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Original Research Article

Microbiological Quality of *Gadus morhua* Treated With Silver Nanoparticles Synthesized Using *Launaea taraxacifolia* Leaves

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ABSTRACT

A number of shelf life-promoting strategies have been adopted for fish and fisheries products. *The in vitro* preservative effect of Ag-nanoparticles synthesized using *Launaea taraxacifolia* leaves on Atlantic cod fish (*Gadus morhua*) was investigated. The treated (TRD) fish samples were dipped in 25% silver nanoparticles (AgNPs) preparation for 30 minutes and analyzed at four hours intervals for 24 hours. Samples were cut into Head (H), Trunk (TR), and Tail (TL) parts and evaluated for total heterotrophic bacterial counts (THBC) and total yeast counts (TYC) all expressed in log CFU/g. Untreated (UTD) served as control. The concentrations of silver in both treated and untreated fish samples were determined. Data were analyzed using descriptive statistics and ANOVA at $\alpha_{0.05}$. The treated samples recorded lower THBC values than the untreated: head (6.18±0.27 vs 6.55±0.65); trunk (6.22±0.11 vs 6.61±0.41) and tail (6.40±0.34 vs 6.57±0.83). The treated samples had lower TYC values than the untreated: head (6.34±0.58 vs 7.44±0.38), trunk (6.59±0.66 vs 7.05±0.46) and tail (6.47±0.30 vs 6.81±0.95). Both the treated and the untreated samples were dominated by *Enterococcus* spp. (569 vs 957) and *Micrococcus* spp. (13 vs 18). *Bacillus* spp. (36) was isolated only in the untreated sample. The silver concentrations were highest in untreated and treated trunk samples respectively (1.12 and 2.13µg/g), followed by head samples (0.58 and 1.41µg/g) while the tail samples had the least (0.27 and 0.29 µg/g). The Ag-nanoparticles synthesized from *Launaea taraxacifolia* leaves exhibited both antibacterial and antifungal activities and could be useful in preserving fish and fishery products.

Keywords: Fish preservation, *Gadus morhua*, *Launaea taraxacifolia*, Silver nanoparticles.

Introduction

Fish products are among the most perishable commodities mainly due to microbial spoilage.[1] Microorganisms are known to be responsible for 25-30% of the major deterioration of fish quality, causing loss of such products. Enzymatic and chemical reactions are usually responsible for the initial loss of freshness whereas microbial activity is responsible for the obvious spoilage.[2] In recent years, many studies have been made into searching for natural preservatives that could inhibit the growth of food spoilage microbes.[3] Meanwhile, a growing number of consumers are aware of the potential negative health effects of chemical preservatives and this has prompted research into alternative natural products to promote safe use.[4] Moreover, the introduction of nanotechnology in food preservation may offer potential solutions for the challenge presented by short shelf life of fish and fishery products, improving their quality and keeping them free of microbial adhesion.[5]

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Plant-derived silver nanoparticles (AgNPs) have been demonstrated to possess effective antibacterial effects and have been vastly used in medicine.[6] Green synthesis method has been studied because of their renewability,[7] simplicity and eco-friendliness,[8] inherent antimicrobial activities, and preservative properties of the obtained AgNPs.[9] Plant components including leaves, fruits, roots, stems and leaves have been widely utilized to synthesize different NPs which are currently being used in different applications,[10] including fish preservation. The presence of a wide array of phytochemicals in their extracts may function as natural stabilizing and/or reducing agents for AgNPs production.[11] It is generally accepted that plant-derived nanoparticles (NPs) are also less likely to have harmful side effects in humans when compared to chemically synthesized NPs.[5] This is because the reductants, which are phytochemicals also possess beneficial biological effects, making them biocompatible. Thus, plants provide a far better alternative for nanoparticles synthesis as they are free from toxic chemicals that are associated with the chemical method of synthesis.[12] Furthermore, the inherent biomolecules in plants serve as natural capping agents, which prevent oxidation of the synthesized NPs.[13]

Launaea taraxacifolia of the family Asteraceae used for this study is a green leafy vegetable (Figure 1) locally cultivated and widely consumed in Nigeria, Senegal, Ghana, Dahomey, and Sierra Leone.[14] It is also known as African lettuce or wild lettuce. In Nigeria, Hausas call it 'Namijindayii, Nomenbarewa, and NonanBarya' while Yorubas call it 'EfoYanrin and Odundun odo. It is a wild plant that grows individually or in clusters on fields, rocky soil, fallows, and abandoned areas. The economic values of this plant were highlighted by Adebisi & Ladipo,[14] for its utility as food, fodders for animals, lactogen in cattle, and induction of multiple births in livestock. In Nigeria, it is employed as a cure for several complaints such as eye diseases (conjunctivitis), yaws, and measles.[14] The leaf extract mixed with the breast milk of a nursing

mother is also employed in the treatment of partial blindness resulting from snake bite.[15]

The leaf of the plant is a rich source of secondary metabolites including tannins, flavonoids, phenolic acids, and alkaloids which have been indicated to be responsible for its biological activities.[16] *Launaea taraxacifolia* has also been reported to exhibit a wide variety of pharmacological activities such as antioxidants,[15,17-19] antimalarial[20,] and anti-peroxidation.[19] The bactericidal potential of *Launaea taraxacifolia* investigated by Ololade *et al*,[18] and Tayman *et al*,[21] showed that extracts and compounds isolated from the plant significantly inhibited the growth of *Staphylococcus aureus, Escherichia coli, Enterococcus faecalis, Micrococcus varians, and Streptococcus agalactiae*. This demonstrates that the plant possesses a broad spectrum potency against gram-positive and gram-negative bacteria. Moreover, Essien *et al*,[13] also demonstrated that the silver nanoparticles synthesized using the leaf extract of *Launaea taraxacifolia* exhibited high potency against *P. aeruginosa* and *P. mirabilis* at low concentrations. However, there is a dearth of information on the effect of AgNPs synthesized using *Launaea taraxacifolia* in fish preservation. Given the promising antimicrobial properties of *Launaea taraxacifolia,* our aim in this study, therefore was to investigate the preservative potential of the silver nanoparticles synthesized using the leaf extract of *Launaea taraxacifolia* on Atlantic cod fish (*Gadus morhua*), a delicacy enjoyed in many parts of the world including Nigeria.

Figure 1: *Launaea taraxacifolia* plant

Materials and Methods

Silver Nanoparticle synthesis using ***Launaea taraxacifolia leaves***

Launaea taraxacifolia leaves were collected in the month of March 2021 from the premises of Bells University of Technology, Ota, Ogun State, Southwest Nigeria, with geographical coordinates of 6.689606 North and 3.165886 East and identified at the Herbarium section of Botany Department, University of Lagos, Nigeria (LUH10055). The leaf extract was prepared using a method described by Essien *et al*.[22] The leaves were thoroughly washed with distilled water, air-dried, and pulverized with a high-speed blender. Precisely 4 g was weighed with an EK-410 Portable Balance, (A&D South Korea), into a conical flask containing 100 mL of sterile distilled water and heated at 65 °C in a thermostatic water bath for 15 minutes. The extract was filtered using Whatman 1 filter paper and held at 4 °C. Aqueous solution (1 mM) of silver nitrate (MLYCHEM, 169.87g mw, 99% purity) was prepared and 10 mL of the leaf extract was added to 90 mL of 1 mM $AgNO_3$ aqueous solution at room temperature. A magnetic stirrer (SB162, Stuart, UK) was used to properly mix the aqueous solution and kept in a dark room for 2 weeks. The aqueous solution was poured into a beaker, allowed to sediment, and subsequently filtered. The filtrate was centrifuged with an SM112 Uniscope Centrifuge (Surgifriend Medicals; England) to sediment the silver nanoparticles which were poured into a glass Petri dish and dried in a hot air oven (SM9053, Uniscope Laboratory Oven, Surgifriend Medicals; England) set at
45 °C for 24 h. The crystalline nature as well as the shape and size dimensions of the synthesized AgNPs were investigated using X-ray Diffraction (XRD) (Empyrean, Malvern Panalytical; The_Netherlands) and Scanning electron microscopy (SEM) (Phenom ProX, Phenomworld; Japan) respectively.

Fish sample preparation

Twenty (20) frozen Atlantic cod fish (*Gadus morhua*), weighing 80 to 90g, were allowed to thaw and treated by the dipping method earlier described by Kester & Oyelese[23] in a solution containing 25% AgNP previously obtained by sonicating the AgNPs in de-ionized water. The fish were dipped for 30 minutes, thereafter drained and held in a clean basket at ambient conditions for 24 h. Untreated fish served as control. Samples were taken at four-hour intervals and cut into Head (H), Trunk (TR), and Tail (TL) and subjected to microbiological analysis.

Microbiological Analysis

The culture media employed in this study were products prepared by Hi-Media, India. The methods of microbiological analysis described by Harrigan & McCance[24] were adopted. The prepared codfish samples were assessed for total heterotrophic bacterial count (THBC) and total yeast count (TYC). Each of the prepared fish parts (10g) was aseptically weighed into 90 mL of sterile distilled water and serially diluted in ten folds up to 10^{-6}. Using the pour plate method, an aliquot of 1mL was inoculated into sterile Petri dishes, and about 20 mL of Nutrient Agar and Potato Dextrose Agar (PDA) were separately poured for enumeration of THBC and TYC respectively. All the inoculated plates were inverted and incubated at about 37 $^{\circ}$C for 24-48 h while the PDA plates were incubated at room temperature for 48-72 h. Developed colonies were counted and expressed as log $CFUg^{-1}$ of fish. The isolates were identified based on their cultural characteristics, microscopic appearance, and reactions to biochemical tests.

Determination of concentration of Silver in Treated and Untreated codfish samples

The treated and untreated fish samples were properly mashed using a clean mortar and pestle and mixed homogeneously. Thereafter, 0.05g of the mashed fish sample was weighed and digested in glass tubes with Aqua regia (w/v), prepared from concentrated nitric acid (BDH Analar, 1.42g/mol. Sp.g, 63.0/g mw, 65% purity) and perchloric acid (DH Daryagan, Delhi, India) in the ratio 4:1. The tubes were kept in boiling water for 20 minutes and a clear yellow solution was obtained. The resulting solution was filtered and used for the determination of the concentration of silver in the control and treatment groups using a Perkin Elmer optima 8000 (USA) inductive coupled plasma atomic emission spectroscopy. The parameters used during the analysis of silver included: limit of detection (0.0002ppb), limit of quantification (103 ppb), R^2-value (0.9999), recovery (99.9894), and wavelength (328 nm).

Statistical Analysis

Analysis of Variance (ANOVA) was applied to data obtained from the treated and untreated fish samples which were analyzed in sub-sets (H, Tr, and Tl). Duncan's Multiple Range test was then used to determine the significant differences between the means of the values. Both statistical analyses were conducted using SPSS (Version 17.0) for Windows Inc, Chicago, U.S.A.

Results and Discussion

The diffraction pattern of the silver nanoparticles is shown in Figure 2 to be amorphous-like, judging from the diffusive nature and broadening of the peaks. The peaks were indexed using the standard reference file (JCPDS No. 4-0783). The peaks corresponded to hkl reflection indices at 111, 200, and 220 planes, thus indicating the formation of silver nanoparticles.[22] The morphology, surface characteristics, and distribution of the synthesized AgNPs as observed in the micrograph presented in Figure 3 show that the particles displayed heterogeneous shapes with slightly rough surfaces and uneven edges, all forming scattered flakes. The scattered flakes observed in this study as opposed to the agglomerated particles observed by Bello *et al*.[20] in NPs synthesized from the same plant may be attributed to the differences in the methods of synthesis. The slightly rough surfaces and uneven edges of the synthesized nanoparticle can offer a better particle surface to

bacterial cell orientation at the molecular level. Hence, this causes the opening of the membrane, leaking of cytoplasmic contents, allowing the penetration of the synthesized AgNPs into the cells, and resulting in apoptosis. This agrees with the explanation of Seong *et al*.[26] and Khalandi *et al*.[27] in their review of the potential role of silver nanoparticles and the possible mechanisms of their actions on bacteria. The microcracks noticed on the surface of the synthesized AgNPs could also enhance surface reaction with the bacterial cell membrane. In addition, the surface roughness of the nanoparticle could promote bacterial cell attachment and antimicrobial activities.

The THBC values of treated and untreated fish samples are shown in Figure 4. By 24 h, the values were comparatively lower in the treated (TRD) than the untreated (UTD) samples for the head (6.18±0.27 vs 6.55±0.65 log CFU/g); trunk (6.22±0.11 vs 6.61±0.41 log CFU/g) and tail (6.40±0.34 vs 6.57±0.83 log CFU/g), although statistical significance was not reached at P<0.05. The lower values recorded in the treated samples are suggestive of the antibacterial and preservative effects of AgNPs synthesized from *Launaea taraxacifolia*. The potential inhibition of *Launaea taraxacifolia* leaf extract investigated against gram-positive and gram-negative bacteria by Ololade *et al.*[18] and Tayman *et al*.[21] accords the findings of this study. A similar effect of AgNPs on THBC values was reported by AbdelRahim *et al*.[25] The study of Elshahawy *et al*.[28] which defined the optimal concentration of AgNPs for sterilization of Tilapia skin grafts also established the sensitivity of bacteria to 25% minimum concentration of AgNPs. Though the actions of the intrinsic bacteria were impaired, the lack of attainment of statistical significance could possibly be attributed to the difference in the types of fish used in both studies.

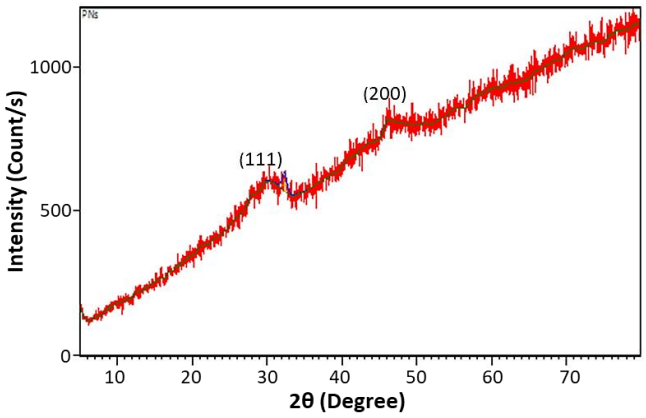

Figure 2: X-ray diffraction pattern of silver nanoparticles synthesized from *Launaea taraxacifolia* leaves

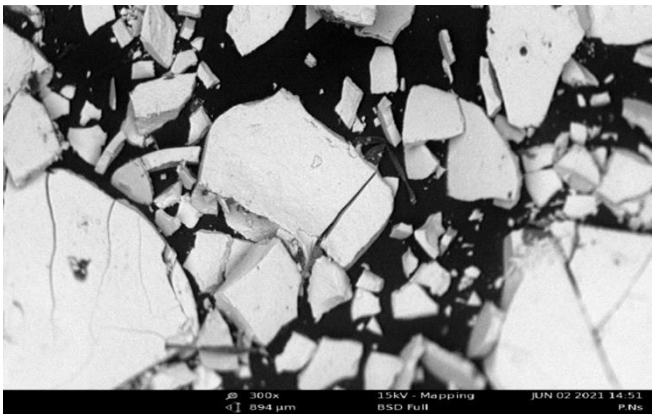

Figure 3: Scanning electron microscopic (SEM) image of silver nanoparticles synthesized from *Launaea taraxacifolia* leaves

Despite the fact that the fish samples were not eviscerated and were stored for 24 h at ambient conditions, the highest THBC value of 6.40±0.34 log CFU/g in the treated TL sample did not exceed the maximum recommended bacterial count for marginally acceptable quality product (M) which is 10^7 (7 log 10 CFU/g) for fish and fishery products.[29] The findings of this study agree with the observations of Daniel *et al*,[30] who worked on Nano ice based on silver nanoparticles for fish preservation and concluded that the fish preserved in antimicrobial nano ice had less CFUs and growth.

The results of TYC after 24 h of storage are presented in Figure 5. TYC values of 6.34±0.58 vs 7.44±0.38 log CFU/g (Head), 6.59±0.66 vs 7.05±0.46 log CFU/g (Trunk), 6.47±0.30 vs 6.81±0.95 log CFU/g (Tail), were respectively recorded in treated and untreated samples. The higher yeast count recorded in the untreated than the treated samples indicated the antifungal properties of AgNPs synthesized from *Launaea taraxacifolia*. This result is in agreement with the findings of Sang *et al*,[31] in their study on antifungal effects of AgNPs against various plant pathogenic fungi. In line with this study, a recent observation was made that yeasts and molds were more sensitive than bacteria in Tilapia skin strips immersed in AgNPs solutions[28].

The morphological and biochemical characteristics of the bacterial isolates that dominated the treated and untreated fish part samples are summarized in Table 1, showing *Bacillus spp., Micrococcus* spp. and Enterococcus spp. as the major contaminants of the study fish. These bacterial isolates were among the genera detected by Arafat[32] in skins and intestines from fresh fishes sold in Ed Dueim, Sudan.

The frequency of occurrence of the bacterial isolates in all the fish part samples is displayed in Table 2. The untreated samples recorded higher occurrence of the bacterial isolates than the treated. *Bacillus spp.* was not detected in all the samples except UTDTL that had 36 colonies. The notable absence of *Bacillus* spp. in the treated samples could imply that the AgNP synthesized from *Launaea taraxacifolia* was antagonistic to the bacteria, thereby inhibiting their growth.[33] The untreated TL part had 18 colonies of *Micrococcus* spp. as compared to the treated samples in which no growth was detected. The untreated TR samples recorded a higher occurrence of *Micrococcus* spp. (93) in comparison with the treated (13). The occurrence of *Enterococcus* spp. was remarkable in all the samples, ranging between 375 and 959. The untreated samples recorded higher occurrence of 51.2%, 60.3% and 65% respectively in TL, TR and H fish parts as compared to treated which showed in the 48.8%, 39.7% and 35% in the TL, TR and H fish parts respectively. The highest occurrence of *Enterococcus* spp. recorded in the both treated and untreated samples of this study can be attributed to the hardy nature of Enterococci, which enables them to survive in a range of stressful and hostile environments.[34,35] The lower frequency of occurrence of *Micrococcus* spp. and *Enterococcus* spp. recorded in the treated as compared to the untreated fish samples further concerts the claims of Morones *et al*.[33] on the antibacterial effect of AgNP synthesized from *Launaea taraxacifolia* leaves.

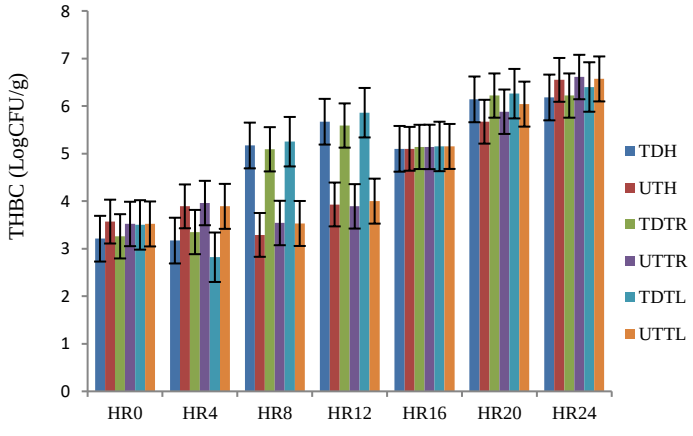

Figure 4: Total heterotrophic bacterial count for treated and untreated *Gadus morhua* samples stored for 24 hours

Table 1: Colonial and Biochemical characteristics of *Listeria* spp. isolated in smoked fish

Colony Morphology	Gram rxns	Microscopic examination	Ca	Co	Mo	G	SFM	SFG	IND	CIT	Ox	H_2S	U	Probable organisms
Rough, indistinct, dull white, rough edge	+	Rod shaped	+	-	+	-	+	+	-	+	-	-	-	*Bacillus* spp.
Smooth, Yellow with circular marging	+	Small cocci, majorly in pairs and clusters	+	-	-	-	-	-	-	-	+	+	+	*Micrococcus* spp.
Transparent with dark centers	+	Cocci in short chains and pairs	-	-	+	+	+	+	-	-	-	+	-	*Enterococcus* spp.

Ca- Catalase, Co- Coagulase, Mo- Motility, G - Gas Production, Ind- Indole, CIT- Citrate,
H_2S- Hydrogen sulphide, U- Urease SFG- Sugar Fermentation Glucose, SFM- Sugar Fermentation Maltose, Ox- Oxidase, + = Positive, - = Negative

Table 2: Frequency of occurrence of bacterial isolates in treated and untreated codfish samples

h part	*Bacillus* spp.		*Micrococcus* spp.		*Enterococcus* spp.	
	Treated	Untreated	Treated	Untreated	Treated	Untreated
TL	0	36	0	18	914	959
TR	0	0	13	93	375	569
H	0	0	0	0	504	938

TL = Tail, TR = Trunk, H = Head, TD = Treated, UTD = Untreated

The concentrations of silver in the different parts of the untreated and treated fish samples after 24 h of ambient storage are shown in Figure 6 to differ significantly ($p<0.05$). The untreated and treated trunk samples respectively recorded the highest values of 1.12±0.05 and 2.13±0.16 µg/g, followed by head samples (0.58±0.08 and 1.41±0.09 µg/g) while the tail samples had the least values of 0.27±0.04 and 0.29±0.05 µg/g. The large mass of tissues in the fish trunk could be responsible for the accumulation of the highest silver concentration than the head and tail parts with less tissue mass. The trace of silver in the untreated codfish samples could have occurred as a contaminant of the fishing ground due to human and anthropogenic activities including smelting operations, manufacture, and disposal of certain photographic and electrical supplies, coal combustion, and cloud seeding.[36] Moreover, US EPA[37] opined that silver is usually found in extremely low concentrations in natural water bodies as a result of the low crustal abundance and low mobility in water. Generally, the silver concentrations obtained in the study were, however, significantly lower than the EPA's reference dose maximum daily exposure of <350µg/g in food. This suggests that the fish preserved with *Launaea taraxacifolia* leaf nanoparticles is relatively safe for consumption.

Conclusion

Silver nanoparticles (AgNPs) synthesized from *Launaea taraxacifolia* possess potent antimicrobial properties that could limit the growth of fish spoilage bacteria, yeasts, and molds. In addition, fish treated with green synthesized AgNPs from *Launaea taraxacifolia* are relatively safe from silver toxicity in humans. Therefore, this study established that the synthesized AgNPs from *Launaea taraxacifolia* could be beneficial in reducing fish spoilage and can be used as a preservative for fish and fishery products as well as other food products.

Conflict of Interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

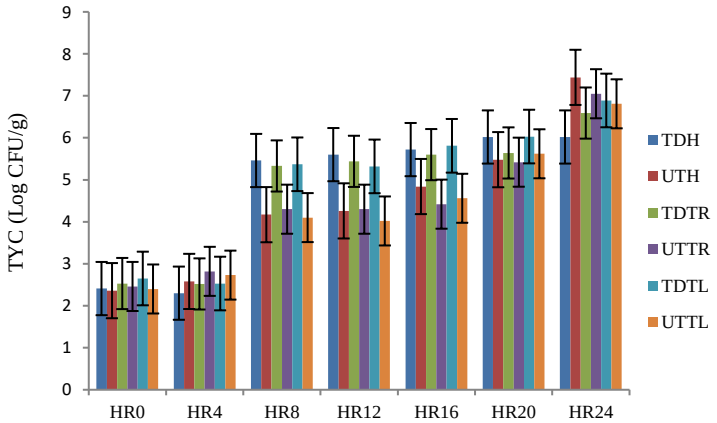

Figure 5: Total yeast count for treated and untreated *Gadus morhua* samples stored for 24 hours

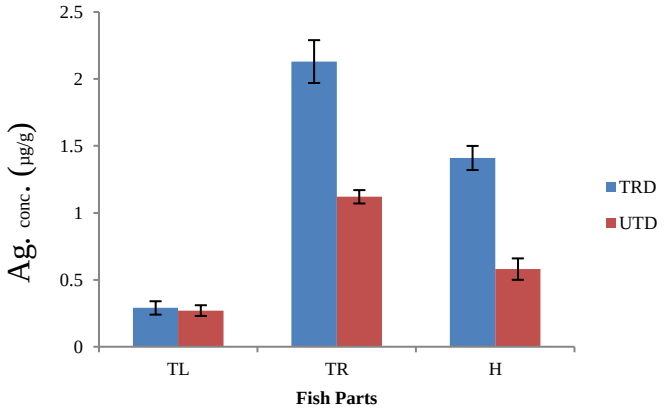

Figure 6: Concentrations of silver in treated and untreated cod fish samples

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