg	20.07	7.45	15.07	26.18
	Eucalyptus bark powder (*Eucalyptus grandis*)	Variation – I	4 mg	11.29	8.83	8.99	16.91
		Variation – II	5 mg	13.96	11.48	13.78	22.72
		Variation – III	6 mg	16.61	10.16	13.52	23.70
Chilli Chicken	**BU(SFC**)**	**Standard**	**1 mg (pinch)**	**10.40**	**11.94**	**6.88**	**17.26**
	Annatto seeds powder (*Bixa orellana*)	Variation – I	1 mg	9.46	8.96	7.11	14.84
		Variation – II	2 mg	8.01	10.35	7.89	15.28
		Variation – III	3mg	9.93	8.46	9.89	16.37
	Eucalyptus bark powder (*Eucalyptus grandis*)	Variation – I	4 mg	7.78	3.86	4.49	9.77
		Variation – II	5 mg	9.28	6.54	7.08	13.37
		Variation – III	6 mg	7.27	10.22	6.50	14.12
Aloo Bajji	**BU (SFC**)**	**Standard**	**1 mg (pinch)**	**47.41**	**9.80**	**12.81**	**50.07**
	Annatto seeds powder (*Bixa orellana*)	Variation – I	1 mg	46.95	8.95	13.28	49.60
		Variation – II	2 mg	47.44	10.00	12.29	50.07
		Variation – III	3mg	50.80	7.64	8.51	52.07
	Eucalyptus bark powder (*Eucalyptus grandis*)	Variation – I	4 mg	49.31	7.45	10.44	50.95
		Variation – II	5 mg	49.69	8.26	9.67	51.29
		Variation – III	6 mg	50.23	6.41	9.07	51.44
Aloo Bonda	**BU (SFC**)**	**Standard**	**1 mg (pinch)**	**51.09**	**6.17**	**7.76**	**52.04**
	Annatto seeds powder (*Bixa orellana*)	Variation – I	1 mg	49.38	9.07	10.05	51.20
		Variation – II	2 mg	49.87	8.26	9.48	51.43
		Variation – III	3mg	51.21	7.80	7.87	52.39
	Eucalyptus bark powder (*Eucalyptus grandis*)	Variation – I	4 mg	47.34	9.33	12.07	49.73
		Variation – II	5 mg	48.58	11.63	10.65	51.07
		Variation – III	6 mg	50.17	7.94	9.24	51.62

**Synthetic Food Colourant
L* lightness of the product
a*represents red or green colour
b* represents yellow or blue colour

Chilli gobi prepared using cauliflower was checked for the intensity of colours in adding the developed natural food colourants of annatto seeds (*Bixa orellana*) powder and eucalyptus bark powder (*Eucalyptus grandis*) powder.

The standard recipe prepared using the synthetic food colourant namely BU had its hue of 26.50 as its limit. Whereas, the variations had their increase in hue as the amount of natural food colourants were increased by 1mg each in each recipe, Variations I, II and III of annatto seeds (*Bixa orellana*) powder had 15.15, 19.44 and 26.18 hue respectively. The variation I, II and III added with eucalyptus bark powder (*Eucalyptus grandis*) and the colour intensity of the recipes increased as 16.91, 22.72 and 23.70 hue, respectively.

The recipes of chilli chicken were prepared using the standard procedure (*Thangam Philips., 2010*) and the standard recipe was prepared using the synthetic food colourant, BU, where the colour intensity range of standard chilli chicken was 17.26 hue. Likewise, in the variations I, II and III of annatto seeds (*Bixa* orellana) had the colour intensity of 14.84, 15.28 and 16.37 hue. Likewise, the variations I, II and III prepared using eucalyptus bark powder (*Eucalyptus grandis*), the range of intensity were with the hue of 9.77, 13.37 and 14.12 respectively.

Similarly, the colour intensity range of standard recipe prepared using synthetic food colourant was 50.07 hue. Likewise, the variations I, II and III of annatto seeds (*Bixa* orellana) had the colour intensity of 49.60, 50.07 and 52.07 hue, respectively. On the account of the hue, the standard recipe and the variation II recipe incorporated with 2mg of annatto seeds (*Bixa* orellana) powder had the identical colour and appearance. The variations – I, II and III of eucalyptus bark powder (*Eucalyptus grandis*), the range of intensity of colour was 50.95, 51.29 and 51.44 hue.

The recipe of aloo bonda which was also considered as an Indian traditional recipes were also prepared with a standard synthetic food colourant of BU brand and the other six variations with selected natural food colourants such as annatto seeds (*Bixa orellana*) and eucalyptus bark (*Eucalyptus grandis*) powder. The standard recipe had its hue value of 52.04, whereas in the variation – I, II and III, the hue had increased from 51.20 to 51.43 and 52.39 respectively. Likewise, in the variation – I, II and III of eucalyptus bark (*Eucalyptus grandis*) also an increase in hue from 49.73, 51.07 and 51.62 respectively.

Table – XLV

Intensity of Food Colour of selected Fruit Preserves Incorporated with Synthetic and Natural Food Colourants

Selected Fruit Preserves Recipes	Type of Colourants	Variations	Amount of Colourants used	L*	a*	b*	Result (ΔE* ab)
Tutti Frutti	**BU (SFC**)**	**Standard**	**1 mg (pinch)**	**10**	**5.21**	**5.42**	**12.51**
	Annatto seeds powder (*Bixa orellana*)	Variation – I	1 mg	8.92	1.15	7.02	11.40
		Variation – II	2 mg	11.36	0.64	7.26	13.48
		Variation – III	3mg	11.94	0.80	7.02	13.87
	Eucalyptus bark powder (*Eucalyptus grandis*)	Variation – I	4 mg	5.64	0.15	6.87	8.88
		Variation – II	5 mg	8.41	1.46	4.29	9.55
		Variation – III	6 mg	8.32	3.45	7.41	11.66
Apple Jam	**BU (SFC*)**	**Standard**	**1 mg (pinch)**	**49.61**	**5.42**	**10.30**	**50.95**
	Annatto seeds powder (*Bixa orellana*)	Variation – I	1 mg	50.68	7.07	8.04	51.79
		Variation – II	2 mg	52.48	5.09	6.69	53.14
		Variation – III	3mg	52.73	4.83	6.36	53.36
	Eucalyptus bark powder (*Eucalyptus grandis*)	Variation – I	4 mg	52.66	4.82	6.51	53.17
		Variation – II	5 mg	52.55	4.97	6.51	53.24
		Variation – III	6 mg	52.64	4.83	6.39	53.27
Jelly	**BU (SFC*)**	**Standard**	**1 mg (pinch)**	**52.15**	**4.92**	**7.03**	**52.85**
	Annatto seeds powder (*Bixa orellana*)	Variation – I	1 mg	52.27	5.09	6.57	52.91
		Variation – II	2 mg	52.29	5.08	6.74	52.96
		Variation – III	3mg	52.47	4.96	6.57	53.11
	Eucalyptus bark powder (*Eucalyptus grandis*)	Variation – I	4 mg	52.57	4.82	6.53	53.19
		Variation – II	5 mg	52.66	4.82	6.51	53.27
		Variation – III	6 mg	52.67	4.82	6.63	53.30

**Synthetic Food Colourant
L* lightness of the product
a*represents red or green colour
b* represents yellow or blue colour

Tutti frutti is a preservative prepared using raw papaya. The colour of the prepared standard and the variations in the recipe were analyzed for intensity of colours, where the standard food colourant used was BU, which had the hue of 12.51. The variations I, II and III of annatto seeds (*Bixa* orellana) had slight increase in the range of hue from 11.40 to 13.48 and then to 13.87 hue. The variations – I, II and III of eucalyptus bark powder (*Eucalyptus grandis*) had the colour intensity increased from 8.88, 9.55 and 11.66, respectively.

For, Apple jam, the standard recipe had its hue valued to 50.95. In variation – I, II and III of annatto seeds (*Bixa* orelaana), the hue has increased from 51.79, 53.14 and 53.36 respectively. Likewise, in the of variations I, II and III of eucalyptus bark (*Eucalyptus grandis*), the hue has increased from 53.17, 53.24 and 53.27, respectively. The intensity of colour increase indicates that the variations incorporated with natural food colourants of annatto seeds (*Bixa orellana*) and eucalyptus bark (*Eucalyptus grandis*) powder has an identical emission of colourants when being used in food.

Jelly, a favourite dessert for people of all age groups and it is also a preservative for consuming the seasonal fruits all round the year. The standard recipe of jelly prepared using BU as synthetic food colourant had 52.85 hue. The intensity of colour in variations I, II and III incorporated with annatto seeds (*Bixa orellana*) range above the standard food colourant when added to jelly, where the colour intensity was 52.91, 52.96 and 53.11 hue respectively. The variations I, II and III of eucalyptus bark (*Eucalyptus grandis*) had the intensity of colours ranging from 53.19, 53.27 and 53.30 hue respectively.

4.5.3 Organoleptic Evaluation of the selected Recipes Incorporated with Natural Food Colourants

The selected recipes incorporated with synthetic and natural food colourants were evaluated by ten trained panel members between the age group of 20 to 30 years. The panel members were young adults, using individual score card of five point hedonic scale rating evaluated the selected recipes according to their preferences. In the hedonic rating score card, the sensory attributes such as appearance, consistency, texture, flavour and taste were scored with five which indicates excellent, four for very good, three for good, two for fair and one for poor (Appendix-X).

Table – XLVI

Computed Organoleptic Evaluation Score of the Traditional Sweets Incorporated with Food Colourants

Sweet Recipes	Type of Colourants	Variations	Score for the Sensory Attributes				
			Appearance	Consistency	Texture	Flavour	Taste
Kesari	**BU (SFC*)**	Standard	4.5 ± 0.8	3.8 ± 0.6	4.2 ± 0.6	4.2 ± 0.8	3.9 ± 0.9
	Annatto seeds powder (*Bixa orellana*)	Variation – I	4.2 ± 0.7	3.8 ± 0.4	3.7 ± 0.5	4.1 ± 0.9	3.9 ± 0.7
		Variation – II	**4.5 ± 0.8**	**4.1 ± 0.6**	**4.3 ± 0.7**	**4 ± 0.8**	**4.1 ± 0.7**
		Variation – III	4.4 ± 0.7	4.1 ± 0.7	3.8 ± 0.6	3.6 ± 0.7	3.5 ± 0.8
	Eucalyptus bark powder (*Eucalyptus grandis*)	Variation – I	3.9 ± 0.7	4 ± 0.7	3.8 ± 0.6	3.6 ± 1.0	3.6 ± 0.7
		Variation – II	**4.2 ± 0.6**	**4 ± 0.5**	**3.8 ± 0.4**	**4 ± 0.5**	**3.6 ± 1.0**
		Variation – III	3.9 ± 0.9	4 ± 0.7	4 ± 0.5	3.8 ± 0.8	3.4 ± 0.8
Motichoor Ladoo	**BU (SFC*)**	Standard	4.3 ± 0.8	3.5 ± 0.8	3.8 ± 0.8	3.9 ± 0.7	3.8 ± 0.6
	Annatto seeds powder (*Bixa orellana*)	Variation – I	4.4 ± 0.7	3.9 ± 1.0	3.8 ± 0.9	4.1 ± 0.6	4 ± 0.8
		Variation – II	**4.6 ± 0.7**	**4.2 ± 0.4**	**3.9 ± 0.9**	**4.2 ± 0.6**	**3.8 ± 0.9**
		Variation – III	4.1 ± 0.6	3.9 ± 0.7	3.6 ± 0.8	3.5 ± 0.7	3.8 ± 0.8
	Eucalyptus bark powder (*Eucalyptus grandis*)	Variation – I	3.3 ± 0.9	3 ± 0.7	2.7 ± 0.7	3.2 ± 0.4	3.2 ± 0.8
		Variation – II	**4.1 ± 0.7**	**3.8 ± 0.6**	**3.9 ± 0.7**	**4 ± 0**	**3.9 ± 0.6**
		Variation – III	3.5 ± 1.0	3.5 ± 0.8	3.3 ± 0.8	3.4 ± 1.0	3.3 ± 0.9
Jalebi	**BU (SFC*)**	Standard	3.1 ± 0.6	3.3 ± 0.5	2.9 ± 0.6	3 ± 0.7	3.2 ± 0.6
	Annatto seeds powder (*Bixa orellana*)	**Variation – I**	**3.1 ± 0.7**	**3.4 ± 0.7**	**3.2 ± 0.6**	**3.1 ± 0.9**	**3.4 ± 1.0**
		Variation – II	3 ± 0.7	3.3 ± 0.8	3.1 ± 0.7	3 ± 0.7	3.3 ± 1.1
		Variation – III	3.1 ± 0.7	3.2 ± 0.8	3.3 ± 0.8	3.3 ± 0.8	3.2 ± 0.8
	Eucalyptus bark powder (*Eucalyptus grandis*)	**Variation – I**	**3.1 ± 0.7**	**3.2 ± 0.8**	**3.1 ± 0.6**	**3.4 ± 0.7**	**3.1 ± 1.0**
		Variation – II	2.9 ± 0.7	3.1 ± 0.9	3.1 ± 1.0	2.9 ± 0.6	3 ± 0.8
		Variation – III	3.2 ± 0.8	3 ± 0.7	3 ± 0.7	3 ± 0.5	3 ± 0.8

***Synthetic Food Colourant**

From the table, the sensory attributes of each traditional sweets recipe were calculated using the organoleptic evaluation scores given by the ten semi trained panel members, according to their preference in appearance, consistency, texture, flavour and taste of the products using five point hedonic scale rating.

The traditional sweets recipe, kesari prepared with BU as standard synthetic food colourant had very good acceptability scores, according to the scores in regard with their appearance, consistency, texture, flavour and taste. Likewise, the variations of the natural food colourants were calculated, variation – II of both annatto seeds (*Bixa orellana)* and eucalyptus bark (*Eucalyptus grandis)* had the highest score in all the attributes of appearance (**4.5 ± 0.8**annatto seeds**, 4.2 ± 0.6**eucalyptus bark**)** , consistency (**4.1 ± 0.6**annatto seeds**, 4± 0.5**eucalyptus bark**)**, texture (**4.3 ± 0.7**annatto seeds**, 3.8 ± 0.4**eucalyptus bark**)**, flavour (**4 ± 0.8**annatto seeds**, 4 ± 0.5**eucalyptus bark**)** and taste (**4.1 ± 0.7**annatto seeds**, 3.6 ± 1.0**eucalyptus bark**)** with excellent and very good likeness, respectively.

Motichoor ladoo prepared using the brand BU as standard along with other variations I, II and III incorporated with annatto seeds (*Bixa orellana)* and eucalyptus bark (*Eucalyptus grandis)* were organoleptically evaluated, where the variation – II of both the natural food colourants scored the highest acceptance with appearance (**4.6 ± 0.7**annatto seeds**, 4.1 ± 0.7**eucalyptus bark**)** , consistency (**4.2 ± 0.4**annatto seeds**, 3.8 ± 0.**eucalyptus bark **6)**, texture (**3.9 ± 0.9**annatto seeds**, 3.9 ± 0.7**eucalyptus bark**)**, flavour (**4.2 ± 0.6**annatto seeds**, 4 ± 0**eucalyptus bark**)** and taste (**3.8 ± 0.9**annatto seeds**, 3.9 ± 0.6**eucalyptus bark**)** with excellent and very good rating.

The standard jalebi prepared using the brand BU and other variations I, II and III were organoleptically evaluated, where the variation – I of both the natural food colourants annatto seeds (*Bixa orellana)* and eucalyptus bark (*Eucalyptus grandis)* scored good acceptability with appearance (**3.1 ± 0.7**annatto seeds**, 3.1 ± 0.7**eucalyptus bark**)** , consistency (**3.4 ± 0.7**annatto seeds**, 3.2 ± 0.8**eucalyptus bark**)**, texture (**3.2 ± 0.6**annatto seeds**,3.1 ± 0.6**eucalyptus bark**)**, flavour (3.1± **0.9**annatto seeds**, 3.4 ± 0.7**eucalyptus bark**)** and taste (**3.4 ± 1.0** annatto seeds**, 3.1 ± 1.0**eucalyptus bark**)** rating respectively, proving it to be better in the sensory aspects when compared to the other two variations.

Table – XLVII

Computed Score for the Organoleptic Evaluation of the Snacks incorporated with Food Colourants

Snack Recipes	**Type of Colourants**	**Variations**	**Scores for the Sensory Attributes**				
			Appearance	**Consistency**	**Texture**	**Flavour**	**Taste**
Chilli Gobi	**BU (SFC*)**	Standard	4.8 ± 0.4	4.6 ± 0.5	4.5 ± 0.5	4.6 ± 0.5	4.8 ± 0.4
	Annatto seeds powder (*Bixa orellana*)	**Variation – I**	**4.2 ± 0.8**	**4.1 ± 0.7**	**4.3 ± 0.7**	**4.3 ± 0.7**	**4.3 ± 0.7**
		Variation – II	3.9 ± 0.9	4.3 ± 0.5	4.3 ± 0.7	4.1 ± 0.6	4.1 ± 0.9
		Variation – III	3.6 ± 1.0	3.7 ± 0.9	3.6 ± 0.8	3.7 ± 0.9	3.7 ± 0.8
	Eucalyptus bark powder (*Eucalyptus grandis*)	**Variation – I**	**4.3 ± 0.7**	**3.9 ± 0.6**	**4.2 ± 0.6**	**4.2 ± 0.6**	**4.2 ± 0.8**
		Variation – II	4.2 ± 0.6	4 ± 0.7	3.9 ± 0.6	4 ± 0.8	4 ± 0.7
		Variation – III	3.9 ± 0.7	3.9 ± 0.7	3.7 ± 0.7	3.8 ± 0.6	3.7 ± 0.8
Chilli Chicken	**BU (SFC*)**	Standard	4.7 ± 0.5	4.4 ± 0.5	4.5 ± 0.5	4.4 ± 05	4.4 ± 0.5
	Annatto seeds powder (*Bixa orellana*)	Variation – I	4.6 ± 0.5	4.5 ± 0.5	4.5 ± 0.5	4.4 ± 0.7	4.5 ± 0.7
		Variation – II	**4.7 ± 0.5**	**4.7 ± 0.5**	**4.7 ± 0.5**	**4.6 ± 0.5**	**4.8 ± 0.4**
		Variation – III	4.2 ± 0.6	4.5 ± 0.7	4.5 ± 0.7	4.2 ± 0.6	4.2 ± 0.4
	Eucalyptus bark powder (*Eucalyptus grandis*)	Variation – I	4.2 ± 0.6	4.5 ± 0.5	4.2 ± 0.8	4.2 ± 0.8	4.4 ± 0.7
		Variation – II	**4.4 ± 0.5**	**4.5 ± 0.5**	**4.4 ± 0.7**	**4.5 ± 0.5**	**4.4 ± 0.7**
		Variation – III	4.3 ± 0.5	4.5 ± 0.5	4.4 ± 0.5	4.4 ± 0.5	4.4 ± 0.5
Aloo Bajji	**BU (SFC*)**	Standard	4.6 ± 0.5	4.4 ± 0.7	4.3 ± 0.8	3.3 ± 0.7	4.6 ± 0.7
	Annatto seeds powder (*Bixa orellana*)	Variation – I	4.2 ± 0.6	4.2 ± 0.6	4.1 ± 0.7	4.1 ± 0.7	4.2 ± 0.8
		Variation – II	**4.2 ± 0.8**	**4.2 ± 0.7**	**4.2 ± 0.8**	**4.2 ± 0.6**	**4.2 ± 0.9**
		Variation – III	3.4 ± 0.8	3.6 ± 0.8	3.5 ± 0.7	3.5 ± 0.8	3.6 ± 0.7
	Eucalyptus bark powder (*Eucalyptus grandis*)	Variation – I	4.2 ± 0.6	3.8 ± 0.6	4 ± 0.7	3.9 ± 0.7	3.8 ± 0.6
		Variation – II	**4.2 ± 0.6**	**3.9 ± 0.6**	**3.7 ± 0.5**	**3.9 ± 0.8**	**3.9 ± 0.9**
		Variation – III	3.8 ± 0.6	3.7 ± 0.7	3.6 ± 0.7	3.5 ± 0.5	3.4 ± 0.7
Aloo Bonda	**BU (SFC*)**	Standard	4.1 ± 0.7	3.8 ± 0.6	3.6 ± 0.8	3.6 ± 0.5	3.3 ± 0.5
	Annatto seeds powder (*Bixa orellana*)	Variation – I	4.1 ± 0.9	4 ± 0.7	3.8 ± 0.6	3.7 ± 0.7	3.8 ± 0.6
		Variation – II	**4.2 ± 0.6**	**3.9 ± 0.9**	**4 ± 0.9**	**4.2 ± 0.6**	**4 ± 0.8**
		Variation – III	4.1 ± 0.9	3.7 ± 0.7	3.7 ± 0.8	3.8 ± 0.8	3.6 ± 1.2
	Eucalyptus bark powder (*Eucalyptus grandis*)	**Variation – I**	**3.8 ± 0.6**	**3.3 ± 0.7**	**3.4 ± 0.6**	**3.5 ± 0.4**	**3.4 ± 0.7**
		Variation – II	3.6 ± 0.7	3.1 ± 0.6	3.1 ± 0.6	3.3 ± 0.5	3.1 ± 0.3
		Variation – III	3.4 ± 0.5	3.1 ± 0.6	3.2 ± 0.8	3.2 ± 0.7	3.2 ± 0.4

***Synthetic Food Colourant**

The sensory attributes of the selected snack recipes are tabulated in the *table – XLVI.* The popular brand BU is used as standard synthetic food colourant, whereas the Variations I, II and III of both the natural food colourants of annatto seeds (*Bixa orellana)* and eucalyptus bark (*Eucalyptus grandis)* of 1 mg, 2mg and 3mg, 4mg, 5 mga and 6 mg respectively are used.

Chilli gobi is a favourite snack consumed by people of all age groups, where variation – I of both the natural food colourants were preferred by the semi trained panelists with scores of appearance (**4.2 ± 0.8**annatto seeds**, 4.3 ± 0.7**eucalyptus bark**)** , consistency (**4.1 ± 0.7**annatto seeds**, 3.9 ± 0.6**eucalyptus bark**)**, texture (**4.3 ± 0.7**annatto seeds**, 4.2 ± 0.6**eucalyptus bark**)**, flavour (**4.3 ± 0.7**annatto seeds**, 4.2 ± 0.6**eucalyptus bark**)** and taste (**4.3 ± 0.7**annatto seeds**, 4.2 ± 0.8**eucalyptus bark**)** , indicating the product to be very good and acceptable to consume.

Likewise, in the variations of chilli chicken, variation – II of annatto seeds (*Bixa orellana*) scored excellent in overall acceptance, in similar to variation – II of eucalyptus bark (*Eucalyptus grandis*) scored very good acceptability rate with appearance (**4.7 ± 0.5**annatto seeds**, 4.4 ± 0.5**eucalyptus bark**)** , consistency (**4.7 ± 0.5**annatto seeds**, 4.5 ± 0.5**eucalyptus bark**)**, texture (**4.7 ± 0.5**annatto seeds**, 4.4 ± 0.7**eucalyptus bark**)**, flavour (**4.6 ± 0.5**annatto seeds**, 4.5 ± 0.5**eucalyptus bark**)** and taste (**4.8 ± 0.4**annatto seeds**, 4.4 ± 0.7**eucalyptus bark**)** and were preferred by most of the panelists.

The recipes prepared using potatoes as main ingredient is aloo bajji and aloo bonda. The variation – II of both the natural food colourants such as annatto seeds (*Bixa orellana)* and eucalyptus barks (*Eucalyptus grandis)* added to aloo bajji scored an overall acceptability of very good with scores in regard to appearance (**4.2 ± 0.8** annatto seeds**, 4.2 ± 0.6** eucalyptus bark**)** , consistency (**4.2 ± 0.7** annatto seeds**, 3.9 ± 0.6** eucalyptus bark**)**, texture (**4.2 ± 0.8** annatto seeds**, 3.7 ± 0.5)**, flavour (**4.2± 0.6** annatto seeds**, 3.9 ± 0.8** eucalyptus bark**)** and taste (**4.2 ± 0.9** annatto seeds**, 3.9 ± 0.9)** likeness proving it to be better for consumption. Likewise, in aloo bonda variation – II of annatto seeds (*Bixa orellana*) and variation – I of eucalyptus bark (*Eucalyptus grandis)* scored an overall acceptability of good, respectively, where the taste of the products were not very much likable by the semi trained panel members.

Table – XLVIII

Computed Score for the Organoleptic Evaluation of the Fruit Preserves incorporated with Food Colourants

Fruit Preserves Recipes	Type of Colourants	Variations	Score for the Sensory Attributes				
			Appearance	Consistency	Texture	Flavour	Taste
Tutti Frutti	**BU (SFC*)**	Standard	4.3 ± 0.8	3.5 ± 0.8	3.8 ± 0.8	3.9 ± 0.7	3.8 ± 0.6
	Annatto seeds powder (*Bixa orellana*)	Variation – I	4.4 ± 0.7	3.9 ± 1.0	3.8 ± 0.9	4.1 ± 0.6	4 ± 0.8
		Variation – II	**4.6 ± 0.7**	**4.2 ± 0.4**	**3.9 ± 0.9**	**4.2 ± 0.6**	**3.8 ± 0.9**
		Variation – III	4.1 ± 0.6	3.9 ± 0.7	3.6 ± 0.8	3.5 ± 0.7	3.8 ± 0.8
	Eucalyptus bark powder (*Eucalyptus grandis*)	Variation – I	3.3 ± 0.9	3 ± 0.7	2.7 ± 0.7	3.2 ± 0.4	3.2 ± 0.8
		Variation – II	**4.1 ± 0.7**	**3.8 ± 0.6**	**3.9 ± 0.7**	**4 ± 0**	**3.9 ± 0.6**
		Variation – III	3.5 ± 1.0	3.5 ± 0.8	3.3 ± 0.8	3.4 ± 1.0	3.3 ± 0.9
Apple Jam	**BU (SFC*)**	Standard	3.8 ± 0.6	3.7 ± 0.9	3.5 ± 1.0	3.7 ± 0.8	3.5 ± 1.0
	Annatto seeds powder (*Bixa orellana*)	Variation – I	4.3 ± 0.7	4.4 ± 0.7	4.1 ± 0.7	3.7 ± 0.7	3.6 ± 0.7
		Variation – II	**4.5 ± 0.7**	**4 ± 0.7**	**4.2 ± 0.6**	**4 ± 0.9**	**4 ± 1.1**
		Variation – III	4.2 ± 0.6	4.2 ± 0.6	4.1 ± 0.6	4.1 ± 0.6	4.1 ± 0.7
	Eucalyptus bark powder (*Eucalyptus grandis*)	**Variation – I**	**4.1 ± 0.7**	**4.2 ± 0.6**	**4.2 ± 1.0**	**4.1 ± 0.9**	**3.8 ± 0.8**
		Variation – II	4 ± 0.8	4 ± 0.8	4.2 ± 0.6	3.7 ± 0.8	3.8 ± 0.8
		Variation – III	3.9 ± 0.9	4.3 ± 0.8	4.1 ± 1.0	4.1 ± 1.0	3.9 ± 1.0
Jelly	**BU (SFC*)**	Standard	3.9 ± 0.9	3.7 ± 0.7	3.7 ± 0.7	3.5 ± 0.5	3.4 ± 0.7
	Annatto seeds powder (*Bixa orellana*)	**Variation – I**	**3.7 ± 0.8**	**3.7 ± 0.8**	**3.4 ± 0.7**	**3.5 ± 0.8**	**3.4 ± 0.7**
		Variation – II	3.5 ± 1.0	3.7 ± 0.8	3.4 ± 0.8	3.3 ± 0.7	3.3 ± 0.5
		Variation – III	3.4 ± 1.0	3.6 ± 0.8	3.3 ± 0.8	3.2 ± 0.6	3.3 ± 0.5
	Eucalyptus bark powder (*Eucalyptus grandis*)	**Variation – I**	**3.2 ± 0.7**	**3.6 ± 0.8**	**3.5 ± 0.7**	**3.3 ± 0.6**	**3.2 ± 0.6**
		Variation – II	3.1 ± 0.6	3.5 ± 0.8	3.5 ± 0.7	3.2 ± 0.6	3.1 ± 0.9
		Variation – III	3.1 ± 0.4	3.5 ± 0.8	3.5 ± 0.7	3.2 ± 0.5	3.1 ± 0.7

***Synthetic Food Colourant**

Fruit preservatives are mostly liked by children and adolescents of all age groups, as these fruit preserves are added as toppings and consumed as desserts by most of them. From the table, the sensory attributes of each preservative was calculated using the organoleptic evaluation scores given by the ten semi trained panel members, according to their preference in appearance, consistency, texture, flavour and taste of the products using five point hedonic scale rating.

The above table shows that the preservatives prepared, where the variations of tutti frutti using BU as standard synthetic food colourant had very good sensory attributes. Likewise, the other variations incorporated with natural food colourants were calculated and variation – II of both annatto seeds (*Bixa orellana)* and eucalyptus bark (*Eucalyptus grandis)* had the highest score in all the attributes of appearance (**4.6 ± 0.7** annatto seeds**, 4.1 ± 0.7** eucalyptus bark**)**, consistency (**4.2 ± 0.4** annatto seeds**, 3.8 ± 0.6** eucalyptus bark**)**, texture (**3.9± 0.9** annatto seeds**, 3.9 ± 0.7** eucalyptus bark**)**, flavour (**4.2 ± 0.6** annatto seeds**, 4 ± 0** eucalyptus bark**)** and taste (**3.8 ± 0.9** annatto seeds**, 3.9 ± 0.6** eucalyptus bark**)** with excellent and very good likeness, respectively.

Whereas for Apple jam, The variation – II of annatto seeds (*Bixa orellana)* and variation – I of eucalyptus bark (*Eucalyptus grandis)* added as natural food colourants scored the highest acceptance with scores of appearance **(4.5 ± 0.7** annatto seeds**, 4.1 ± 0.7** eucalyptus bark**)** , consistency (**4± 0.7** annatto seeds**, 4.2 ± 0.6** eucalyptus bark**)**, texture (**4.2 ± 0.6** annatto seeds**, 4.2 ± 1.0** eucalyptus bark**)**, flavour (**4± 0.9** annatto seeds**, 4.1 ± 0.9** eucalyptus bark**)** and taste (**4 ± 1.1** annatto seeds**, 3.8 ± 0.8** eucalyptus bark**)** of excellent and very good rating.

The jelly prepared with 1mg annatto seeds (*Bixa orellana)* and 4 mg of eucalyptus bark (*Eucalyptus grandis)* Variation – I scored good acceptability rating of appearance (**3.7 ± 0.8** annatto seeds**, 3.2 ± 0.7** eucalyptus bark**)**, consistency (**3.7 ± 0.8** annatto seeds**, 3.6 ± 0.8** eucalyptus bark**)**, texture **(3.4 ± 0.7** annatto seeds **3.5 ± 0.7** eucalyptus bark**)**, flavour (**3.5± 0.8** annatto seeds**, 3.3 ± 0.6** eucalyptus bark**)** and taste (**3.4 ± 0.7** annatto seeds**, 3.2 ± 0.6** eucalyptus bark**)** respectively, proving it to be better in the sensory aspects when compared to the other two variations.

Thus, the natural food colourants incorporated in selected recipes suchas traditional sweets, snacks and fruit preserves had good acceptability on all sensory attributes. Majorily, the variations I and II (1mg and 2mg of annatto, 4mg and 5mg of eucalyptus) are highly preferred in comparison with the variation I.

PHASE – VI

4.6 Development of Food Colour Sensor

4.6.1 Developing a Food Colour Sensor to Identify the Level of Food Colourants in Selected Common Recipes

Actualizing blockchain innovation for information capacity and to improve exact traceability within the supply chain for each step of colourant detection. Measuring the toxicity is recorded on a permanent record, giving an unquestionable data for the quality of selected common recipes. On creatinguser-friendly interface and informative visualization apparatuse is related to the webpage that would encourage the information translation for being user friendly with shifting levels of ability.

Real-time caution frame work on webpage gives information regarding any peculiarities or deviations in toxicity of colourant levels the exceeds the permissible limitations. The remedial solution of the device is to preserve the quality and food security in the selected recipes. Sensor frame work related to webpage is adaptable and customized that would permit the adjustment of the selected recipes during fabricating situations. This adaptability would cater to the particular needs of detecting toxicity in selected recipes.

The toxicity of food colourantshas paved way for the utilization of sensors and webpages holding a guaranteed effectiveness, exactness and accuracy over the supply chain of data set grouped for specific recipe, eventually profiting and regulating the need for the usage of the device among common people and food vendors to organize their preparation of foods using food colourants.

4.6.2 Specific Application of Software in the Developed Food Colour Sensor to Find out the Level of Toxicity in the Selected Recipes

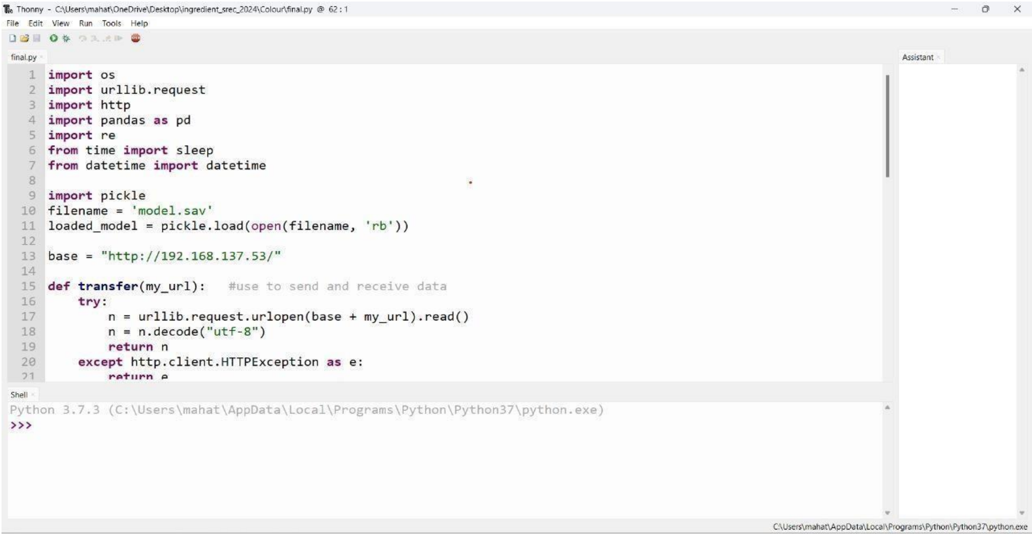


Plate – 4.6.2 (a)

Data Set Source Code Executed

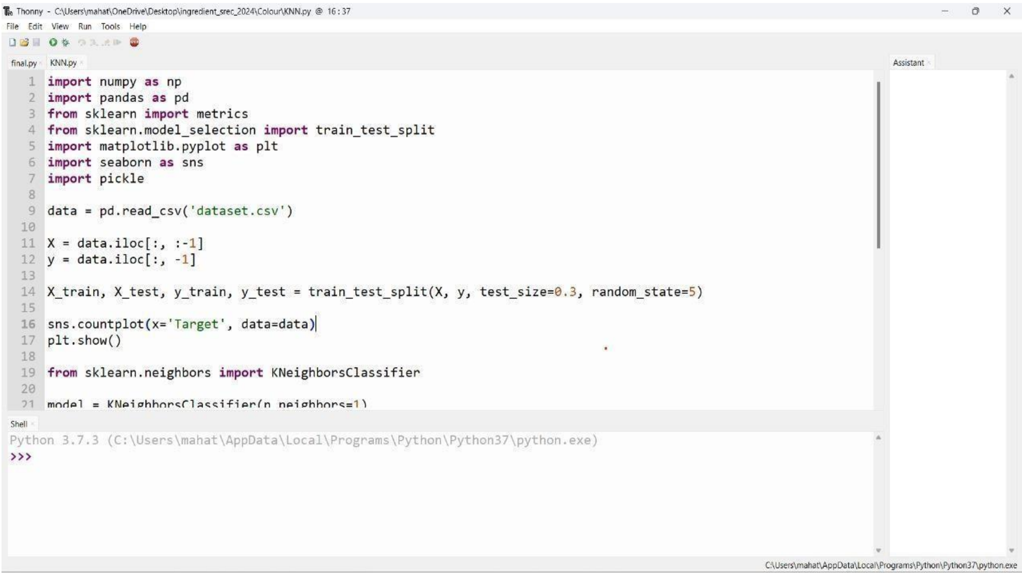


Plate – 4.6.2 (b)

Implemented KNN Algorithm for the Device Execution

Plate – 4.6.2 (c)

Graph set for the Data base Executed to Detect Toxicity in Food Colourants

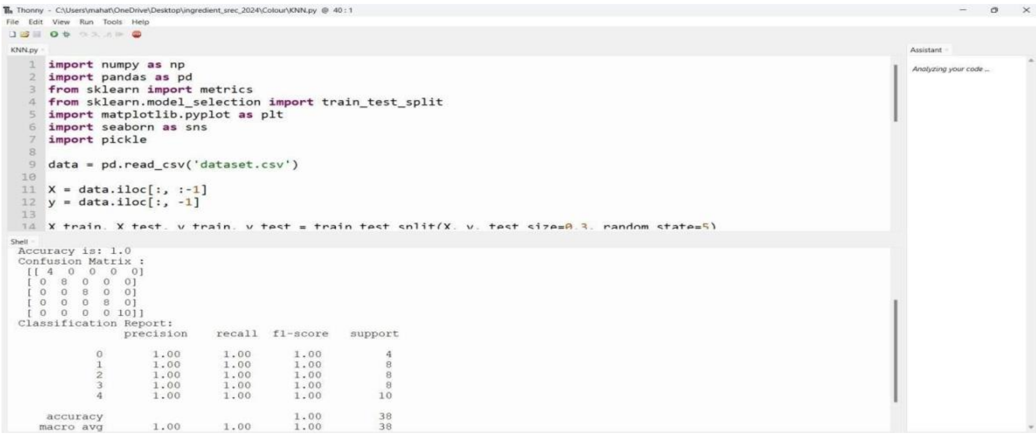


Plate – 4.6.2 (d)

Values Executed to Detect Toxicity in Food Colourants

From the data set built using the limitations of FSSAI (2011, 2021), the source code (Appendix – XI) are being used to run the program implementing KNN algorithm to detect and execute the values of toxicity. The implemented algorithm were used to construct the graph set that is being used to determine the toxicity values in selected foods classifying it as mild, moderate and severe level of toxicity.

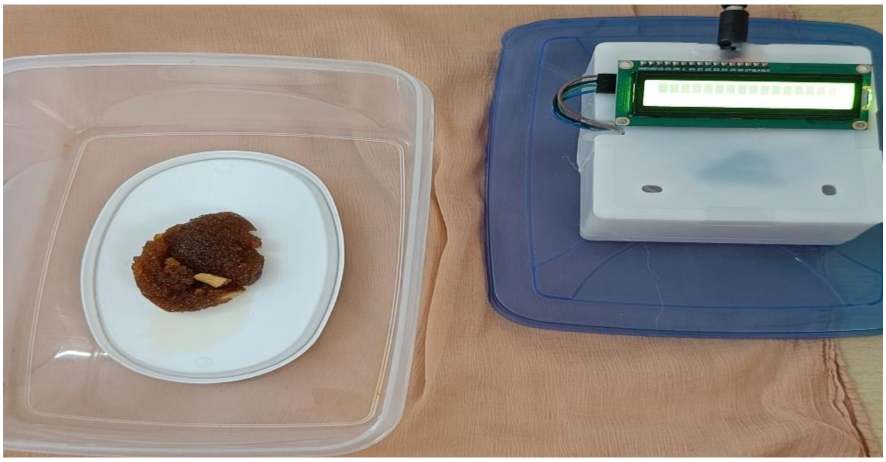


Plate – 4.6.2 (e)

Food Placed in the Sensor Box to Detect the Level of Food Colour added

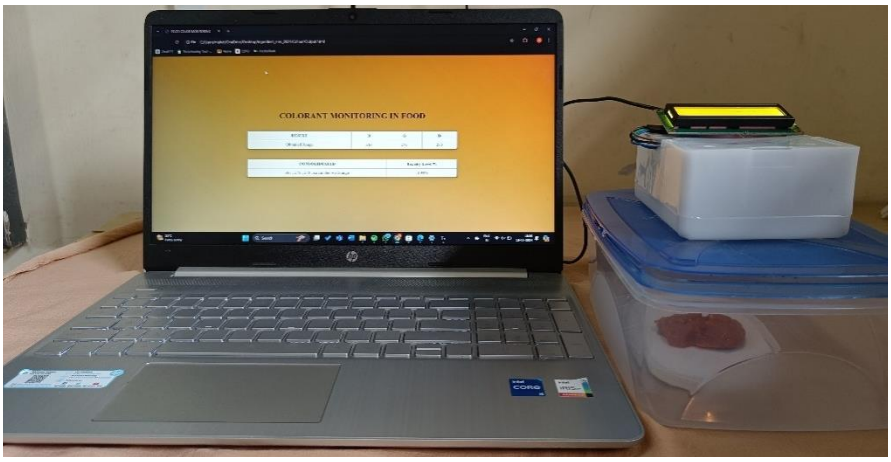


Plate – 4.6.2 (f)

Sensor Box Connected to the System for Result

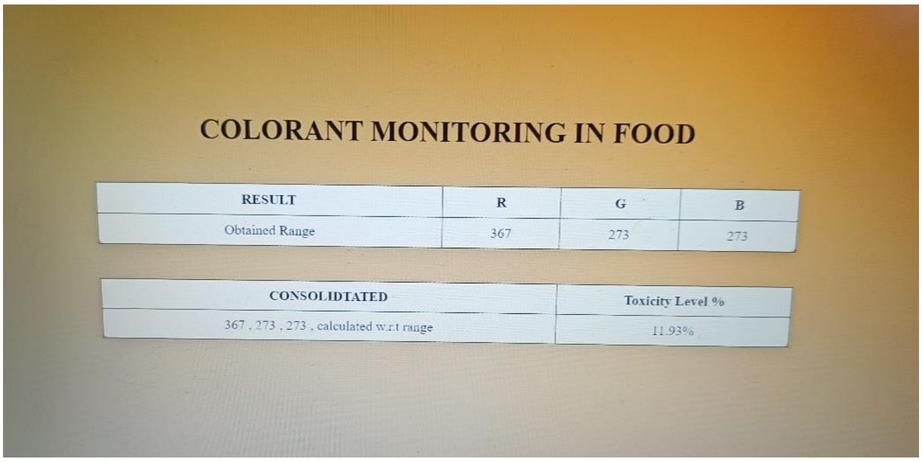


Plate – 4.6.2 (g)

Designed Webpage with the Result

Table – XLIX

Level of Food Colours Detected in the Selected Foods

Recipes used for Testing the Food Colour Sensor	Amount Detected by the Sensor(mg)	Permissible Amount Prescribed by FSSAI, 2021 (mg)	Safe or Toxic for Consumption
Kesari (1) (a pinch of SFC*)	**4.5**	4 to 7	**Safe**
Kesari (2) (three pinches of SFC*)	**7.8**	**4 to 7**	**Toxic**
Chilly Chicken (1) (a pinch of SFC*)	**4.61**	4 to 7	**Safe**
Chilly Chicken (2) (two pinches of SFC*)	**4.64**	4 to 7	**Safe**
Chilly Chicken (3) (two pinches of SFC*)	**4.84**	4 to 7	**Safe**
Chilly Gobi	**4.20**	4 to 7	**Safe**
Jalebi	**4.76**	4 to 7	**Safe**
Ladoo (1) (Yellow SFC*)	**5.08**	4 to 7	**Safe**
Ladoo (2) (Orange SFC*)	**5.45**	4 to 7	**Safe**
Recipes based on Natural Colourants			
Vegetable Cutlet	**3.8**	-	**Safe**
Cut Mango Pickle	**3.39**	-	**Safe**
Tomato Powder	**3.39**	-	**Safe**

The synthetic food colourants added to the commercial foods were detected through the food colour sensor and the results were tabulated. Recipe 1 of kesari was added with orange colourant in the quantity that is prepared at home and the level of toxicity was 4.5 mg, when compared to the FSSAI guidelines, it is permissible limit. But when an extra amount was added it showed as toxic, exceeding the permissible level. Likewise, chilly chicken prepared using three variations of 1, 2 and 3 has all shown to be safe for consumption with the range of 4.61, 4.64 and 4.84 respectively. Commercially available chilly gobi, jalebi and ladoo added with yellow and orange synthetic food colourants were checked for permissible level for consumption, where the results were 4.20, 4.76, 5.08 and 5.45 mg, respectively. To check the efficacy with the natural colourants, vegetable cutlet, mango pickle and tomato powder of natural form were place in the sensor and it showed the results below the permissible limit proving its efficacy.