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# Antimicrobial Activity of Astaxanthin Pigment Extracted from Shrimp Waste against Microorganisms and to Study the Effect of Gamma Radiation on The Production Yield of Astaxanthin

**Rajesh C. Patil[1], Shreya T. Raut[2], Vijay N. Waghmare[3] and Vijay N.Pawar4**
[1],[2]Department of Microbiology, M. M. College of Arts, N. M. Institute of Science, H.R.J. College of Commerce (Bhavan's College), Andheri (West), Mumbai-400058.
[3]Department of Biotechnology, M. M. College of Arts, N. M. Institute of Science, H.R.J. College of Commerce (Bhavan's College), Andheri (West), Mumbai-400058.
4,Associate Professor, Siddharth College of Arts, Science and Commerce, Fort, Mumbai.
email- rajesh.patil@bhavans.ac.in

**ABSTRACT**
*The present study is undertaken to determine the antimicrobial activity of shrimp shell wastein Mumbai, Maharashtra, India. Total two sets of samples were taken from Andheri market each having dried shrimp and tiger shrimp shell waste. The shrimp shells contain astaxanthin pigment which is antioxidant, antimicrobial and anticancer in nature. The extraction of the pigment is carried out by solvent extraction method using hexane as a solvent. The samples were also exposed to gamma irradiation which increased the yield of the astaxanthin pigment. The pigment extracted was effective against Gram negative bacteria than Gram positive bacteria and yeast. Salmonella typhi was found to be more susceptible to the extracted astaxanthin pigment. The pigment was effective at 0.08µg/ml concentration inhibiting both Gram negative as well as Gram positive microorganisms.*
***KEY WORDS:*** *Astaxanthin, gamma-radiation, MIC, Gray.*

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## INTRODUCTION

The control of infectious diseases is severely threatened by the steady increase in number of microorganisms that are resistant to the antimicrobial agents. Initially, microorganisms resistant to multiple drugs were found mostly in hospitals, where antimicrobial agents are used most extensively, but resistance is currently found almost as frequently in the community. Community acquired infections-particularly respiratory and Gastro-intestinal infections, the leading causes of death in USA before the advent of antibiotics continue to be the leading cause of death in the developing countries. Diarrheal disease, skin infections, fungal disease are most important causes of illness and death in young and elder population in the developing countries [1-3]. There are few studies carried out to determine antimicrobial effect of natural pigment on pathogenic microorganisms. Since, shrimp shell waste contains a natural antimicrobial agent,its study against pathogenic microorganisms is important. Also, the results obtained can help us to compare which pathogen is more sensitive or resistant to the natural astaxanthin pigment.

Astaxanthin ($C_{40}H_{52}O_4$), is a keto-carotenoid having molecular mass 596.84 g/mol. Astaxanthin was first described in aquatic crustaceans as an oxidized form of β-carotene, which gives the carapace of these animals its pinkish colour. It was later found that this pigment is very common in certain species of fish and birds, in which it plays a role in colouration during the mating season [4].

In 2000, India produced 2.10 million mt through aquaculture, 72% more than in 1990. This boom in aquaculture has caused several problems [5]. Shrimp farming is highly profitable and brings considerable socio-economic benefits to the communities, environmental and social conflicts in shrimp farming in India. In India, total marine shrimp production is 1.087 million mt, freshwaters crustaceans is 0.386 million mt, total crustaceans production is 1.647 million mt. The highest ranking state for production of shrimp in India is Andhra Pradesh followed by West Bengal, Orissa, Maharashtra, Kerala, Karnataka, Tamil Nadu and Gujarat. [6].

***Source of Astaxanthin:***

Only a few microorganisms produce natural astaxanthin, including non-photosynthetic bacteria, *Brevibacterium, Mycobacterium lacticola, Agrobacterium auratim, green microalgae, Haematococcus pluvialis* and the red yeast *P.rhodozyma*.[7].

***Gamma radiation and its applications:***

Study of effect of gamma- irradiation on production of astaxanthin is already done on microorganism producing astaxanthin such as *Haematococcus pluvialis*, *Phaffiarhodozym* [8]. *Xanthophyllomyces dendrorhous* which are main microorganisms for production of astaxanthin on industrial scale. But there is no such research done to study the effect of gamma-irradiation on astaxanthin production which is produced from shrimp shell waste. Shrimp shell waste not only can prove to be a good source of astaxanthin but, using shrimp shell waste also has its another significant importance in waste management. Thus, production of best from waste which can be helpful for human mankind can be achieved through this study.

A study was carried out to evaluate the application of food irradiation technology as a method for reducing food allergy. Milk beta-lactoglobulin, chicken egg albumin, and shrimp tropomyosin were used as model food allergens for experiments on allergenic and molecular properties by gamma irradiation. The quantity of intact allergens in an irradiated solution was reduced by gamma irradiation depending upon the dose. These results showed that epitopes on the allergens were structurally altered by radiation treatment and that the irradiation technology can be applied to reduce allergenicity of allergic foods (6).

**MATERIAL AND METHOD**

***Methods of extraction of astaxanthin*:**

Astaxanthin extraction from crustacean wastes would imply in large quantities of this by-product. Various alternative methods have been suggested to solve this problem, such as silage, which consist of treating crustaceans' wastes organic and inorganic acid, and astaxanthin extraction with vegetable or fish oils, which can be directly incorporated into the feeds [9]. Astaxanthin is a lipophilic compound and can be dissolved in solvents and oils. Solvents, acids, edible oils, microwave assisted and enzymatic methods are used for astaxanthin extraction [10]. H.pluvialis mutated with 4000 Gray gamma-ray irradiation showed increased in the amount of astaxanthin by 15% [11].

Extraction of astaxanthin from shrimp shell waste was achieved by modifying the method published by L.Senthamil, 2015 [12], which was a modified version of recently published methodology for extraction of astaxanthin. We used only hexane solvent instead of hexane and acetone mixture for extraction of astaxanthin introduced by [13].

Some pathogenic Gram positive, Gram negative microorganisms like *Staphylococcus aureus, Bacillus cereus, Escherichia coli, Salmonella typhi, Pseudomonas aeruginosa and fungi like Aspergillusniger, Candida albicans*were used as target microorganisms to evaluate antimicrobial potential of extracted astaxanthin pigment against them. All these microorganisms were taken from Bhavan's Microbiology Department.

***Sample collection:***

Sample of waste tiger shrimp and dried shrimp was collected from local Andheri market in an ice box in 1kg quantity each and was transported to Bhavans college, Andheri (west). Further, it was stored in refrigerator at -4°C.

***Sample processing:***

Both the samples were weighed 25g and washed under running tap water and was kept for drying in hot air oven for 24-48 hours until there was no moisture present. Both the samples, 5 mg each were irradiated to dose of Gamma irradiation from 1 to 3Gray from Bhabha Atomic Research Centre (BARC, Mumbai). One set of each sample was kept for control activity.

**Figure 1. Dried shrimp shell waste and Tiger shrimp shell waste in petri-plate for drying.**

***Extraction of Astaxanthin:***
The dried sample was grinded in a motor pestle with 50ml hexane and solvent. 5mg glass beads were placed in the solvent mixture and was vortexed for 30 seconds. The vortexed mixture was heated in boiling water bath at 50°C for 10 minutes. The organic layer was separated by centrifugation at 3000 rpm for 5 minutes. This step was repeated until hexane turned colourless. 6 mL DMSO ( Di-methyl sulfoxide) was added and mixture was again vortexed. This mixture was placed in boiling water bath for 10 minutes and again vortexed. By this process, crude astaxanthin solution was extracted. The extracted solution with highest dose treatment of gamma radiation i.e. 3Gray was subjected to GCMS. Unexposed sample to gamma radiation was set as control.

***Quantitative analysis of Astaxanthin by UV-Spectrometry:*** The analysis was performed in Bhavans Research Centre (BRC). The total extracted solution containing astaxanthin was subjected to UV-Spectrometer and was subjected for absorbance at 468nm and the total carotenoidcontent (astaxanthin) was calculated as µg astaxanthin per gram sample.

Formula for quantifying Astaxanthin (µg/g sample) = $A_{468\ nm} \times V_{extract} \times df / 0.2 \times W_{sample}$

Where, $A_{468\ nm}$ = Absorbance used

$V_{extract}$ = Volume of extract

0.2 = $A_{468nm}$ value of 1µg/ml standard astaxanthin

$W_{sample}$ = Weight of the sample.

**RESULTS**


**Figure 2. Antimicrobial activity by Agar-well diffusion method against: *S.aureus***


**Figure3. Antimicrobial activity by Agar-well diffusion method against:*B.cerus***

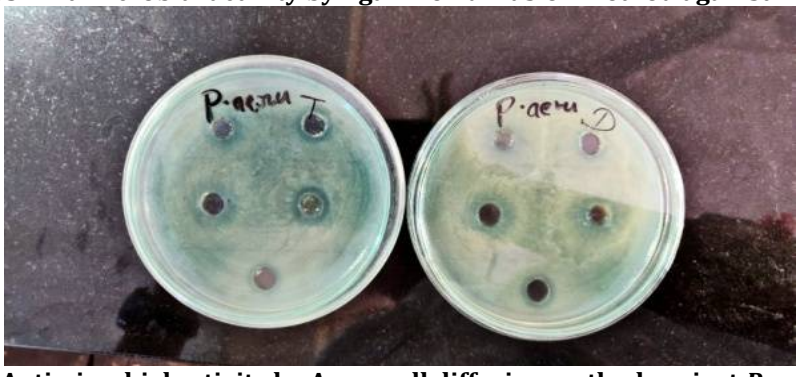



**Figure 4. Antimicrobial activity by Agar-well diffusion method against:*P.aeruginosa***

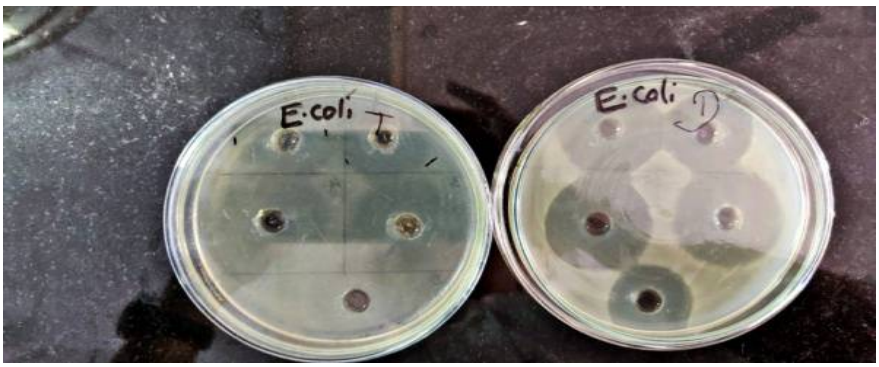



**Figure 5. Antimicrobial activity by Agar-well diffusion method against:*E.coli***


**Figure 6. Antimicrobial activity by Agar-well diffusion method against:***C.albicans*

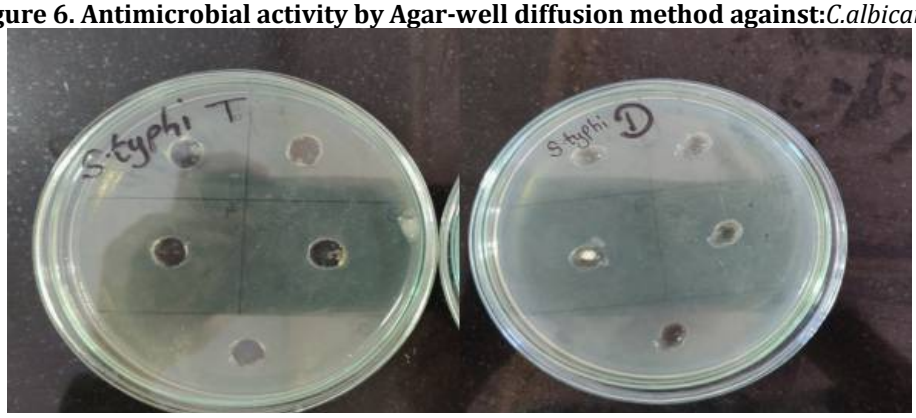
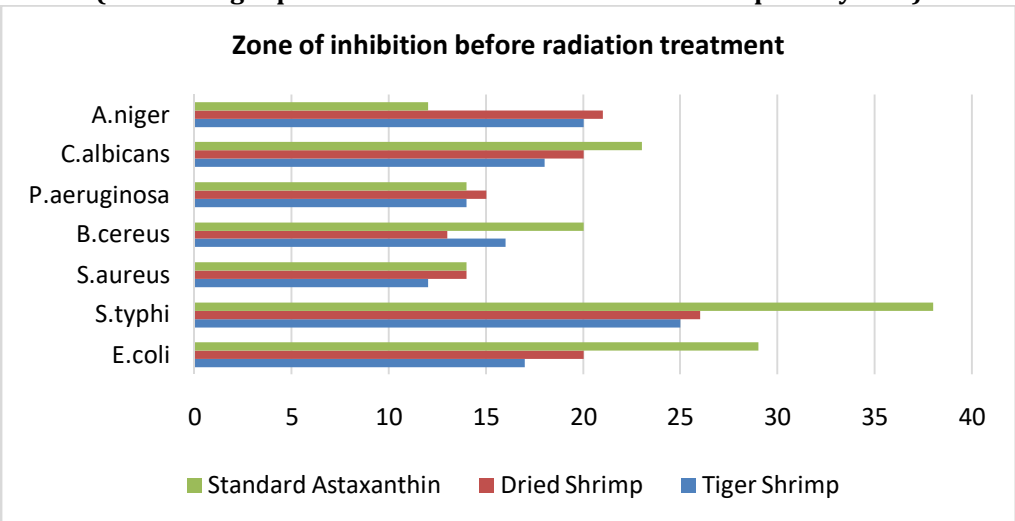


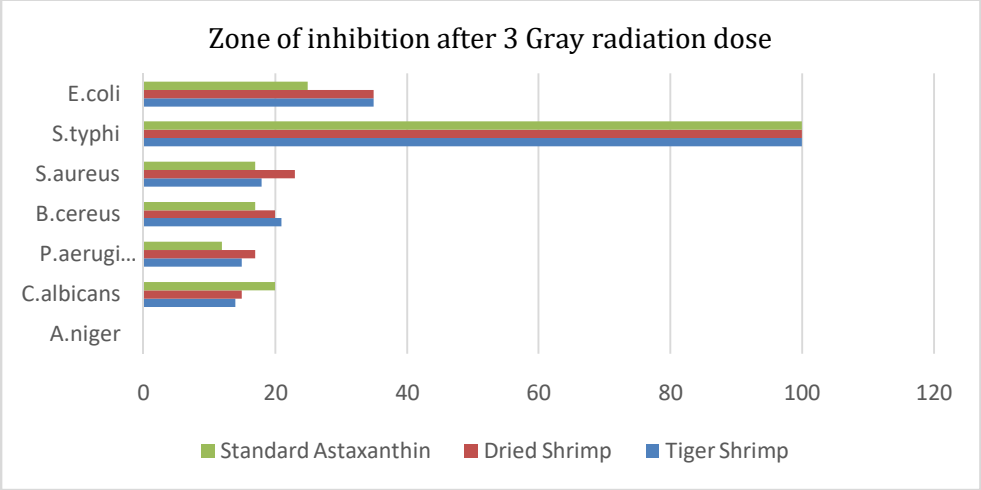

**Figure 7.Antimicrobial activity by Agar-well diffusion method against:***S.typhi*

**[4. .***S.aureus***, 5.** *B.cereus***, 6.** *P.aeruginosa***, 7.** *E.coli***, 8.** *C.albicans***, 9***. S.typhi***.]**

**(Nutrient agar plates were used for Astaxanthin Susceptibility Test)**




Graph 1. Zone of inhibition before radiation treatment




Graph 2. Zone of inhibition after 3 Gray radiation dose.

**Table1. Amount of astaxanthin after exposure to 3 Gray gamma radiation by UV-Spectrometry method:**

| Sample | Non-radiated | | Radiated | |
|---|---|---|---|---|
| Absorbance | 470 nm | 480 nm | 470 nm | 480 nm |
| Dried Shrimp | 0.85 | 0.739 | 0.22 | 0.157 |
| Tiger Shrimp | 0.041 | 0.043 | 0.204 | 0.206 |
| Standard | 1.421 | 1.393 | 0.017 | 0.017 |

Tiger Shrimp: AST(ug/ml) = 0.204 x 50 x 106 ÷ 5 x 1 x 2100 = 0.5148 ug/5g
Dried Shrimp: AST(ug/ml) = 0.220 x 50 x 106 ÷ 5 x 1 x 2100 = 0.5552 ug/5g

**Table 2. Determination of Minimum Inhibitory Concentration (MIC)**

| Stock Concentration (ug/ml) | Absorbance (470nm) | |
|---|---|---|
| | **S.aureus** | **P.aeruginosa** |
| 0.02 | 0.291 | 0.385 |
| 0.04 | 0.37 | 0.414 |
| 0.06 | 0.277 | 0.453 |
| 0.08 | 0.254 | 0.242 |
| 0.1 | 0.213 | 0.278 |
| Media Control | 0.135 | 0.132 |
| Negative Control | 1.19 | 0.146 |
| Positive Control | 0.349 | 0.356 |

Hexane solvent control did not have any zone of inhibition indicating validation of the solvent used in extraction of astaxanthin. The antimicrobial activity of astaxanthin extract from dried and tiger shrimp waste samples were tested against the seven different microorganisms including Gram negative and Gram-positive bacteria, yeast, fungi. The Standard astaxanthin solution (4 mg capsule), Standard amoxycillin antibiotic and solvent control (hexane) was also assayed against every microorganism for better comparison and references and also for evaluating better effect of the extracted product under study.

As dried shrimp sample is expected to contain more product in concentrated form than the tiger shrimp sample, it will exhibit more antimicrobial activity than the tiger shrimp sample extract would exhibit the same. The extract obtained from dried shrimp sample showed comparatively more zone of inhibition measured in mm against every microorganism, except for *B.cereus* , which was less susceptible to the dried sample extract as compared to the tiger shrimp sample extract.

The highest zone of inhibition was found in *S.typhi* w.r.t. both the samples and the standards. So, S.typhi is found to be the most susceptible organism for the extracted astaxanthin pigment. *Candida albicans* was least susceptible to the standard antibiotic. Butits susceptibility increased with astaxanthin extracted. *Pseudomonas aeruginosa* also showed comparatively less susceptibility against amoxycillin, but, increased the susceptibility when exposed to astaxanthin [14-17].

In all organisms, dried shrimp is more effective in inhibiting microorganisms as compared to the tiger shrimp extract as expected, except, *B.cereus* which showed susceptibility more to the tiger shrimp extract than to dried shrimp sample.

The zone of inhibition increased as the dosage increased. This indicates that the amount of product yield increased when the samples were exposed to gamma irradiation. Samples irradiated with 3Gray dosage showed maximum number of zone of inhibition which is directly proportional to the antimicrobial activity [18].

The zone of inhibition for *S.typhi* could not be measured as it showed inhibition throughout the plate indicating highest susceptibility. Whereas, *A.niger* failed to show proper growth. *C.albicans* was least susceptible to the extract when it was unexposed to gamma-irradiation, but, when it was exposed to gamma rays with 2Gray dosage for tiger shrimp sample extract, it showed susceptibility [19].

The control sample of tiger shrimp extract which was left unexposed to gamma rays showed to inhibit *S.typhi* followed by *A.niger, C.albicans, E.coli, B.cereus, P.aeruginosa* and *S.aureus*. 3kGy gamma rays irradiated sample also showed highest inhibition for *S.typhi* followed by *E.coli, B.cereus, S.aureus , P.aeruginosa* and *C.albicans*. Thus, the zone of inhibition for *E.coli , B.cereus* and *S.aureus* increased when the sample was exposed to gamma radiation, indicating that the concentration of astaxanthin increased after exposure to gamma radiation. The standard astaxanthin purchased from HealthVit also showed maximum inhibition against *S.typhi ,E.coli, C.albicans, B.cereus, P.aeruginosa, S.aureus* in descending order of zone of inhibition [20-22].

The standard antibiotic amoxycillin also showed highest zone of inhibition for *S.typhi* followed by *E.coli, C.albicans, B.cereus, P.aeruginosa, S.aureus*. It is also observed that the standard astaxanthin used showed similar antimicrobial effect as compared to the antibiotic amoxicillin used in this study. Also, when sample was exposed to gamma rays showed to be more effective in exhibiting antimicrobial activity as compared to the non-exposed sample. It is also observed that astaxanthin is more effective against Gram negative bacteria than Gram negative bacteria as Gram negative lack outer cell membrane and therefore astaxanthin easily penetrates interior of the cells of Gram-negative bacteria and kill them.

Both the extracts showed maximum antimicrobial activity against *S.typhi* followed by *E.coli, S.aureus, B.cereus, P.aeruginosa and C.albicans*. It can also be interpreted that dried shrimp showed more antimicrobial effect than tiger shrimp extract against every target organism used.

The amount of astaxanthin present was quantified using Double beam UV-Spectrophotometry for both dried shrimp sample and tiger shrimp sample extract at 470 nm as well as 480 nm where readings at 470nm showed maximum absorbance. The quantity of the product extracted which showed maximum antimicrobial activity with 3Gray gamma-irradiation dosage was calculated using the above mentioned formula [23]. Tiger shrimp extract showed 0.5148 ug/5g astaxanthin whereas, Dried shrimp extract showed 0.5552 ug/5g astaxanthin present in the sample. Quantitative analysis is performed by making use of the fact that certain chromophores absorb at specific wavelength although, corrections are usually necessary to account for interfering substances. Such corrections, commonly require measuring the absorbance, by the interfering substance, at a wavelength remote from that for the compound under test, plus the knowledge of the absorbance at the test wavelength. If the ratio of the absorbances of the interfering substance is known for the remote and test wavelength, then the correction is simple [24].

Minimum Inhibitory Concentration (MIC) of the extracted astaxanthin obtained by exposure of 3Gray Gamma irradiation from dried shrimp (0.5552 ug/5g) showing highest antimicrobial activity was tested against a Gram positive and a Gram negative organism i.e., *S.aureus* and*P.aeruginosa.*It is observed that the absorbance increases till 0.06 µg/ml concentration of astaxanthin extracted from dried shrimp sample which is exposed to 3KGy dose of gamma rays. The absorbance decreases at 0.08 µg/ml concentration, meaning, that astaxanthin extracted is effective in inhibiting both Gram positive *S.aureus* and Gram negative *P.aeruginosa* at 0.08 µg/ml minimum concentration. Media control showed least absorbance value followed by negative control. In fact, negative control must show 0.0 absorbance but, the absorbance was observed greater than 0.0 as the 96- well plate used was not available sterile and therefore, biofilm must have occurred on the plate prior to the addition of the test sample.

## CONCLUSION

Astaxanthin from shrimp shell waste can be efficiently extracted by solvent extraction method using hexane as a solvent system. The astaxanthin extracted from shrimp shell waste either from dried sample or tiger shrimp sample is efficiently antimicrobial against microorganisms where, *S.typhi*is highly susceptible to astaxanthin and *C.albicans* is least susceptible. Thus, this product can be used to treat various types of bacterial infections especially typhoid without any side effect as it is extracted from a natural source. When irradiated with gamma rays the production yield of the astaxanthin pigment increased linearly as the dosage increased. It can thus be concluded that extraction of astaxanthin from shrimp shell waste is efficient, simple, economic and less time consuming and beneficial study for human mankind as it also helps in management of waste produced from hotels, industries etc.

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