Important biological activities of papaya peel extracts and their importance in formulation of a low cost fish feed to enhance the skin colour and the healthiness of guppies

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Abstract: *Different plant extracts of Carica papaya (papaya) peels were investigated for their antioxidant capacity (AOC), antibacterial activity, antidiabetic activity and concentration of carotenoids content. An investigation was carried out to identify the best extraction conditions to obtain a suitable extract that can be used to formulate a fish feed with important bioactivities. Three different papaya peel drying methods and a suitable solvent to use with the maceration technique was tested. According to the UV-Visible spectra of the extracts, the highest percentage of carotenoids was obtained for the ethyl acetate extract of air-dried sample (5.4 µg/mL). According to the Folin–Ciocalteu assay, the highest AOC (22 PGE µg/mg) was displayed by the ethanolic extract of air-dried sample. Highest DPPH radical scavenging activity of 96% was found in the ethanolic extract of oven-dried (50 °C), air-dried and freeze-dried samples. According to disk diffusion assay, the ethanolic extract of the air-dried sample exhibited antibacterial activity against Pseudomonas aeruginosa. Highest α-amylase inhibitory activity was obtained for the ethyl acetate extract of air-dried papaya peel (37%). An economic fish feed prepared incorporating 4% of the ethanolic extract of air-dried papaya peels effectively enhanced the skin colour and the healthiness of guppies within a much shorter period of time compared to a commercially available fish feed. This study reveals important bioactivities and a useful application of papaya peel waste. Findings of this research can be used for value addition and efficient management of papaya peel waste.*

Keywords: Papaya, Antioxidant, Antibacterial, Antidiabetic, Carotenoids

1. Introduction

With the advancement of screening programmes, exploring the therapeutic potential of edible plant materials has become a wide research area and a promising path for novel applications [1]. There is a high demand towards the naturally isolated bio-active compounds or standardized crude extracts from plant materials over synthetic substances due to their biological, industrial applications and adverse side effects of synthetic drugs [2]. Papaya which belongs to *Caricaceae* family is reported to contain important bioactive ingredients not only in its fruit but also in the other plant parts including leaves, roots, bark, peel, seeds and flesh [3]., It has been reported that papaya is a rich source of protein, fat, fibre, vitamin C, thiamine, riboflavin, niacin, carotene, amino acids, citric and malic acids [4]. In addition, papaya peel contain fibre, phenolic compounds, vitamin C, soluble solids, titratable acidity, and the minerals copper, sulphur, and potassium [5]. The red and the yellow colours of papaya is a result of the presence of pigments such as β-carotene, violaxanthin, lycopene and some other unresolved pigments [6]. Papaya peel has the antidandruff, skin soothing and moisturizing ability which is important in cosmetic industry [3]. The ability of eliminating intestinal parasites, anti-helminthic and anti-amoebic properties of bioactive compounds found in seeds pave a promising path for new drug discoveries [3]. Additionally, papaya peel waste is generated in many countries throughout the year and proper management of this waste product could gain commercial interest in various industries. Since the annual global average production of papaya is about 10.0 million metric tons[7], it is important to add value to the left over waste materials of papaya fruit which could in turn be commercially beneficial as well as be useful to the environment due to reduction of waste [8]. However, careful consideration has to be put into the extraction conditions used to isolate these valuable natural products in an intact and useful manner. The extraction method is crucial in terms of obtaining intact and high yield of particular compound from the plant materials in order to show its effective biological activities. Therefore, it

is important to optimize the conditions to get maximum of these ingredients in an intact manner via proper extraction without decomposing and economically favourable method as they can be used in other applications [9],[10]. The extraction methods and bio activities of papaya peel extracts compared during this study were not analysed before and this type of a bio prospecting analysis on crude extracts before isolation of pure compounds are isolated from crude mixtures will help to provide a prior clue on what sort of properties it possess that can be used to decord problematic issues.

2. Experimental methodology

2.1. Preparation of papaya peel extracts

Peels of papaya which were left behind after consumption were identified to extract the natural products. Natural products were extracted in to three different solvents by maceration after drying the papaya peels via three different drying methods (air drying, oven drying, freeze drying).

2.2. Drying methods of papaya peel samples

Papaya peels were cut in to small pieces and weighed separately. Samples were exposed to air dry [11] for three days, lyophilized [12] for 8 hours using a freeze dryer and exposed to temperatures of 50 °C and 80 °C using a laboratory oven [13] for 3 hours respectively. Each sample was ground in to a coarse powder.

2.3. Extraction of natural products by maceration

Weights of 2 g of each dried samples of papaya peel were separately put in to amber colour bottles containing 10 mL of ethanol, ethyl acetate (EtOAC) and hexane. Each sample was subjected to maceration overnight at 37 °C for overnight. Then samples were filtered and extracts were concentrated with the aid of a rotary evaporator. After the solvent evaporation the extracts were stored in the refrigerator until further use.

2.4. Characterization of the crude plant extracts using photochemical analysis

2.4.1. Thin Layer Chromatography (TLC)

Since all papaya peel extracts were coloured and papaya was reported as a rich source of carotenoids, thin layer chromatograms were developed using petroleum ether: acetone (7:3) as the mobile phase to investigate the existence of the carotenoid pigments in each extracts [14].

2.4.2. UV-Visible spectrometry

The various plant pigments absorb light in overlapping spectral regions depending on their colour. Therefore, the concentration of the carotenoid pigment mixture was calculated using Beer-Lambert law with the maximum absorption at 470 nm using Ɛ value as 221 L/g/cm in methanol [15].

2.5. Phytochemical analysis of papaya peel extracts

2.5.1. Test for phenols and tannins

A volume of 500 µL of each plant extract was added to 1 mL of distilled water. A 5% of solution of $FeCl_3$ was added drop wise to this mixture and checked for the appearance of a dark green or purple colour which indicates the presence of phenols [9].

2.5.2. Lead acetate test

A volume of 1 mL of each plant extract was dissolved in 1 mL of distilled water. A 1% of lead acetate was added drop wise and checked the appearance of a precipitate [9].

2.5.3. Saponification for saponins

A volume of 1 mL of each plant extract was added to a test tube containing 20 mL of distilled water and was agitated for 15 minutes to check the formation of foams [9].

2.5.4. Test for flavonoids

Diluted NaOH was added to the 1 mL of each plant extract drop wise to check the appearance of the intense yellow colour and then diluted HCl was added to the solution until the yellow colour disappears [9].

2.5.5. Test for steroids and terpenes

A volume of 500 µL of each plant extract was mixed with 2 mL of conc.H_2SO_4 and 2 mL of chloroform. The development of red colour at the interface indicates the presence of steroids and the development of the reddish brown colour at the interface indicates the presence of the terpenes [9].

2.5.6. Test for alkaloids

Diluted HCl was added to a 500 µL of each plant extract drop wise and the mixture was stirred and filtered. Hager's reagent (1% picric acid) was added to the filtrate to check the formation of a yellow colour precipitate which indicates the presence of alkaloids [9].

2.5.7. Test for reducing sugar

Few drops of Fehling's solution were added to the 500 µL of each plant extract and it was then heated to check the formation of brick red colour precipitation [9].

2.6 Antioxidant capacity

2.6.1. Folin-Ciocalteu's method

A volume of 100 µL of methanolic extract was mixed with 2 mL of 2% (w/v) sodium bicarbonate and was incubated at room temperature for two minutes. Then 0.100 mL of Folin-Ciocalteu reagent was added and was incubated for 30 minutes under dark conditions. Absorbance values were measured at 750 nm. A pyrogallol standard curve was developed using a series of pyrogallol standard solutions in methanol. The antioxidant capacity of each extract was determined in terms of pyrogallol equivalents (PGE) using the pyrogallol standard curve. This protocol was carried out in triplicates for each sample [9], [16].

2.6.2. DPPH radical scavenging assay

Known concentrations of DPPH stock solution and crude extracts were prepared in methanol. Test blank was prepared by mixing 1950 µL of methanol and 50 µL of the extract. Test sample was prepared by mixing 1950 µL of DPPH with 50 µL of extract. After incubating under dark conditions for 30 minutes the absorbance of the test sample was measured against the test blank and the absorbance of the control sample was obtained by measuring the absorbance of the control sample (2 mL of DPPH solution) against the control blank (2 mL of methanol) at 517 nm. All samples were tested in triplicate. The percentage of Radical Scavenging Activity (% RSA) was calculated for each sample using the following equation (1) [11].

$$RSA\ \% = \frac{Absorbance_{control\ ample} - Absorbance_{test\ sample}}{Absorbance_{control\ sample}} \times 100\% \quad (1)$$

2.7. Antibacterial activity

Disk diffusion assay was carried out against *Bacillus cereus* (ATCC11778), *Staphylococcus aureus* (ATCC 25923), *Salmonella typhimurium* (ATCC 14028) and *Echerichia coli* (ATCC 35218). Negative control was prepared by loading the solvent to dissolve the plant extract and 1 mg/mL gentamycin solution was as the positive control. After 24 hours of incubation at 37 °C, the diameters of the inhibition zones were measured using a vernier caliper. All experiments were carried out in triplicate. Investigation of cytotoxic effects of selected plant extracts.

2.8. Investigation of the antidiabetic activity of the plant extracts using α-amylase inhibitory assay

A volume of 200 µL of each plant extract was placed in a test tube and 200 µL of alpha amylase in 0.02 M phosphate buffer (pH 6.9) was added to each extract. Then the mixture was kept at room temperature for 15 minutes and 1% starch in phosphate buffer was added. Again, the mixture was kept at room temperature for 15 minutes. A volume of 400 µL of 3,5-Dinitrosalicylic acid (DNS) was added to the solution and then the test tube was kept in a boiling water bath for 5 minutes. (DNS solution was prepared by mixing 0.1 g of DNS, 0.16 g of NaOH and 2.99 g of sodium potassium tartarate in 10 ml of phosphate buffer.) The reaction mixture was cooled to room temperature and each was diluted by adding 1 ml of distilled water. This test sample was blanked with the same mixture which was made by adding 400 µL of buffer instead of DNS reagent. Control sample was prepared by making the same mixture but adding acetone instead of plant extract which was the solvent used to dissolve the extractions. Control sample was blanked with the same mixture where 400 µL of buffer used instead of DNS reagent. Absorbance of the test sample and the control sample was measured at 540 nm using a UV- visible spectrophotometer and the percentage inhibition was calculated using following equation (2) [9].

$$Percentage\ inhibition\ \% = \frac{Absorbance_{control\ ample} - Absorbance_{test\ sample}}{Absorbance_{control\ sample}} \times 100\% \quad (2)$$

2.9. Formulation of fish feed using air-dried papaya peel ethanolic extract

Fish feed was formulated using bread crumbs (carbohydrate source), gelatin (protein source and as a binder), ethanolic papaya peel extract (pigments, antioxidant and antibacterial compounds) and corn oil (lipid source). Incorporation of corn oil in to the fish feed has been reported to help in higher retention of pigments in guppies. A clear difference of pigmentation of the body colour to the naked eye is an important criterion when buying ornamental fish by customers, a visual sensory evaluation was carried out to grade the skin coloration of the treated fish groups of this study.

3. Results and discussion

3.1. Investigation of the effect of drying methods and extraction conditions used on papaya peel

3.1.1. Percentage yield of papaya peel extracts

Table 3.1. Percentage yield of papaya peel extracts with different drying methods and solvents.

Percentage yield (%)			
Drying method	**solvent**		
	EtOAC	**Ethanol**	**Hexane**
Oven dried (50 °C)	0.3	24.3	0.3
Oven dried (80 °C)	0.7	6.2	1.7
Air dried	1.8	11.2	2.4
Freeze dried	0.1	1.5	0.3

Since the quality and the quantity of natural product that are extracted from papaya peels could be dependent on the processing and extraction conditions that are utilized, the yields and bioactivity data for differently obtained extracts were compared (Table 3.1). Highest percentage of yield (24.3%) was obtained for the ethanolic extract of oven-dried (50 °C) papaya peels. Out of the four different drying methods, the lowest percentage yields (less than 2%) were recorded for the freeze dried samples. Ethanolic extract of air-dried sample also showed a significant percentage yield of 11.2%.

3.2 Characterization of the crude plant extracts using photochemical analysis

3.2.1. Thin Layer Chromatography (TLC)

Table 3.2. R_f values of pigments on TLC

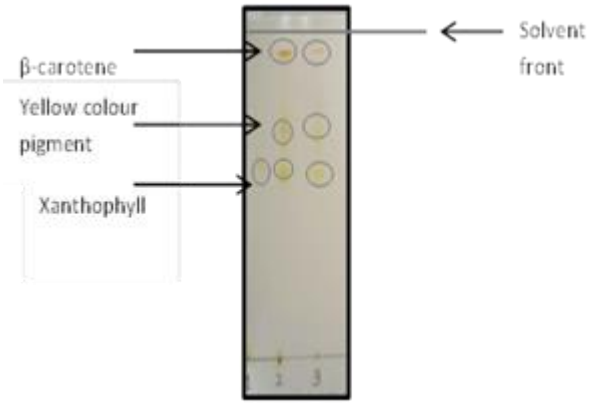

Figure 2.1- A sample thin layer chromatogram of papaya extracts (lane 1- ethanol extract, lane 2- EtOAc extract, lane 3- hexane extract)

Well separated band pattern, colours of bands and variation of R_f values of each extract were different from each other and it proved that different compositions and amounts of pigments in extracts have given rise to the distinctive colours observed. In addition, it was observed that drying method and solvent polarity clearly affects the extraction efficiency of different pigments. However, any of the extracts obtained from freeze drying did not give rise to any significant band pattern with colour spots suggesting that the amounts of pigments in the freeze dried samples was low.Band patterns and colour intensities were also different from solvent to solvent proving the doctrine of "like dissolves like" where polar pigments dissolve more in polar solvents and non-polar pigments prefer to dissolve in non-polar solvents more However, β- carotene (Rf = 0.94), xanthophyll (Rf = 0.50), and lycopene (Rf = 0.81) which might found in papaya peel having hydrocarbons prefer to dissolve in EtOAc and hexane where the yellowish orange colour bands were observed.

3.2.2. UV-Visible spectrometry

Highest value of 5.2 µg/mL was shown by ethyl acetate extract of air- dried sample. Ethanolic extract was found to contain 2.5 µg/mL of carotenoids (Table 3.3).

Table 3.3. Concentration of carotenoids in papaya peel extracts

Method of drying	Solvent	Concentration of carotenoids (µg/mL)			
		Oven dried (50 °C)	Oven dried (80 °C)	Air dried	Freeze dried
Papaya peel	EtoAC	0.2	1.3	5.4	0.2
	Ethanol	0.2	0.9	2.5	0.3
	Hexane	0.6	0.8	1.7	0.0

Band	R_f values obtained	Literature published R_f value [12]
β-carotene	0.94	0.94
Lycopene	-	0.81
Xanthophyll	0.43-0.63	0.50

The concentration of carotenoids of all the extracts was higher for the air-dried samples. The reason for the low carotenoid concentration in oven-dried (50 °C) could be because of the less extractability of carotenoids due to the breaking of protein-carotenoid complexes at low temperatures is significantly low. However, when oven dried at 80 °C method compared with higher concentrations of air-dried extracts, it can be assumed that probably the degradation of pigment prevails than the enhancement of extractability. Negligible amount of carotenoids in the extracts obtained from for the freeze dried samples again suggests the fact that freeze drying method is not a good drying method of extractions of carotenoids.

3.3. Investigation of selected biological activities of the extracts obtained from avocado and papaya waste materials

3.3.1. Antioxidant activity

Table 3.4. Antioxidant capacity (AOC) of papaya peel extracts

Solvent	Antioxidant Capacity-(AOC) (PGE µg/mg)			
	Oven dried (50 °C)	Oven dried (80 °C)	Air dried	Freeze dried
EtOAc	5 ± 2	3 ± 1	10 ± 1	2 ± 0
Ethanol	18 ± 6	5 ± 1	22 ± 2	12 ± 4
Hexane	5 ± 4	2 ± 0	3 ± 0	1 ± 0

Table 3.5. Percentage Radical Activity of papaya peel extracts

Highest antioxidant capacity of 22 PGE µg/mg in the Folin-Ciocalteu's assay as well as the highest DPPH radical scavenging activity of 96% was recorded for the ethanolic extract of the air-dried peels whereas the lowest AOC and DPPH radical scavenging activity was shown by the hexane extract of the freeze-dried sample (Table 3.4 and 3.5). Among the tested solvents, ethanol is the best solvent to extract natural antioxidants present in papaya peels compared to other solvents. Furthermore, air drying method

Solvent	Percentage Radical Scavenging Activity (RSA) (%)			
	Oven dried (50 °C)	Oven dried (80 °C)	Air dried	Freeze dried
EtOAc	26 ± 8	57 ± 24	92 ± 27	35 ± 10
Ethanol	96 ± 28	44 ± 13	96 ± 28	96 ± 28
Hexane	18 ± 6	25 ± 7	55 ± 16	18 ± 6

can be considered as the best drying method to retain the best percentage of antioxidant compounds compared to other methods. Therefore, air drying method was selected to prepare the necessary papaya extracts for further investigations since the initial parameters such as the carotenoid content, total AOC and radical scavenging activity were higher for the air dried samples compared to other three drying conditions and air drying could be easier and more economical approach that can be commercially effective during value addition process to these papaya peel.

3.3. Phytochemical analyses of the papaya extracts

Table 3.6. Phytochemical analysis of air-dried papaya extracts

Phytochemical test	solvent		
	Ethanol	EtOAc	Hexane
Tannins and phenols (a) FeCl3 test	†	–	–
(b) Lead acetate test	–	–	–
Saponins	†	–	–
Flavonoids	–	–	†
Terpenes and steroids	†	†	†
Alkaloids	–	–	–
Reducing sugars	†	†	–

(†) - positive result - presence of particular phytochemical compound and (–) - negative result - absence of the particular phytochemical compound)

The phytochemical analyses is considered effective in discovering the secondary metabolites present in plants which give rise to bioactive profile of plants. Several different phytochemical tests were carried out to investigate the presence and absence of corresponding bioactive compounds in air dried papaya peel. Ethanoilc extract gives positive results for saponins, tannins or phenols, terpenes or steroids and reducing sugar except for flavonoids and alkaloids. Due to the absence of the most of the phytochemical compounds tested, it can be considered that the differential extraction of plant metabolites depend on the solvent of choice.

3.3.2. Antibacterial activity

Only the ethanolic extract of air-dried papaya peels showed a small inhibition zone against *Pseudomonas aeruginosa*. Even though, the papaya peel extracts tested during this study did not result in significant antibacterial activities against the tested bacterial species, previous studies have reported the inhibitory activity of papaya peel ingredients against *Staphylococcus aureus* [13]. This difference might be due to differences in the extraction process or the regional difference of plant source.

3.4.3. Antidiabetic activity

Table 3.4. Percentage inhibition of α-amylase in papaya peel extracts

Solvent	Percentage inhibition of α- amylase (%)
Ethanol	32 ± 5
EtOAc	37 ± 6
Hexane	34 ±15

The antidiabetic activity of the air-dried papaya peel extracts was investigated using the α-amylase inhibitory assay. The highest percentage inhibition of α-amylase enzyme that corelates to the antidiabetic activity was obtained for the ethyl acetate extract of papaya peels (37%). (Table 3.7) However, the other papaya extracts also displayed comparable α-amylase inhibition. It has been reported that aqueous extract of papaya unripe fruit has antidiabetic activity[14]. But these moderate antidiabetic activity displayed by the papaya peel extracts could be an additional benefit if these extracts are utilized in food supplements.

3.5. Formulation of a fish feed based on the ethanolic extract of air-dried papaya peels to enhance the skin colour and healthiness of guppies

Figure 3.1-Three guppy groups used for the visual sensory evaluation ((a) - Fish group fed with feed 1 (feed with no pigment), (b) - Fish group fed with feed 2 (feed with 2% of extract), (c) - Fish group fed with feed 3(feed with 4% of extract)

Table 3.5. Results of the visual sensory evaluation of the fish fed with different fish feed

The ethanolic air dried papaya peel extract with the higher amount of pigment (2.5 µg/mL) was selected to prepare the fish feed as it showed both high antioxidant (AOC - 22 PGE µg/m and RSA of 96%) and antibacterial activity.

According to the results indicated in Table 3.8, after each fish group was fed with differently formulated feeds for 10 days, it was evident that the feed enriched with the ethanolic papaya peel extract was capable of significantly enhancing
the pigmentation and skin colour of guppies. A remarkable percentage of evaluators (99%) agreed that the fish fed with a feed containing either 2% or 4% papaya extract displayed a higher visible enhancement of skin colour than the fish group fed with a feed lacking the papaya extracts (Figure 3.1). These results also suggested that the extent of pigment enhancement clearly depends on the concentration of carotenoids containing extract that was added to their feed.

During the feeding period, a 13% mortality was observed with the fish group fed with the feed lacking any pigment source, whereas, no mortality was observed in the fish groups that were fed with a feed containing the natural papaya pigment extract. This indicates that the incorporation of carotenoid rich papaya peel extract could additionally enhance the healthiness of fish. This could be due to the presence of antioxidant and antibacterial agents in these natural extracts as observed during our tests for different classes of natural products. Formulation of a fish feed using the papaya peel extracts as stated above could be beneficial towards enhancing the skin colour of fish as well as their healthiness.
As previously explained due to the deteriorating effect and higher cost of synthetic pigments, natural colouring agents are being promoted for the formulation of fish feed.[15] Therefore, the fish feed formulated with papaya peel waste could be a great solution for aquaculture as well as for the waste management process of papaya.

Table 3.6. The visual sensory evaluation results for the fish fed with commercial and papaya extract

Statement on the intensity of skin colour of guppies	Percentage of evaluators in agreement (%)
Fish fed with feed 1 (feed with no pigment) has the most intense skin colour	3
Fish fed with feed 2 (feed with 4% of the pigment) has the most intense skin colour	94
Fish fed with feed 3 (commercial fish feed) has the most intense skin colour	3

The newly formulated fish feed based on papaya peel extract was further compared to a commonly available commercial fish feed in Sri Lankan aquaculture market in order to understand its applicability and compatibility in the current competent market. Results of this study are indicated in Table 3.9.

statement about the intensity of skin colour of guppies	Percentage of evaluators in agreement (%)	Mortality percentage (%)
Fish fed with feed 1 (feed with no pigment)has the most intense skin colour	1	13
Fish fed with feed 2 (feed with 2% of the pigment) has the most intense skin colour	9	0
Fish fed with feed 3 (feed with 4% of the pigment) has the most intense skin colour	90	0

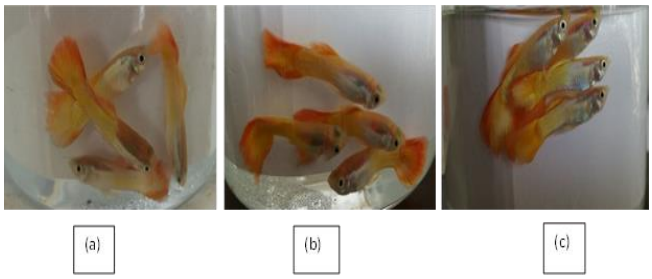

Figure 3.2 - Three guppy groups used for the evaluation assay ((a) - Fish group fed with feed 1(feed with no pigment), (b) - Fish group fed with feed 2 (feed with 4% of extract), Fish group fed with feed 3 (commercial fish feed))

A significant percentage (94%) of the evaluators agreed that there was a clear improvement in the skin colour of the fish group fed with feed 1 that was prepared using the pigment rich papaya peel extract (Figure 5). Only 3% of the evaluators ranked that the fish fed with the commercial feed had the most intense skin colour. This result indicates the effectiveness and the reliability of the of the newly developed papaya peel extract based fish feed towards colour enhancement of ornamental fish in a cost effective manner.

Conclusion

Different drying methods were compared to identify the best method which removes the highest water content with maximum retention of important bioactive components intact. Dried plant materials are easy handle due to prolong storage capability. The chance of bacterial and fungal effect on dried plant parts will be less compared to fresh plant parts and also the removal of excess water concentrates the metabolites inside the cell. Since the extraction was carried out using organic solvents, presence of water can create an immiscibility lowering the extraction efficiency.Air drying methods would be commercially effective and at same time the bioactivity of air-dried extracts were relatively high. Formulation of a low cost fish feed using these important biological activities of papaya peel extracts was succeed to enhance the skin colour and the healthiness of guppies.
Pigmentation is one of the important quality attributes of the fish for consumer acceptability. The proper formulation of

food that can ensure the pigmentation and the healthiness of fish is one of the significant requirements in the aquarium fish market. Today, fish feeds are formulated using different types of pigments such as carotenes (synthetic β- carotene), animal based natural carotenoids (shrimp meal, shrimp oil) and plant based natural carotenoids (marigold, sea weed) [15][16]. Due to the chemical residues that remain with synthetic pigments and higher cost associated with fish feed prepared using synthetic carotenoids, use of natural extracts that are rich in carotenoids, antioxidants and antibacterial compounds would be more desirable towards formulation of a fish feed with promising benefits.

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