OPEN ACCESS Freely available online

Medicinal & Aromatic Plants

Research Article

Accelerated Shelf Life Study of Fish Oil Stored in Medicinal Plant Extracts

Jithu Paul Jacob*[1] and Saleena Mathew[2]

[1]*St. Alberts College (Autonomous) Ernakulam, Kochi, Kerala, India*; [2]*School of Industrial Fisheries (CUSAT), Kerala, India*

ABSTRACT

Stability studies provide evidence on in what way the quality of a drug or its product varies with time under influence of changing environmental factors such as humidity, temperature and light. Present study is an attempt to study accelerated stability of fish oil incorporated with three medicinal plant extracts, which can be used for preserving it from further oxidation. The results of accelerated shelf life studies of fish oil treated with *O. sanctum* extracts (2 mg %). It is found that *E.coli*, *Salmonella* and *P. aeruginosa* were absent throughout the storage period of 6 months in the treated sample. Though TPC was found high at initial stage (1250000 cfu/gm.), by 6 months the value was reduced to <05 cfu/gm. Fungus and yeast contents with a high value during 2nd month (1000 cfu/gm) decreased to a value of 110 cfu/gm in 6 months. Rancidity index showed absent in all the days. Contrary to *O. sanctum* treated fish oil, the total fungus and yeast content which was minimum at the initial period (<10 cfu/mL) increased to a maximum value of 2110 cfu/gm in *A. barbadensis* and *B. diffusa* treated samples. Rancidty was absent throughout the storage period in both the treatments. Thus total fungus and yeast content was significantly lowered in *O. sanctum* treated fish oil when compared with *A. barbadensis* and *B. diffusa* treated fish oil.

Keywords: Lipids; Accelerated shelf life; Medicinal Plant Extracts; Fish oil

INTRODUCTION

India is considered as a gold mine of traditional medicinal plants with an established record and people have a good knowledge of its use. The challenge is concerning their formulations due to its complex nature and the absence of its complete constituent evaluations. For the endurance of its quality, purity and stability a complete evaluation of the constituents is required. It also establishes a retest period for the particular drug substance or its product for the recommended storage conditions. Thus, it is proved that the stability study is unavoidable for the assessment of product quality.

In general, pharmaceutical products are studied for its stability profile in an accelerated humidity and temperature, and these investigational findings can be very helpful for predicting reliable expiry date or shelf-life at room temperature by assuming certain criteria and assumptions [1]. Each and every product has a definite shelf-life and it depends on different physical, chemical, biological and environmental factors. Real time study is a time consuming and a long procedure. Therefore, it is difficult for the manufacturer to wait till the drug degrades naturally to about 90% of labeled amount at room temperature. Taking this into account the stability study is normally supported for assigning shelf-life of any drugs. A series of guidelines which are acceptable to multiple countries for the approval of a drug have been established and known as Quality Guidelines or ICH (International Council for Harmonization of technical requirements for pharmaceutical for Human use) guidelines. By using this method we can predict the shelf life of any drug product in a very short period of time.

In contrast to conventional preparations of medicinal plants, products incorporated with their extracts lead to a number of unique problems with regard to quality and stability. So as to ensure good reproducibility, adequate control is essential. A key part of quality control is to guarantee the chemical stability of the final product during its storage. Present study is an attempt to study accelerated stability of fish oil incorporated with three medicinal plant extracts, which can be used for preserving it from further oxidation.

***Corresponding author:** Jithu Paul Jacob, St. Alberts College (Autonomous) Ernakulam, Kochi, Kerala, India, Tel: 083308 87774; E-mail: jithupaul007@gmail.com

Received: June 13, 2019; **Accepted:** June 21, 2019; **Published:** June 29, 2019

Citation: Jacob JP, Mathew S (2019) Accelerated Shelf Life Study of Fish Oil Stored in Medicinal Plant Extracts. Med Aromat Plants (Los Angeles) 8:334. doi: 10.35248/2167-0412.19.8.334

MATERIALS AND METHODS

Accelerated shelf life study of fish oil

The accelerated stability study of fish oil incorporated with medicinal plant extracts was done at accelerated humidity and temperature conditions, viz. accelerated stability study taking ICH (International Council for Harmonization of Technical requirements for pharmaceutical for Human use) guidelines as reference. Fish oil was treated with 70% ethanolic extract of IMP at an optimum concentration of 2 mg% of O. sanctum extracts and 5 mg% of B. diffuse and 5 mg% of A. barbadensis. All samples were prepared in dark bottles and stored under nitrogen by passing the gas into it. The samples were kept at 400 ± 20°C and 70 ± 5% humidity in a humidity chamber, and at 37 ± 20°C and normal humidity. The samples were withdrawn after one, two, three and six months in triplicate for analysis.

Detection of E. coli (API Part II, 2008)

1 mL of the fish oil sample was homogenized with 1-2 g of polysorbate 80R and heated up to 40°C in a water bath. Added 50 mL nutrient broth into it and maintained the same temperature till emulsion formed within 30 minutes. From this took 1 gm. sample, added 50 mL nutrient broth which was kept in a sterile screw-capped container, shaken well. Allowed it to stand for 1 hr. and after shaking, loosened the cap and incubated at 37°C for 18-24 hrs.to get the enrichment culture.

By means of an inoculating loop, streaked a portion from the enrichment culture on the surface of MacConkey agar medium. Covered and inverted the dishes and incubated. Upon examination, if none of the colonies are brick-red in colour and have a surrounding zone of precipitated bile, the sample meets the requirements of the test for the absence of Escherichia coli.

If the colonies described above are found, transferred the suspect colonies individually to the surface of Levine eosin-methylene blue agar medium, plated on Petri dishes. Covered and inverted the plates and incubated. Upon examination, if none of the colonies exhibits both a characteristic metallic sheen under reflected light and a blue-black appearance under transmitted light, the sample meets the requirements of the test for the absence of Escherichia coli. The presence of Escherichia coli was confirmed by further suitable cultural and biochemical tests.

Detection of pseudomonas aeruginosa (API Part II, 2008)

1 mL of the fish oil sample was homogenized with 1-2 g of Polysorbate 80R. Then heated up to 40°C in a water bath and mixed carefully. Inoculated homogenized sample in to 100 mL Soybean casein digest medium. Mixed thoroughly and incubated at 35-37°C for 24-48 hours. Examined the medium for growth and if growth is present, streaked a portion of the medium on the surface of Cetrimide agar medium each plated on Petri dishes. Covered and incubated at 35-37°C for 18-24 hours. If growth of colonies on Cetrimide agar plate are gram negative rods revealed by gram staining, usually with a greenish fluorescence occurs, then performed oxidase and pigment test.

Streaked representative of the suspected colonies from the agar surface of Cetrimide agar to the surfaces of Pseudomonas agar medium (F) for detection of fluorescein and Pseudomonas agar medium (P) for detection of pyocyanin.

Incubated for not less than 3 days at 33-37°C. Examined the plates under UV (365 nm) for yellow and blue fluorescence respectively. If growth of suspect colonies occurs, a pinch of colony is transferred to the filter paper and placed 2-3 drops of 1% w/v solution of N,N,N',N'- tetramethyl -4 phenylene diamine dihydrochloride. If there is no development of a pink color, changing to purple within 5-10 seconds, the sample meets the requirements of the test for the absence of *Pseudomonas aeruginosa*.

Interpretation: If there is purple color formation within 10 seconds, after adding 1% oxidase reagent on a filter paper, it is confirmed as *Pseudomonas aeruginosa* and depending upon the pigment color produced on *Pseudomonas* agar F and P medium, it is reported as *P. flourescens* and *P.pyocyanin*.

Limit of detection: *Pseudomonas aeruginosa*=Presence/Absence/gm/mL

Detection of *staphylococcus aureus* (API Part II, 2008)

1 mL of the fish oil sample was homogenized with 1-2 g of Polysorbate 80R. Then heated up to 40°C in a water bath and mixed carefully. Inoculated the homogenized sample in 100 mL of Soybean casein digest medium. Mixed thoroughly and incubated at 35-37°C for 48hours. Prepared a subculture on Baird Parker agar, and incubated at 35-37°C for 24-48 hours.

Black, shiny, surrounded by clear zones of 2 to 5 mm colonies of gram positive cocci on BP agar plate and gram positive *cocci* in clusters revealed by gram staining, indicate the presence of *Staphylococcus aureus*.

Confirmed if bacteria is present by tests such as coagulase test by using rabbit plasma. Coagulase test is carried out by transferring suspected colonies from BP agar medium to 0.5 mL of rabbit plasma with or without additives. Incubated it in water bath at 37°C examined the tubes at every 3 hours and subsequently at suitable intervals up to 24 hours. Negative coagulation test confirms the absence of *Staphylococcus aureus*.

Limit of detection: *Staphylococcus aureus*=Presence/Absence/g/mL

Detection of *Salmonella spp.* (API Part II, 2008)

1 mL of the fish oil sample was homogenized with 1-2 g of Polysorbate 80R. Then heat up to 40°C in water bath and mixed carefully. Added 100 mL of Nutrient broth and heated up to 40°C. This temperature was maintained for the shortest time until the formation of an emulsion within 30 minutes. Shaken and allowed to stand for 4 hrs. and shaken again.

Loosened the cap and incubated at 35°C to 37°C for 24 hrs.

Primary test: Added 1 mL of the enrichment culture to a mixture of 10 mL Selenite F broth and 10 mL Tetrathionate bile brilliant green broth. Incubated the preparation at 36°C-38°C for 48 hrs. Subcultured to Bismuth sulphite agar and Xylose

Lysine Deoxycholate agar. Incubated at 36°C to 38°C for 18-24 hrs.

If no growth of colonies on the agar plates, *Salmonella* spp. is absent. If growth is present on BSA with black or green colonies and Red colonies with or without black centers on XLD, done the secondary test.

Secondary test: Sub cultured from the agar plates to TSI by first inoculating the surface of the slope and then making a stab culture with the same inoculating needle, and at the same time inoculated a tube of urea broth. This was incubated at 36°C to 38°C for 18-24 hrs. The formation of acid and gas in the stab culture (with or without concomitant blackening) and the absence of acidity from the surface growth in the triple sugar iron agar, together with the absence of a red color in the urea broth, indicated the presence of *Salmonella* spp. If acid but no gas is produced in the stab culture, the identity of the organisms should be confirmed by agglutination tests.

Agglutination test procedure: From the test culture on nonselective media, transferred a loopful of growth to a drop of sterile 0.85% saline on clean slide and emulsified the organism. Rotated the slide for 1 minute and then observed for agglutination. If agglutination (auto agglutination) occurs, the culture is rough and cannot be tested. Subcultured to non-selective agar, incubated and tested the organism again as described in step 1 and 2. If no agglutination occurs, it was proceeded with testing. Carried out the control test in parallel by repeating the primary and secondary tests using 1 mL of the enrichment culture and a volume of broth containing 10-50 *Salmonella* abony (NCIM 2257) organisms prepared from a 24 hr culture nutrient broth, for the inoculation of the tubes (a) and (b). The test is not valid unless the results indicate that the control contains *Salmonella*.

Limit of detection: *Salmonella* spp=Present/Absent/gm./mL

Method of analysis for total yeast and mold count (API Part II, 2008)

10 mL of the fish oil sample was homogenized with 5 g of Polysorbate 80R. Then heated it not more than 400°C in a water bath and mixed carefully. Added 85 mL of buffered sodium chloride peptone solution (pH-7) and heated up to 400°C. Maintain this temperature for the shortest time till the formation of an emulsion within 30 minutes. Adjusted the pH to 7. From the pretreated suspension, serial dilution was carried out till 10-5 dilution. Poured 1 mL each of the homogenized samples to labeled petri plates taken in duplicate. 15-20 mL of Sabouraud dextrose agar was added with Chloramphenicol cooled to 45°C, into petri dishes. Immediately mixed the sample homogenate/dilutions and agar thoroughly by alternate rotation and rocking to and fro of the plates on a level surface. Allowed the agar to solidify. The solidified agar plates were incubated in the dark at 20-25°C for 3-5 days. A negative control was carried out by using sterile distilled water in the place of sample homogenate.

Counting of plates: Reported results in colony forming units based on average count of duplicate set, if no growth of colonies on all plates is less than 1 for the corresponding lowest dilution used.

Limit of detection: TPC=>1 CFU /mL

Permissible Limit: >10 CFU/gm.

Method of analysis for total aerobic plate count (API Part II, 2008)

10 mL of the fish oil sample was homogenized with 5 g of polysorbate 80R. Then heated it not more than 40°C in water bath and mixed carefully. Added 85 mL of buffered sodium chloride peptone solution (pH-7) and heated up to 40°C.This temperature was maintained for the shortest time until the formation of an emulsion within 30 minutes. Adjusted the pH to 7. From the pretreated suspension, serial dilution was carried out till 10-5 dilution. Dispensed 1 mL each of sample homogenate into petri plates. Added 15-20 mL of Soybean Casein Digest agar, cooled to 45°C, into petri dishes. Immediately rotated the plates in a back and forth motion. The agar plates were kept for 5-10 minutes. The agar plates were incubated at 35°C for 5 days. A negative control was conducted by using sterile distilled water in the place of sample homogenate.

Counting of colonies: The colonies on the plate was counted with highest number of colonies but not more than 300 per plate as the maximum consistent with good evaluation, by using colony counter. For plates with 300 colonies, reported results in colony forming units based on average count of duplicate set.

Limit of detection: TPC=>1 CFU /mL

Permissible Limit: 105 CFU/gm

Statistical analysis

All of the experiments were done in triplicate. The data were recorded as means ± standard deviations and were analyzed with SPSS (version 11.0 for Windows, SPSS Inc., Chicago, IL, USA), and the statistical significance was determined at $P<0.05$.

RESULTS AND DISCUSSION

Accelerated shelf-life study of Fish oil incorporated with medicinal plant extracts

Table 1 shows the results of accelerated shelf life studies of fish oil treated with *O. sanctum* extracts (2 mg%). It is found that *E.coli*, *Salmonella* and *P. aeruginosa* were absent throughout the storage period of 6 months in the treated sample. Though TPC was found high at initial stage (1250000 cfu/gm.), by 6 months

the value was reduced to <05 cfu/gm. Fungus and yeast contents with a high value during 2nd month (1000 cfu/gm) decreased to a value of 110 cfu/gm in 6 months. Rancidity index showed absent in all the days.

Table 1: Changes in microbial and fungal parameters and rancidity index during accelerated shelf life study of fish oil with added *Oscimum sanctum* extracts (2 mg%).

Parameters	0 (initial)	1st month	2nd month	3rd month	6th month
Salmonella Spp/g	Absent	Absent	Absent	Absent	Absent
E. coli/g	Absent	Absent	Absent	Absent	Absent
P. aeruginosa/g	Absent	Absent	Absent	Absent	Absent
TPC	1250000 cfu/gm	16300 cfu/gm	110 cfu/gm	<10 cfu/gm	<05 cfu/gm
Fungus & Yeast	<10 cfu/gm	900 cfu/gm	1000 cfu/gm	200 cfu/gm	110 cfu/gm
RancidityIndex	Absent	Absent	Absent	Absent	Absent

The results of accelerated shelf life study of fish oil treated with *A. barbadensis* extracts (5 mg%) and for *B. diffusa* extracts (5 mg %) are shown in Table 2 and Table 3 respectively. Contrary to *O. sanctum* treated fish oil, the total fungus and yeast content which was minimum at the initial period (<10 cfu/mL) increased to a maximum value of 2110 cfu/gm in *A. barbadensis* and *B. diffusa* treated samples. Rancidty was absent throughout the storage period in both the treatments. Thus total fungus and yeast content was significantly lowered in *O. sanctum* treated fish oil when compared with *A. barbadensis* and *B. diffusa* treated fish oil.

Storage stability studies provide evidence on in what way the quality of a drug or its product varies with time under influence of changing environmental factors such as humidity, temperature and light. In general, pharmaceutical products are studied for its stability profile in an accelerated humidity and temperature, and these investigational findings can be very helpful for predicting reliable expiry date or shelf-life at room temperature by assuming certain criteria and assumptions. It also establishes a retest period for the particular drug substance or its product for the recommended storage conditions. Thus, it is proved that the stability study is unavoidable for the assessment of product quality. In contrast to conventional preparations of medicinal plants, products incorporated with their extracts lead to a number of unique problems with regard to quality and stability. A key part of quality control is to ensure good reproducibility and guarantee chemical stability of the final product during its storage.

Table 2: Changes in microbial and fungal parameters and rancidity index during accelerated shelf life study of fish oil with added *Aloe barbadensis* extracts (5 mg %).

Parameters	0 (initial)	1st month	2nd month	3rd month	6th month
Salmonella Spp/g	Absent	Absent	Absent	Absent	Absent
E. coli/g	Absent	Absent	Absent	Absent	Absent
P. aeruginosa/g	Absent	Absent	Absent	Absent	Absent
TPC	150000cfu/gm	13900 cfu/gm	210 cfu/gm	<10 cfu/gm	<05 cfu/gm
Fungus & Yeast	<10 cfu/gm	<10 cfu/gm	200 cfu/gm	1700 cfu/gm	2110 cfu/gm
RancidityIndex	Absent	Absent	Absent	Absent	Absent

Table 3: Changes in microbial and fungal parameters and rancidity index during accelerated shelf life study of fish oil with added *B. diffusa* extracts (5 mg%).

Parameters	0 (initial)	1st month	2nd month	3rd month	6th month
Salmonella Spp/g	Absent	Absent	Absent	Absent	Absent

E. coli/g	Absent	Absent	Absent	Absent	Absent
P. aeruginosa/g	Absent	Absent	Absent	Absent	Absent
TPC	1050000cfu/gm	1590 cfu/gm	90 cfu/gm	<10 cfu/gm	<05 cfu/gm
Fungus & Yeast	<10 cfu/gm	400 cfu/gm	800 cfu/gm	3900 cfu/gm	4710 cfu/gm
Rancidity Index	Absent	Absent	Absent	Absent	Absent

In this study, results are reported based on accelerated stability study of fish oil incorporated with the three medicinal plant extracts. As per the results of the previous studies, *Ocimum* extract has shown antimicrobial properties against both gram negative bacteria (*Salmonella enteritica*, *Vibrio parahaemolyticus*, *Escherichia coli*) and gram positive bacteria (*Listeria monocytogenes*) with higher antibacterial activity against gram negative bacteria compared to gram positive bacteria [2]. These results are in agreement with the results of present study. Tulsi extract has also been shown to be effective against filamentous fungi such as *Aspergillus Niger*, *Aspergillus fumigatus*, *Aspergillus flavus*, *Rhizopus stolonifera* and *Penicillium digitatum* [3-6]. Other clinically important filamentous fungi like *Fusarium solani*, *Penicillium funiculosum*, *Rhizom ucortauricus* and *Trichoderm areesi* are also susceptible to Tulsi extract [3]. The leaf extract has also been effective against fungi such as Rizopous, Cladosporium, Curvularia and Lunata. These effects against fungus got similar action in the present study on fish oil storage. Ibrahim et al. [7] investigated the phytoconstituents and antimicrobial activity of aqueous, ethanol and acetone extracts of *A. vera* gel against some human and plant pathogens by disc diffusion method. According to his reports the ethanol and acetone extracts recorded significant antimicrobial activity against many of the pathogens. In general, according to the results of the study, leaves of Ocimum sanctum were found to be containing chemical compounds which can be used as antimicrobial compounds against food borne microbial pathogens [8]. According to Girish and Satish [9] in experiments of *B. diffusa* extracts against certain bacteria, maximum inhibition was observed in *S. aureus* followed by megaterium and bacilu cereus, respectively at 50 microlitre concentration. Therefore, the use of *B. diffusa* was experimentally trailed in fish oil, and the results found to be interestingly good in increasing shelf life of fish oil.

Thus *Oscimum* extract showed high antibacterial activity against gram negative bacteria compared to gram positive bacteria, so also the other medicinal plant extracts. The medicinal plant extracts were also found effective in fungal inhibition when incorporated with stored fish oil. This approach will unquestionably build an innovative way for applying different herbal extracts in maintaining the quality, consistency, safety as well as stability of various food products. These efforts can ensure uniform therapeutic functionality and stability of products. Above studies indicate that the fish oil preserved in medicinal plant extracts were stable at room temperature for more than 2 years. According to "ICH Guidelines, when data show little or no variation with time, then statistical analysis are not required, and proposed shelf life is twice of real time data, but not more than 12 months. By using this method the shelf life of any drug preparation can be predicted in a very short period of time. This approach will unquestionably build an innovative way for maintaining the quality, consistency, safety as well as stability of different products.

CONCLUSION

Storage stability studies provide evidence on in what way the quality of a drug or its product varies with time under influence of changing environmental factors such as humidity, temperature and light. In general, pharmaceutical products are studied for its stability profile in an accelerated humidity and temperature, and these investigational findings can be very helpful for predicting reliable expiry date or shelf-life at room temperature by assuming certain criteria and assumptions. It also establishes a retest period for the particular drug substance or its product for the recommended storage conditions. Thus, it is proved that the stability study is unavoidable for the assessment of product quality. In contrast to conventional preparations of medicinal plants, products incorporated with their extracts lead to a number of unique problems with regard to quality and stability. A key part of quality control is to ensure good reproducibility and guarantee chemical stability of the final product during its storage. In this paper results are reported based on accelerated stability study of fish oil incorporated with the three medicinal plant extracts. *Ocimum* extract showed high antibacterial activity against gram negative bacteria compared to gram positive bacteria, so also the other medicinal plant extracts. The extracts were also found effective in fungal inhibition when incorporated with stored fish oil. Above studies indicate that the fish oil preserved in medicinal plant extracts were stable at room temperature for more than 2 years. According to "ICH Guidelines, when data show little or no variation with time then statistical analysis are not required, and proposed shelf life is twice of real time data, but not more than 12 months. By using this method the shelf life of any drug preparation can be predicted in a very short period of time. This approach will unquestionably build an innovative way for maintaining the quality, consistency, safety as well as stability of different PUFA based products. These efforts can ensure uniform therapeutic functionality and stability of these products.

Practical application

Stability studies provide a key factor in determining the quality of a product. In particular medicinal plant extracts gives a stable product ensuring the oxidative stability of the product. Because

of its high ant oxidative property it can be utilized in various products that had to be prevented from deterioration for a long period. Rather, these compounds have other medicinal uses which gave neutraceutical importance to the manufactures who prescribed a compound preserved in these extracts. There is possibility of future research for the application the medicinal extracts in other compounds which need preservation for long term usage.

REFERENCES

1. Cannors KA, Amidon GL, Kennon L. Chemical Stability of Pharmaceuticals. A handbook of Pharmacists. John Wiley & Sons, New York, USA. 1979.
2. Mishra P, Mishra S. Study of Antibacterial Activity of Ocimum sanctum Extract against Gram Positive and Gram Negative Bacteria. Am J Food Technol. 2011;4:336-341.
3. Dharmagadda VSS, Tandon M, Vasudevan P. Biocidal activity of the essential oils of Lantana camara, Ocimum sanctum and Tagetes patula. J Sci Ind Res. 2005;64:53-56.
4. Bansod RSM. Antifungal activity of essential oils from Indian medicinal plants against human pathogenic Aspergillus fumigatus and A niger. World J Med Sci. 2008;3:81-88.
5. Kumar A, Shukla R, Singh P, Dubey NK. Chemical composition, antifungal and anti-aflatoxigenic activities of Ocimum sanctum L. essential oil and its safety assessment as plant based antimicrobial. Food Chem Toxicol. 2010;48:539-543.
6. Grover G, Rao S. Investigations of antimicrobial efficiency from essential oil from O. sanctum & O. gratissimum. Perfuma, Kosmat. 1977;58:326-329.
7. Ibrahim W, Sarwar Z, Abid S, Munir U, Azeem A. Aloe Vera Leaf Gel Extract for Antibacterial and Softness Properties of Cotton. J Textile Sci. 2017;7:1-6.
8. Mann CM, Cox CD, Markham JL. The outer membrane of Pseudomonas aeruginosa NCTC 6749 contributes to its tolerance to the essential oil of Melaleuca alternifolia (tea tree oil). Lett Applied Micobiol. 2000;30:294-297.
9. Girish HV, Satish S. Antibacterial activity of important medicinal plants on human pathogenic bacteria-a comparative analysis. World Applied Sci J. 2008;5:267-271.