5 SUMMARY

Potential usage of synthetic food colourants extracted or obtained from inedible sources like metals, rocks and toxic elements from the earth have become a concern for replacement with natural origins of colourants in foods. Numerous toxic effects on both short term and long term usage of these synthetic food colourants have been noted in terms of allergic reactions, behavioural changes especially in children and adolescents, neurocognitive effects related to motor impairments have become prominent life changing approaches, globally. To improve the safety, quality of foods and for the betterment of the food producers, replacement of synthetic food colourants is much needed for the hour.

At the industrial level, the demand for natural colourants for food processing is increasing, mostly due to concerns about health risks triggered by some synthetic additives that can be toxic at metabolic, physiological and toxicological levels. Considering, among others, genotoxic and carcinogenic risks, urticaria, asthma, nausea, eczema, bronchitis, bronchospasm, headache, reduced blood coagulation and hyperactivity are more prevalent. In the European Union, only the authorized use of natural food colours and those synthesized equally to the natural colourants are clearly regulated.

The primary objectives focused in this study is to replace synthetic food colourants with natural food colourants that cause ill effects to the living beings. Thus, the findings of the study are summarized and concluded.

In consideration towards demonstration of health improvisation in concern towards the physical and mental wellbeing of the community, the investigator has conducted the study to analyze an alternative for the upraising health issue. The primary focus of this study is to alternate synthetic food colourants with available natural source of colourants. A study on the usage of synthetic food colourants was carried out, where a market survey was conducted on the brands that were most popularly used and purchased in the supermarkets and shops in Coimbatore city. Over 150 supermarkets and shops were monitored for purchase of synthetic food colourants. The preferred brands of synthetic food colourants purchased were bakers, bush and gold camel. Among the three brands BKwere sold in 28 shops, BU were sold in 21 shops and GC were sold in 34 shops. GC was highly and frequently purchased by the customer, which was analyzed in the further proceedings of the study to analyze the presence of heavy metals by Inductively Coupled Plasma Mass Spectrometer.

Through random sampling method, home makers (N=100) between the age group of 25 – 45 years and food vendors (N=100) were the population selected for analyzing the usage of food colourants in the food prepared by them. A questionnaire with 25 questions were personally answered by the selected group of respondents. The attitude of the food vendors towards answering the formulated questionnaire was very reluctant, their responses and attitude were less of concern when compared to the home makers. Thus, the recipes preferred and prepared by the home makers were mainly considered for the analysis of adding natural food colourants to the recipes and for evaluating the selected recipes through trained panel members.

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 the food vendors had the practice of incorporating synthetic food colourants into their foods.

The food vendors had 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.

The natural food colourants preferred to be used mostly by the home makers were considered for analysis. Around 14 easily available and commonly used natural food colourants was answered by the home makers, from which the least preferred natural food colourant of less than 25 percentage of the population were selected for analyzing their colour and usage. 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 that annatto seeds come under natural food colourants. Likewise, the knowledge on eucalyptus bark (*Eucalyptus grandis*), madder root2 (*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.

A brief study on the selected natural food colourants were carried out, their availability was checked in the local market and traditional herbal medical stores. Then their natural form of availability was checked and the natural samples were collected from the Tamil Nadu Forest and Research Centre, Mettupalayam. The naturally obtained raw substances were preserved in zip lock covers and transferred to the laboratory by the researcher, where different extraction methods were carried out. The methods of extraction were: 1. Aqueous method of soaking process; and 2. Powdering of the substance.

During the process of soaking through aqueous method, the tamarind seeds did not leech out colour, likewise, tanner's cassia was a seasonal flower and the colour was not sufficient enough for preservation, whereas the other natural substances of annatto seeds (*Bixa orellana),* eucalyptus bark (*Eucalyptus grandis*), madder roots (*Rubia cordifolia*) and roselle petals (*Hibiscus sabdariffa*) gave out brilliant red, yellow and brown colour that were equivalent to the synthetic food colours of tartrazine (E102), sunset yellow FCF (E110), azorubine (E122), amaranth (E123), ponceau 4R (E124), erythrosine (E127), allura red AC (E129), brown HT (E155) and litholrubine BK (E180).

From the selected natural food colourants, annatto seeds (*Bixa orellana),* eucalyptus bark (*Eucalyptus grandis*), madder roots (*Rubia cordifolia*) and roselle petals (*Hibiscus sabdariffa*) toxicity study, shelf life study and microbial assay were carried out. During the shelf life study, prolonged exposure of the colourants towards room temperature and refrigeration showed the formation of few microbial structure in form of slimy layer. Thus, microbial assay was carried out with Rose Bengal Chlorophenicol medium for fungal identification and nutrient agar medium for bacterial identification. Fungal identification was carried out by analyzing the physical morphology of the fungal growth present in the petric plates, whereas the bacterial medium was streaked and stained with crystal violet solution for the identification of the bacterial species.

Two different toxicity methods were used to thoroughly analyze the natural substances for the presence of toxic bioactive compounds. Brine shrimp assay was carried out as primary toxicity study from which the roselle petals (*Hibiscus sabdariffa*) was found to be extremely toxic in nature in both aqueous, 100 to 1500 µg/ml, all thirty shrimp were found dead and in the powdered form 80 percentage of the shrimp were found dead in 1500 µg/ml. Thus, it was ruled out from further analysis. Likewise, aqueous extracts of annatto seeds (*Bixa orellana)* with 53 percentage, eucalyptus bark (*Eucalyptus grandis*) with 100 percentage and madder root (*Rubia cordifolia*) with 100 percentage also showed high mortality rate in brine shrimp assay. So, aqueous extracts were also ruled out from further processing.

Analysis for antioxidants was carried out, in the annatto seeds (*Bixa orellana*), the percentage of inhibition in aqueous extracts have 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 has decreased from 82.79 percentage to 54.92 percentage. In the eucalyptus barks (*E grandis*) extract, the percentage of inhibition was decreased from 97.39 percentage to 68.70 percentage that is from 10 µl to 750 µl respectively. In eucalyptus barks powder there was decrease in inhibition percentage from 67.21 percentage to 54.10 percentage.

In the test for the phytochemicals, the presence of 17 different metabolites was tested. The madder roots' (*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.

Then secondary toxicity study was carried out through Inductively coupled plasma mass spectrometer (ICPMS), through which heavy metals were analyzed. In this method of detecting toxins, madder roots (*Rubia cordifolia*) proved to have larger amount of lead 3.28560 µg, cadmium 0.05162 µg, chromium 5.48116 µg and mercury 0.15526 µg in its natural form. Thus, madder roots (*Rubia cordifolia*) was not suggested for further studies.

Analysis for characterization of the natural food colourants of annatto seeds (*Bixa orellana)* and eucalyptus bark (*Eucalyptus grandis*) were followed. Field Emission Scanning Electron Microscope (FE-SEM) was technology used in capturing the microstructure image of the natural food colourants. To generate x-rays diffraction (XRD), 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 in annatto seeds (*Bixa orellana)* and eucalyptus bark (*Eucalyptus grandis*).

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. The blue curve in the graph indicates the thermal stability of the annatto seeds (*Bixa orellana*) powder. 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. The eucalyptus bark powder, tested for thermal gravitivity effect have shown a slight loss of mass, when there is increase in temperature for every 100^0C.

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. Photodegradation was monitored as the concentration level of annatto seeds (*Bixa orellana*) and eucalyptus bark (*Eucalyptus grandis*) were measured in zero hour, then gradually increased the time of exposure to the monochromatic light from zero hour to two, four, eight, twelve, eighteen and twenty four hour respectively. From zero hour to twenty-four hour, the degradation in concentration has been 0.32 µg loss of strength in the concentration of annatto seeds (*Bixa orellana*), whereas the same level of degradation is measured as 0.32 µg of loss of concentration due to degradation is found in eucalyptus bark (*Eucalyptus grandis*).

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* study was demonstrated to prove that the selected natural food colourants had 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.

As for the annatto seeds (*Bixa orellana*) powder, the same procedure was carried out, where on the first week 0.01 mg and 0.08 mg, that is two 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 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 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.

Weekly weight gain or loss of the Wistar Albino rats that has been monitored regularly and listed that were fed with eucalyptus barks (*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. 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 range of BMI increases then the level of obesity also gets increased, classifying the weight gain as slight, moderate and severe obesity. The BMI value of all the three groups of rats were calculated and the weight gain have shown slight increase in the body mass of 0.78 g/cm^2. This shows that the rats were slightly obese when compared to the range of BMI in Wistar albino rats.

From the histology of Wistar Albino rats, the organs of the rats that consumed eucalyptus bark (*Eucalyptus grandis)* and annatto seeds (*Bixa orellana*) powder 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 have shown any alteration in cell structure or any unfavourable effects under the light microscope using multiple magnification powers. In the kidney cells of eucalyptus barks (*Eucalyptus grandis)* and annatto seeds (*Bixa orellana*) fed rats, round shaped glomerulus were normal and solid in structure, where proximal tubules with cuboid round cells and villis were visible. This indicated that there was no nephrotoxicity taken place in the wistar albino rats.

The cerebral cross section had displayed no congestion and hemorrhage, proved for no neurotoxicity in the rats of experimental groups. In liver histology, normal nuclei hepatocytes were observed, proved to have no hepatotoxicity in the rats. In the architectural histology of spleen, the zones of the organs were visible with no inflammation, proving it to be with no toxins. There were no disruption or deformation in the myocardiac fibers of the cardiac tissue. Thus, there were no cardiac toxicity found in the rats. Also in the gastro intestinal organs, there were no mucosal degeneration or hemorrhage in the intestinal walls.

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*) powders 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.

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) and 3.70-5.80 (10^3/mm^3) respectively. Here, the control group and the experimental groups RBCs and WBCs measured 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.

The reference range of 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 – 1, whereas experimental group – 2 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 – 1 and 2 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 – 1 and 2 ranges as 6.5, 6.53 and 6.5 g/dL.

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. Serum concentration of total protein and albumin in the experimental group 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*) powders did not affect the hepatocytic functions of the wistar albino rats.

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

The nutrients analyzed for protein content in the annatto seeds (*Bixa orellana*) and eucalyptus bark (*Eucalyptus grandis*) powders were 5.92 g and 2.13 g, respectively, whereas fat content was of 5.92 g and 2.13g, crude fiber was 16.84 g and 15.14 g, carbohydrates was 77.12 g and 83.39 g and the calorific value was 351.33 kcal and 345.59 kcal respectively.

The micronutrients analysed in annatto seeds (*Bixa orellana*) and eucalyptus 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*) and eucalyptus bark (*Eucalyptus grandis*) powders.

The recipes prepared and incorporated with the natural food colourants are as follows: kesari, motichoor ladoo, chilli gobi, chilli chicken, tutti frutti, jalebi, aloo bajji, aloo bonda, apple jam and jelly. Using the standard procedure, these recipes were prepared and their intensity in colour was also recorded. In the standard preparation of all the recipes the highest popular and most commonly (through the survey) used synthetic food colourant BU was used to note the colour difference between the natural and the synthetic food colourants. All the recipes were prepared with one standard commercial synthetic food colourant. 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.

The common recipe of kesari, in comparison to the standard recipe prepared with BU, annatto seeds (*Bixa orellana*) powder of variation – III had bright hue of 27.23, when compared with eucalyptus bark (*Eucalyptus grandis*) of hue 24.69. In comparison to the standard recipe of ladoo, 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.

The variations had their increase in hue as the amount of natural food colourants were increased by 1mg each in each recipe, where the standard procedure was followed. 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 standard recipe and the variation – II of annatto seeds (*Bixa* orellana) powder incorporated recipe of aloo bajji 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 standard recipe aloo banda had its hue value of 52.04, whereas in the variation – I, II and III, the hue has 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 there is an increase in hue from 49.73, 51.07 and 51.62 respectively.

In the fruit preservatives of tutti frutti, 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. In variation of apple jam – 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.

In the sensory evaluation of the products, kesari and motichoor ladoo with variation – II had been evaluated as very good with four points in the hedonic scale rating (4.5±0.8, 4.2±0.6 and 4.6±0.7, 4.1±0.7). Whereas, variation – I of jalebi (3.4±1.0, 3.1±1.0) of both the natural food colourants were preferred by the panellists. In the snacks recipes, variation - I (4.3±0.7, 4.2±0.8) of chilli gobi, variation – II and III of chilli chicken (4.8±0.4, 4.4±0.7), variation – II (4.2±0.9, 3.9±0.9) of aloo bajji and variation – II of annatto seeds (4±0.8) and variation – I (3.4±0.7) of aloo bonda had been favoured by the semi trained panelists. Variation – II of all the fruit preservatives of tutti frutti (annatto seeds – 3.8±0.9 and eucalyptus bark – 3.9±0.6) and apple jam (annatto seeds – 4±1.1 and variation – I of eucalyptus bark – 3.8±0.8), and variation – I of jelly of both the natural food colourants (3.4±0.7 and 3.2±0.6) were highly accepted by the panelists with a very good score through the hedonic scale rating.

The toxicity of food colourants has 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. From the data set built using the limitations of FSSAI (2011, 2021), the source code is being used to run the program implementing KNN algorithm to detect and execute the values of toxicity. The implemented algorithm is 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.

Common commercial recipes were selected and placed in the developed food colour sensor to test its efficacy. The limitations recommended by FSSAI is 4 to 7 mg for the added synthetic food colourants. Kesari was prepared added with orange synthetic colourant in the quantity that is used for preparation at home (a pinch) was tested 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 has also 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 with two variations were 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 between three to four which is below the permissible limit proving its efficacy. Thus, the developed food colour sensor has been proved to be efficient in testing the toxicity of the commercially used synthetic food colourants.