Biological and Pharmacological Evaluation of Crude Venom Extracted from *Tetraodon fahaka strigosus* and *Potamotrygon garouensis* obtained from River Niger, Niger State, Nigeria

[1]Mann, A., [2]Abdulkadir, A., [1]Fadipe, L. A., [1]Ndamitso, M. M., [1]Salihu, A. M., [2]Ogbadoyi, E. O., [3]Daniyan, S. Y. and [4]Bake, G. G.
[1]Department of Chemistry, Federal University of Technology, Minna, Niger State, Nigeria
[2]Department of Biochemistry, Federal University of Technology, Minna, Niger State, Nigeria
[3]Department of Microbiology, Federal University of Technology, Minna, Niger State, Nigeria
[4]Department of Water Resources Aquaculture and Fisheries Technology, Federal University of Technology, Minna, Niger State, Nigeria
Correspondence: Address: abdullahimann@futminna.edu.ng; Tel.no: +2348034295656

Abstract
The present study investigates some biological and pharmacological effects of *Tetraodon fahaka strigosus* (Puffer fish) and *Potamotrygon garouensis* (Stingray) venom extracts. The saline extracts from skin and innards of *T. fahaka strigosus* and *P. garouensis* were used to evaluate haemolytic, enzymatic and *in-vitro* antioxidants activities. *In-vivo* determination of median lethal dose, some blood biochemical parameters and analgesics activities of the extracts in mice were also carried out. The result of this study showed that all extracts from both the puffer fish and stingray showed significantly haemolytic, proteolytic and phospholipase activities. The DPPH scavenging activity of different parts (innard and skin extracts) of puffer fish and stingray demonstrated significantly antioxidant activity at IC_{50} values 0.16 mg/ml and 0.15 mg/ml and 0.20 mg/ml and 0.01 mg/ml respectively; when compared to the negative control. Median lethal dose range between 31-90 mg/kg body weight. Furthermore, there was significant increase ($p<0.05$) in the packed cell volume (PCV) and body weight of animals treated with extracts of *T. fahaka strigosus* and *P. garouensis* compare to control. The crude extracts from *T. fahaka strigosus* and *P. garouensis* administered to mice significantly inhibit acetic acid induced writhing ($p<0.05$). The results of serum biochemical analysis of animals treated with extracts of *T. fahaka strigosus* and *P. garouensis* revealed a significant increase in the activities of alanine transaminase (ALT) and aspartate transaminase (AST) ($p<0.05$), while no significant difference was observed in alkaline phosphatase (ALP)**,** albumin, total protein and bilirubin. The absorption spectra of *T. fahaka strigosus* skin and innards and *P. garouensis* tail and spine sample extracts respectively contains several bands arising from the contribution of different functional groups belonging to proteins, lipids and other biomolecules. The spectral analysis showed variations in composition of biomolecules of the skin, innards, tail and spine samples at a wave number region of 4000–400 cm^{-1}. These studies also suggest that the extracts of skin and innards from *T. fahaka strigosus* (puffer fish) and *P. garouensis* (stingray) have haemolytic, antioxidant and analgesic activities which could be exploited for further therapeutic intervention.
Keywords: *Tetraodon fahaka strigosus,* Puffer fish*, Potamotrygon garouensis,* Stingray, haemolytic, enzymatic and *in-vitro* antioxidants activities

Introduction
Animal venoms have been considered an excellent resource of bioactive molecules for the discovery of novel drug leads (King, 2015). There has been much research on the activities and component of terrestrial venoms such as those from snakes, scorpions and spiders but relatively less into the aquatic organism venoms (Ortiz *et al.,* 2015). This is due in part to the greater convenience in capturing terrestrial animals over marine specimen. Aquatic organisms are viewed as less of a threat (Church and Hodson, 2002). However, many venomous animals can be found in marine environment and many capable of producing severe envenomation in humans that have led to fatalities (Borondo *et al.,* 2001).

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Venomous fishes represent more than 50% of the venomous vertebrates and are often involved in human accidents. Fish venom represent a largely untapped treasure of biologically important compounds (Ortiz *et al.,* 2015). Phylogeny and venom evaluation have shown an estimate of about 2000 species of venomous fishes which include stone fish, puffer fish, scorpion fish (Smith and Wheeler., 2006).

Tetraodon fahaka strigosus (Puffer fish) is a long establish delicacy, in spite of its known potential for toxicity. Tetrodotoxin is the naturally occurring toxin that is mainly responsible for the risk to consumers (Hong *et al.,* 2017). The toxin is named from puffer fish family (Tetraodontidae) where it has been found to be concentrated in the liver, ovaries, intestine and other organs. Over 20 species of puffer fish have been found to harbour the toxin (Noguchi *et al*., 2006). Tetrodotoxin is both water soluble and heat stable, so cooking does not negate its toxicity; rather it increases it toxic effect (Saoudi *et al*., 2010). It is a potent neurotoxin that is over a thousand times more toxic to human than

cyanide (Noguchi *et al.,* 2006; Saoudi *eta l.,* 2010). The toxin binds to the sodium ion through the channel of the excitable tissue of the victim (muscles and nerves). The inhibition of sodium through the channels effectively immobilizes the tissues (Denac *et al*., 2000; Zimmer, 2010). The onset and severity of the symptoms of tetrodotoxin poisoning after ingestion in human is dose dependent (Islam *et al.,* 2011). Initial symptoms include tingling (paraesthesia) of the tongue and lips, followed by or concurrent with headache and vomiting which may progress to muscle weakness and ataxia. In severe cases death may occur due to respiratory and/or heart failure (Hinman *et al.,* 2003).

Potamotrygon garouensis (Stingray) are a group of cartilaginous fishes that are commonly found occupying the demersal zone of tropical and subtropical marine waters, although some species are found in freshwater environments. Most rays have retro serrate spines located on their caudal appendage, the spines are layered by dermis, venom glands and epidermis and then encapsulated by an integumentary sheath, resulting in a structure termed a 'barb' (Rice *et al*., 1970). This venom system is used defensively to protect the animal from predation and potential aggressors. Whilst the venoms of most stingrays remain completely unstudied, the venom activities of the South American freshwater stingrays of the family *Potamotrygonidae* have been partially characterised. *P. garouensis* venoms induce oedema in mice and are capable of causing necrosis and exhibit some proteolytic and hyaluronidase activity (Magalhães *et a*l., 2008). Stingray venom is a cocktail of many compound which include orpotrin, phosphodiesterase, hyaluronidase, serotonin, 5'-nucleotidase. Stingray venom have been demonstrated to interfere with membrane phospholipids through an as yet uncharacterized mechanism that results in pro-inflammatory activity as well as act on large arterioles of the microcirculatory network resulting in vasoconstriction respectively (Kimura *et al*., 2014). Infrared spectroscopy is the most versatile powerful spectroscopic techniques for studying molecular structures and intra molecular interactions in biological tissues and cells; since it covers the details on the functional group as well as chemical composition that are contained in the infrared spectrum of specific substances. The Fourier Transform infrared (FTIR) spectroscopy is used extensively used to probe structural changes in proteins and lipids in order to determine chemical, physico-chemical, structural, morphological, and intermolecular cross-linking of foods and biomaterials (Venkataramana *et al*., 2010). The present work evaluates the toxicological, analgesic and antioxidant activities, and molecular structural interactions of two freshwater fishes: *T. fahaka strigosus* (puffer fish) and *P. garouensis* (stingray) in River Niger, Niger State, Nigeria.

Materials and Methods

Materials

All chemicals and reagents used were of analytical grade

Animals

Mice weighing between 25–30 g were obtained from the animal house, Department of Biochemistry, Federal University of Technology, Minna. The animals were kept and maintained in well ventilated cages. Animals were maintained on grower's mash (Vital Feeds Nigeria Ltd) and provided with water *ad libitum.* They were allowed to acclimatize to the laboratory conditions before the treatment.

Sample Collection and Preservation

The two fish species: *Potamotrygon garouensis* (Stingray) and *Tetraadon fahaka strigosus* (Puffer-fish) were purchased fresh from the local fishermen from the coast of River Niger, Niger State, Nigeria as soon as the boats arrived at Baro, Nupeko, Nantu & Tsoegi riverports in June, 2019. The fish samples were transported alive in an aquarium to the Department of Aquaculture and Fisheries Technology, Federal University Technology, Minna, Nigeria for identification; where the fish samples were identified using the taxonomy keys by Reed *et al.* (1967); Olaosebikan. and Raji (2004). The two sample fish species were prepared and morphometric analysed following a rigid quality assurance/quality control protocols to ensure accurate and reliable analytical data. The samples were taken in an ice-cold box to the laboratory where they were thoroughly washed with distilled water, weighed, measured, rinsed with distilled water again and kept at −20°C for analysis at the Department of Chemistry Laboratory and for further analyses at the Department of Biochemistry Laboratory, Federal University of Technology, Minna, Niger State, Nigeria.

Extraction of Crude Sample

The extraction was carried out following the method described by Baranwal *et al*., (2013). The collected puffer fish was dissected and the visceral organs like liver, intestine, muscle and skin were removed. Then 3 g each of the internal organs and the skin were homogenized with 0.15 M NaCl in a blender and centrifuged at 10,000 rpm at 4 ºC for 30 min as previously reported (Nagasaka *et al.* 2009). The supernatants were filtered by using Whatman no. 1 filter paper. The filtrate solution was referred as crude extract and stored at 4 ºC for further analysis. In the case stingray, 10 g each

of the dorsal spine and the tail of dissected stingray fish were removed and were homogenized with 0.15 M NaCl in a blender and centrifuged at 10,000rpm at 4 ºC for 30 min (Nagasaka *et al.* 2009).

Haemolytic Study

Preparation of Erythrocyte Suspension

Fresh blood of cow, sheep, goat and chicken were collected from the nearby slaughter house in Minna and was added with EDTA solution 2.7 g in 100 mL of distilled water as anticoagulant at 5 % of the volume of blood. The blood was centrifuged at 5000 rpm for 7 min at 4 °C along with normal saline about double the quantity of blood. The supernatant was discarded. 1 mL of the packed RBC thus obtained was resuspended in normal saline to obtain a 1% RBC suspension.

Haemolytic Assay

Crude extracts of parts of puffer and stingray were assayed on cow, sheep goat and chicken erythrocytes followed by the method of Pani and Venkateshvaran (1997). Blood obtained from these animals together with Ethylenediaminetetraacetic (EDTA) solution (2.7%) as an anticoagulant at 5% of the blood volume was centrifuged at 5000 rpm for 10 minutes. 1% erythrocyte suspension was prepared for haemolytic study in microtitre plate. Serial two-fold dilutions of the extract (100 µl; 1 mg puffer and stingray in 1 ml PBS) were made in phosphate buffer saline (PBS) (pH 7.2) starting from 1: 2. An equal volume of 1% blood was added to each well. The plates were shaken for mixing the RBC and extract. The plates were incubated at temperature of 25ºC for 2 h before reading the results. Erythrocytes suspension to which distilled water was added (100 µl respectively) served as blanks for negative control. Formation of a fine "Button cell" with regular margin indicates the negative reaction. A uniform red coloured suspension of the lysed RBC indicates the positive result. Haemolytic activity was expressed as Haemolytic Unit (HU). 1 HU being defined as the reciprocal of the highest dilution of the extracts in which a haemolysis is obtained.

Haemolytic assay on blood agar plate

The haemolytic activity was assayed using blood agar plates by following the method of Lemes-Marques and Yano (2004). Cow, Sheep, Goat and Chicken blood agar plates were prepared by adding 5 ml of blood to 95 ml of sterile blood agar aseptically, with the result poured immediately onto the petri dishes. After solidification, wells were cut into the agar plate-using a corkscrew borer (5 mm diameter). Wells were loaded with 50 µl (1 mg/ml) of samples. The plates were observed for haemolysis after overnight incubation at room temperature.

Protease assay

100 µl of venom was added to 200 µl of 3% gelatine (prepared in phosphate buffer at pH 6.8) and incubated for 90 min at 37ºC to start proteolysis reaction. After incubation, the reaction was stopped by addition of 400 µl of 20% trichloroacetic acid and placed on a rotary shaker for 2 minutes at 30 rpm for complete precipitation of proteins, blank tube was prepared by adding 400 µl of 20% trichloroacetic acid to the venom before the substrate was added. The precipitated protein was removed by centrifugation at 10,000 xg and the absorbance of the supernatant was recorded at 540 nm.

Phospholipase assay (Modification of Haberman and Newman, 1954)

0.5 ml egg yolk suspension (2 mg/ml) was introduced into a clean test tube containing 50 µl of 1 mM $CaCl_2$ and 100 µl of distilled water. To this, 100 µl of 20 mg/ml venom solution was added. This was incubated at 37ºC for 1 h. After incubation the enzyme was inactivated by heating for 2 min. One drop of phenolphthalein was added, this was titrated against 20 Mm NaOH. To determine the volume of NaOH used by the free acid content of the yolk, the titration was carried out on the yolk suspension without the venom. The volume obtained was subtracted from the volume obtained above in the presence of the enzyme. This gives the volume of NaOH used up by the fatty acid released from lecithin content of the yolk by phospholipase.

***In-vitro* Antioxidant determination**

The scavenging activity against DPPH was carried out as reported by Brand-Williams *et al*., (1997). Different concentrations of extract were incubated with solution of DPPH at room temperature for 30 min in a dark to complete the reaction. The absorbance of the solution was measured at 517 nm. The percentage (%) inhibition was calculated using the following equation.

$\{(Ac - As) / Ac\} \times 100$

Where, Ac is the absorbance of the control, and As is the absorbance of the extract/standard. The percentage (%) inhibitions are plotted against concentration and from the graph, the half maximal Inhibitory Concentration (IC_{50}) is calculated.

In vivo Study
Toxicity Assay
Determination of Median Lethal Dose (LD_{50})

The experiment was carried with mice of three groups comprising of three mice each treated by oral gavage with 5, 10 and 100 mg/kg body weight (BW) respectively of crude extracts from both fish. Mice were observed over 48 h and mortality was recorded. The LD_{50} value was determined by calculating the geometric mean of the animals that survived.

$LD_{50} = \sqrt{}$ (Highest non-lethal dose) × (Lowest lethal dose)

Subchronic toxicity Treatment

The extracts were orally administered to the mice daily for a period of 21 days, body weights were estimated weekly. The rats were fasted overnight, anaesthetized with chloroform and sacrificed 24 h after the last treatment. After 21 days, of the treatment, the mice were sacrificed, their blood collected in nonheparinized tubes. The serum obtained was then used for analysis.

Subchronic toxicity Treatment

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Estimation of effect of the extracts on some biochemical parameters

Aspartate Amino Transferase (AST) was determine using the method of Reitman and Frankel (1957). Alanine Amino Transferase (ALT) was determined according to the method of Reitman and Frankel (1957) as described by Sini *et al*. (2006). Activity of alkaline phosphatase (ALP) was determined by a standard method described by Haussament (1977). The serum total protein (TP) concentration was determined as described by Fine *et al*. (1935). The serum ALB concentration was determined by the method described by Doumas *et al*. (1971). Serum total bilirubin concentration was determined by the methods of Sherlock (1951). The serum creatinine concentration was determined by the method described by (Bartels and Bohmer 1973). The serum urea concentration was determined based on the method of Fawcett and Scout, (1960).

Analgesic activity

Writhing was induced in mice by intraperitoneal administration of 0.1 ml of 1% acetic acid Sawadogo *et al* (2006). Six groups (n = 5 mice per group) were formed. Control group (group I) received distilled water, group II, III, IV and V orally received the extract (100 mg kg^{-1} of puffer skin, puffer inner stingray spine and stingray tail respectively) and group VI treated with Paracetamol (100 mg kg^{-1}) 1 h before acetic acid injection. The number of writhing movements was counted for 20 min. The analgesic effect was evaluated by a percentage reduction of writhes in treated group compared to the control group.

FTIR Spectroscopic analysis

Small amount of each sample extract was respectively placed directly on the germanium piece of the infrared spectrometer with constant pressure applied. Data of infrared absorbance was collected over the wave number ranged from 4000 cm^{-1} to 650 cm^{-1}. The reference spectra were acquired from the cleaned blank crystal prior to the presentation of each sample replicate. The FTIR spectrum of all samples was analyzed on the basis of peak values in the region of infrared radiation (Pavia, 2001).

Results
Haemolytic Activity of Crude Fish Extract

The haemolytic activity of the crude extract of *Tetraodon fahaka strigosus* and *Potamotrygon garouensis* against cow, sheep, goat and chicken is shown in Table 1, 2, 3 and 4 respectively. The parts of the fish showed haemolytic activity against all blood group except the spine of *P. garouensis* which show no haemolytic activity. The innards of *T. fahaka strigosus* showed the highest haemolytic activity against cow blood (1024HU) while the skin of *T. fahaka strigosus* showed the lowest haemolytic activity against goat blood (16HU).

Table 1: Haemolytic activity of fish extracts against cow blood

Serial no	Crude extract	Sample concentration (500 µg/ml)	Total haemolysis up to dilution	Haemolytic titre value (HU)
1	Puffer fish innards	500	10	1024
2	Puffer fish skin	500	7	128
3	Stingray fish tail	500	5	32
4	Stingray fish spine	500	0	0

Table 2: Haemolytic activity of fish extract against Sheep blood

Serial no	Crude extract	Sample concentration (500 µg/ml)	Total haemolysis up to dilution	Haemolytic titre value (HU)
1	Puffer fish innards	500	9	512
2	Puffer fish skin	500	7	128
3	Stingray fish tail	500	8	256
4	Stingray fish spine	500	0	0

Table 3: Haemolytic activity of fish extract against Goat blood

Serial no	Crude extract	Sample concentration (500 µg/ml)	Total haemolysis up to dilution	Haemolytic titre value (HU)
1	Puffer fish innards	500	8	256
2	Puffer fish skin	500	4	16
3	Stingray fish tail	500	7	128
4	Stingray fish spine	500	0	0

Table 4. Haemolytic activity of fish extract against Chicken blood

Serial no	Crude extract	Sample concentration (500 µg/ml)	Total haemolysis up to dilution	Haemolytic titre value (HU)
1	Puffer fish innards	500	9	512

2	Puffer fish skin	500	5	32
3	Stingray fish tail	500	7	128
4	Stingray fish spine	500	0	0

Haemolytic Activity of blood agar

Haemolytic activity of blood agar against cow, sheep, goat and chicken is shown in Figure 1. The different parts of the *T. fahaka strigosus and P. garouensis* extract showed zone of inhibition on the blood agar except the spine of *P. garouensis* which showed no zone of inhibition. The maximum zone of inhibition in blood agar plate were (8.5±0.3 mm), (7.7±0.3 mm) and (6.2±0.3 mm) in the innards of *T. fahaka strigosus* against cow, goat and chicken respectively and minimum was (3.7±0.4 mm), (8.1±0.2 mm) and (4.1±0.2 mm) in skin of *T. fahaka strigosus* against sheep, goat and chicken respectively.

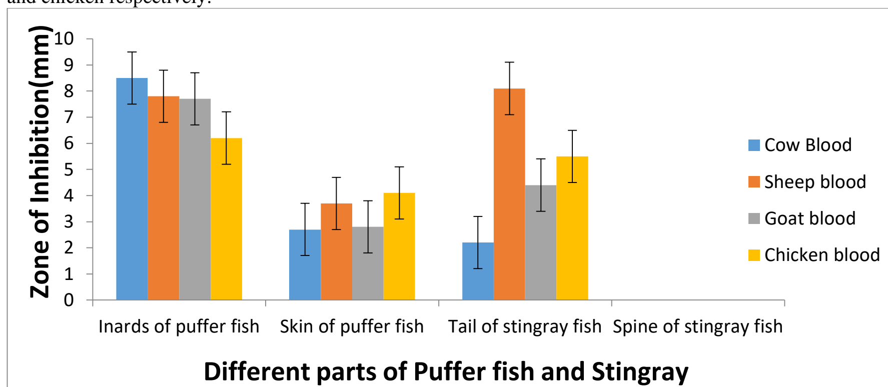

Figure 1: Haemolytic activity of fish extract on blood agar plate

Assay for protease and phospholipase activity

The assay for the protease activity of the crude extract of the different parts of *T. fahaka strigosus* and *P. garouensis* is shown in Table 5. The innards of *T. fahaka strigosus* showed the highest protease activity (0.109 mg/ml/Min). While the spine of *P. garouensis* showed the lowest protease activity (0.023 mg/ml/Min). The assay for the phospholipase activity of the venom is shown in table 6. The innards of *T. fahaka strigosus* showed the highest phospholipase activity (1.07 mg/ml/Min) while the spine of *P. garouensis* showed the lowest phospholipase activity (0.20 mg/ml/Min).

Table 5: Protease activity on stingray and puffer fish

Sample	Activity (mg/ml/Min)
Puffer fish innards	0.109
Puffer fish skin	0.071
Stingray tail	0.039
Stingray spine	0.023

Table 6: Phospholipase activity on stingray and puffer fish

Sample	Phospholipase activity mg/ml/Min
Puffer fish innards	1.07
Puffer fish Skin	0.60
Stingray Tail	0.73

Stingray Spine	0.20

The result obtained in table 1 shows the haemolytic activity of the crude fish extract on cow blood. The result showed the highest haemolytic activity in the innards of *T. fahaka strigosus* (1024HU) and the lowest activity (32HU) in the tail of *P. garouensis*. In sheep blood shown in table 2 the highest haemolytic activity was observed in the innards of *T. fahaka strigosus* (512HU), followed by 256HU in the tail of *P. garouensis* and the least in the skin of *T. fahaka strigosus* (128HU). In goat blood cell the maximum haemolytic activity was observed in innards of *T. fahaka strigosus* (256HU) and the minimum (16HU) in the skin of *T. fahaka strigosus* as shown in figure 1. The haemolytic activity on chicken is shown in figure 1 with the maximum haemolytic activity (512HU) in the innards of *T. fahaka strigosus* and 32HU the minimum in the skin of *T. fahaka strigosus*. The spine of *P. garouensis* showed no haemolytic activity against any of the blood group since Haemolytic Unit (HU) is defined as the amount of protein require to cause 50% haemolysis or the reciprocal of the highest dilution of the toxin in which a haemolytic pattern is obtained. The haemolytic activity shown by *T. fahaka strigosus* correspond to earlier work on other species of puffer fish by Bragadeeswaren *et al*., 2010.

The haemolytic assay on blood agar plate is shown in figure 1. The different parts of the crude fish extract showed good haemolytic activity against various red blood cells except for the spine *P. garouensis* which showed no zone of inhibition. The maximum zone of inhibition in blood agar plate was (8.5±0.3 mm) in the innards of *T. fahaka strigosus* against cow blood and minimum (2.2±0.2 mm) in the tail of *P. garouensis*. Sheep blood agar plate produced highest activity in the tail of *P. garouensis* (8.1±0.2 mm) and lowest in skin of *T. fahaka strigosus* (3.7±0.4 mm). Goat blood agar plate produced maximum zone of inhibition in the innards of in the innards of *T. fahaka strigosus* (7.7±0.3 mm) and the minimum (2.8±0.2 mm) in the skin of *T. fahaka strigosus*. Chicken blood agar plate produced the highest zone of inhibition in innards of *T. fahaka strigosus* (6.2±0.3 mm) and lowest in skin of *T. fahaka strigosus* (4.1±0.2 mm). The result obtained in Table 5 shows the activity of protease enzyme in the crude fish extract. The enzyme activity was determined by measuring the absorbance of the crude fish extract at 540 nm. The innards of *T. fahaka strigosus* showed the highest enzyme activity (0.109 mg/ml/Min) and the lowest enzyme activity (0.023 mg/ml/Min) in the spine of *P. garouensis.* The phospholipase activity of the crude fish extract is shown in Table 6. The innards of *T. fahaka strigosus* showed the highest enzyme activity (1.07 mg/ml/Min) while the spine of *P. garouensis* showed the least enzyme activity (0.20 mg/ml/Min.

Antioxidative activities expressed as DPPH free radical scavenging activity is presented in Figure 2. Both extracts demonstrated reduction in free radical scavenging ability against DPPH when compared to the control. Strong antioxidant activity has been attributed to be the molecular mechanism underlying the mopping up of toxicant induced free radicals. DPPH is a stable free radical which delocalises its spare electron to give a deep violet colour. In the presence of an antioxidant, a hydrogen atom is donated followed by loss of colour.

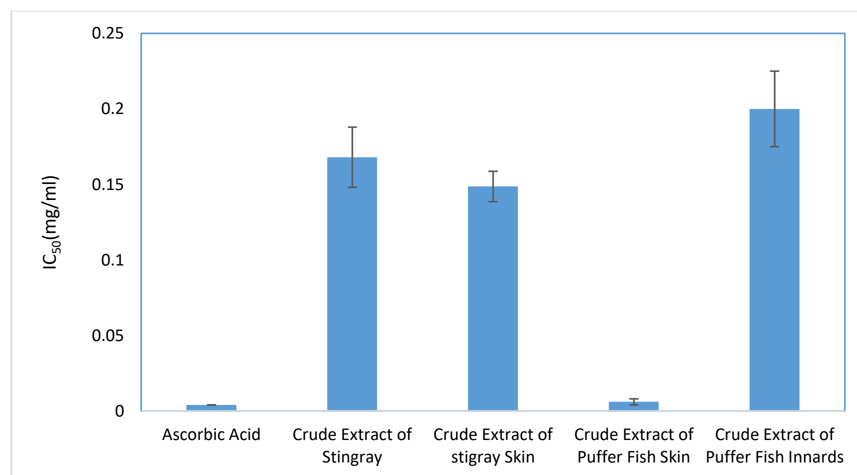

Figure 2: DPPH Scavenging activity of different parts of fish

The median lethal dose extracts from puffer fish in mice shows the effect of this toxin at different dose were the puffer skin extract gavaged at dose 20, 50, 100 in mg/kg and the median lethal dose was recorded to be 31 mg/kg while the puffer innards methanol extract gavage at dose 20, 50 and 100 in mg/kg was recorded to be 70 mg/kg. The changes of body weights revealed that flesh extracts did not affect rat body weight gain in comparison to control. Contrarily, when using puffer innard extracts, the body weight gain was significantly lower than control (Figure 3).

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Table 7: Median Lethal Dose (LD_{50}) of crude extract of *T. fahaka strigosus*

Sample extracts	Dose (mg/kg)	Death After (24 h)	LD_{50} (mg/kg)
	20	0/3	
T. fahaka strigosus (Skin extract)	50	2/3	31.00
	100	3/3	
T. fahaka strigosus (innard extract)	20	0/3	70.00
	50	0/3	
	100	3/3	

Table 8: Median Lethal Dose (LD_{50}) of crude extract of *P. garouensis*

Sample extracts	Dose (mg/kg)	Death After (24 h)	LD_{50} (mg/kg)
	50	0/3	
P. garouensis (tail)	80	1/3	63.00
	100	2/3	
P. garouensis (spine)	50	0/3	90.00
	80	0/3	
	100	3/3	

From the line graph in Figure 3 shows the effect of the extract on the body weight of the mice after two weeks of administration. The puffer skin extract showed more toxic effect compared to the control and also the stingray tail extract showed a great change in weight after 10 days of administration were the mice in that group dropped from 28

g body weight to 18 g and the spine from 28 g to 19 g after 10days. Acetic acid injection-induced writhing was significantly reduced by the oral administration of crude extract by 35, 55 and 59 and 48% for *P. garouensis* skin, *P. garouensis.* innard, *T. fahaka strigosus* spine and *T. fahaka strigosus* tail respectively. Paracetamol used as reference, inhibited the writhing by 80% at 100 mg kg^{-1}

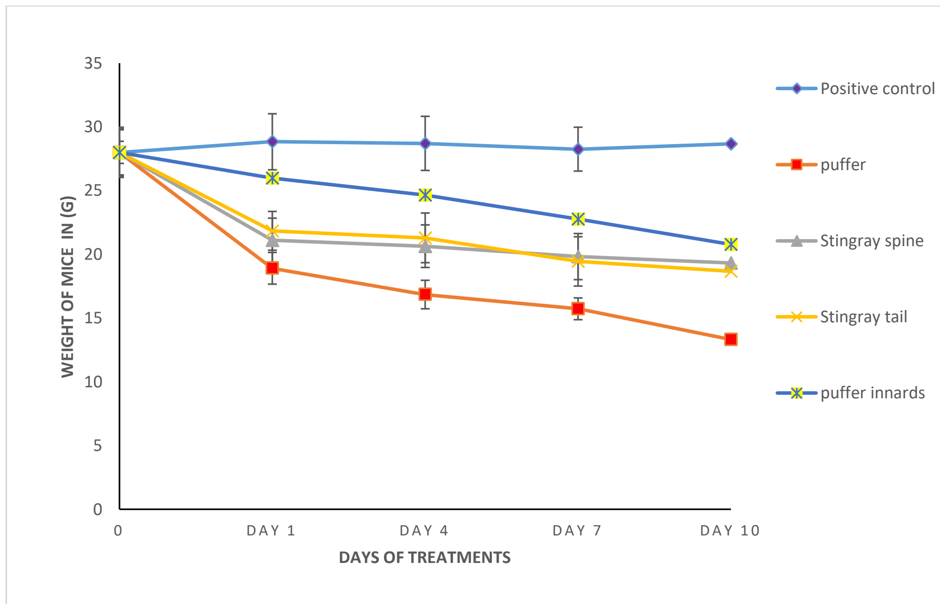

Figure 3: Effect of the crude extract of *T. fahaka strigosus* and *P. garouensis on* Weight changes in mice

Table 9: Effect of the crude extract of *T. fahaka strigosus* and *P. garouensis* on PCV in mice

Sample	PVC (%)
Normal control	47.20±3.56^{ab}
Puffer fish innards	43.40±1.14^{a}
Puffer fish skin	44.00±4.06^{a}
Stingray tail	44.80±3.35^{ab}
Stingray spine	48.60±4.45^{b}

Values are mean ± SD (n = 5) for each group Values are statistically significant compared to control group at p <0.05. Values with different superscript down the group are significantly different (p<0.05).

Table 10: Effects of Crude Extracts of Different parts of *T. fahaka strigosus* and *P. garouensis* biochemical parameters

Treatment n=5	**(IU/L)**			**mg/dl**		
	ALT	**AST**	**ALP**	**Total Protein (TP)**	**Albumin (ALB)**	**Total Bilirubin (TB)**
Normal Control	24.00±1.41	16.60±3.29	59.00±5.00	6.90±0.02	3.36±0.34	0.41±0.04
Puffer spine	26.80±1.64	18.20±0.45	60.20±6.82	6.80±0.02	3.34±0.02	0.44±0.04

Puffer skin	31.80±2.28	20.40±2.30	65.20±2.59	6.16±0.04	3.26±0.14	0.57±0.09
Stingray spine	26.20±2.59	18.40±1.51	62.20±6.01	6.80±1.00	3.32±0.34	0.46±0.07
Stingray Tail	33.80±1.30	24.00±2.35	65.00±4.71	5.82±0.16	3.20±0.16	0.62±0.09

Values are mean ± SD (n=5)

Table 11: Analgesic effect of Crude extract of on acetic acid-induced writhing

S. No	Treatment	Dose mg/kg	No. of writhes	Inhibition of writhing response (%)
1	Control	----	48.66±4.04	-
2	Paracetamol (std)	100	10.05±2.12[b]	80
3	Puffer skin	5	30.65±2.10[a]	35
4	Puffer innard	5	21.95±1.68[c]	55
5	Stingray spine	5	20.10±2.00[c]	59
6	Stingray tail	5	25.45±3.20[c]	48

The absorption spectra of *T. fahaka strigosus* skin and innard sample extracts are shown in Figures 4 and 5. The band in skin extract was observed at 3919.48, 3815.32 and 3479.7 cm^{-1} which represented occurrence of alcohols and phenol compounds. The band at 3398.69, 3329.25 and 3201.94 cm^{-1} represented amine and amide compounds. The peak around 2800.73,2484.4, 2345.91 cm^{-1} and 2179.63 denoted alkanes. Peak at 1643.41 cm^{-1} indicated amino acids. Remaining peaks observed at 1354.07, 1261.49, and 1072.46 cm^{-1} indicated nitro compounds, aromatics, alcohols, carboxylic acids, and esters. In case of *T. fahaka strigosus* innards extract, the peak observed at 3892.48, 3807.61 and 3691.88 cm^{-1} represent alcohols and phenol compounds. The peaks at 3398.69 showed amines and amides. The band at 2881.75, 2800.73, 2522.98, and 2171.92 cm^{-1} indicated alkanes, Carboxylic acid and esters. Peak at 1633.41 cm^{-1} signified amino acids and remaining peaks observed at 1288.49, 1072.46, 933.58 and 756.12 cm^{-1} represented the esters, alkyl halides, ketone and aliphatic amines.

The absorption spectra of *P. garouensis* tail and spine sample extracts are shown in Figure 6 and 7. The band in tail extract was observed at 3919.48, 3765.17 and 3444.98, cm^{-1} which represented occurrence of alcohols and phenol compounds. The band at 3344.68 and 3194.23 cm^{-1} represented amine and amide compounds. The peak around 2823.88, 2553.84, 2341.66 cm^{-1} and 2121.77 denoted alkanes. Peak at 1643.41 cm^{-1} indicated amino acids. The peaks observed at 1365.65 and 1111.03 cm^{-1} indicated carboxylic acids and esters. For *P. garouensis* spine extract the peak observed at 3919.48, 3834.61 and 3614.72 cm^{-1} represent alcohols and phenol compounds. The peaks at 3390.97 and 3225.09 cm^{-1} showed amines and amides. The band at 2661.85, 2800.73, 2411.1, and 2141.06 cm^{-1} indicated alkanes, carboxylic acid and esters. Peak at 1633.41 cm^{-1} signified amino acids while peaks observed at 1303.92, 1084.03, 956.72 and 667.39 indicated carboxylic acids, esters and aliphatic amin

Figure 4: Infra-red spectrum of *Tetraodon fahaka strigosus* (Puffer fish) skin extract

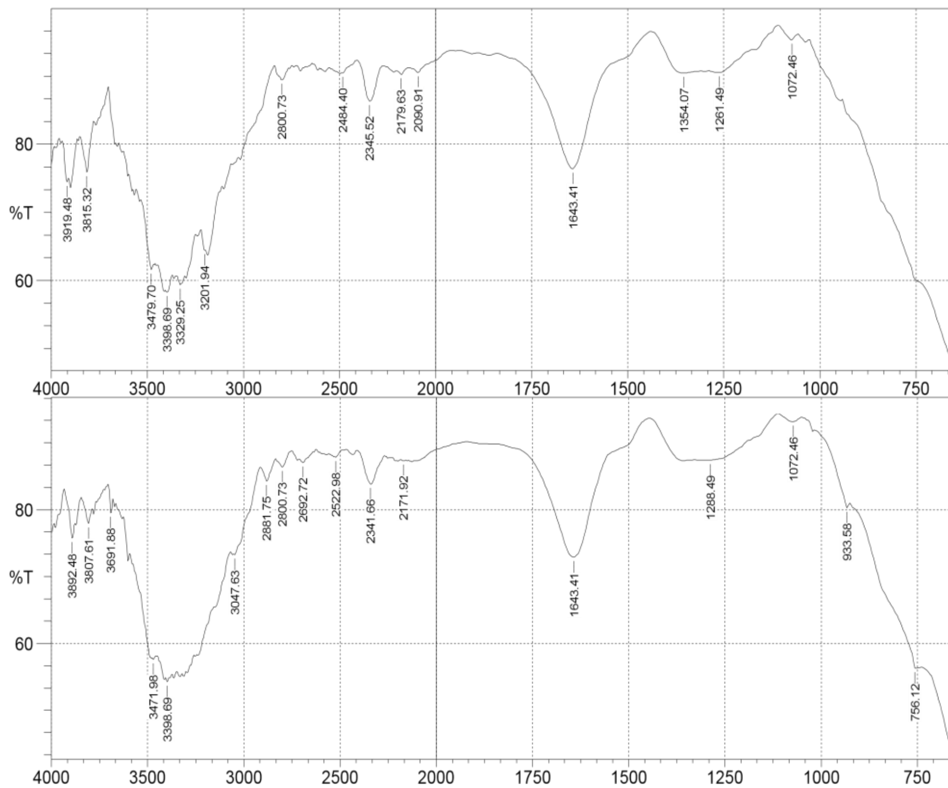

Figure 5: Infra-red spectrum of *Tetraodon fahaka strigosus* (Puffer Fish) Innard Extract

Figure 6: Infra-red spectrum (FTIR) of *Potamotrygon garouensis* (Stingray) tail extract

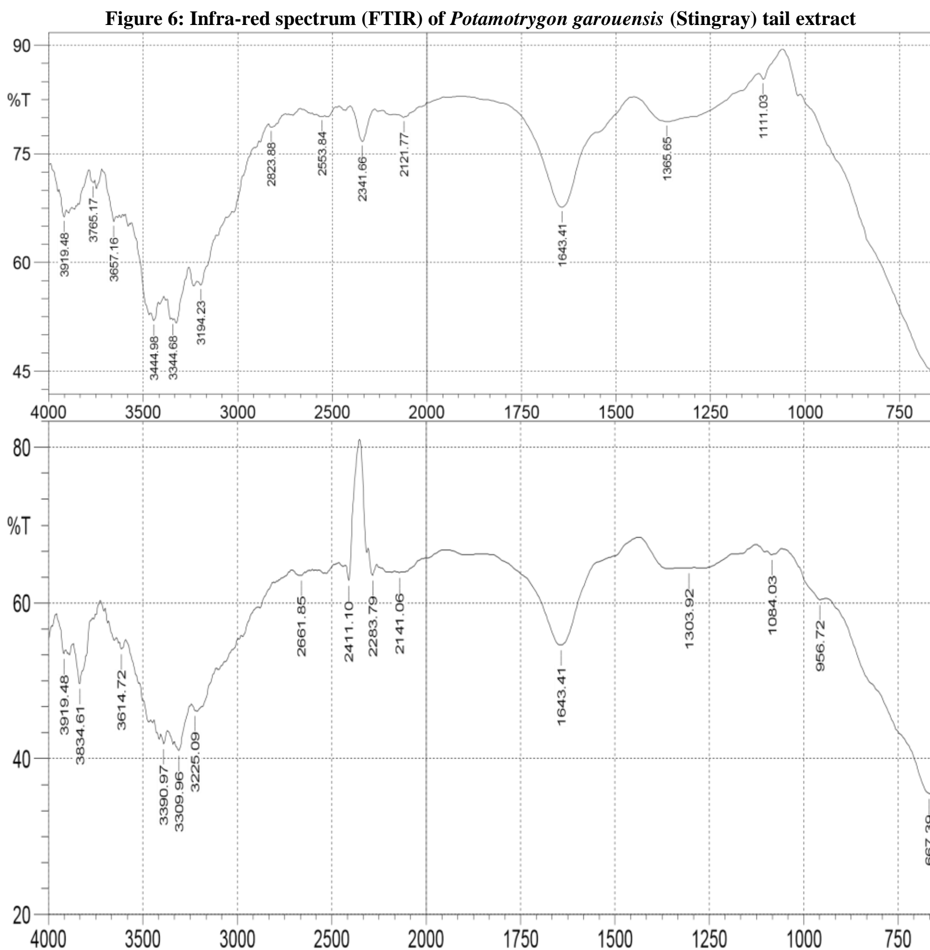

Figure 7: Infra-red spectrum of *Potamotrygon garouensis* (Stingray spine) Extract

Discussion

A large number of marine organisms are known to possess bioactive substances that have tremendous pharmaceutical potential for the future (Qasim *et al*.,1998). Although considerable progress has been made on toxicology of fish venom and most of them have tried to extract its active natural toxic components (Ziegman and Alewood, 2015). Venoms are complex mixtures, including peptides and proteins, some of them with enzymatic activities such as protease, phospholipase A2, and hyaluronidase, among others, as well as non-enzymatic proteins such as pore-forming cytolysins lectins and toxins that bind to ion channels (Chan *et al*., 2016). There are also non-proteinaceous substances, such as biogenic amines and some neurotransmitters, which can be an important part of the envenomation symptomatology which are poorly characterized concerning their roles in the pain induction and inflammatory responses associated with venom toxicity (Campos *et al*., 2016).

This study characterizes some biological and pharmacological activities present in the crude extract from skin and innards of *T. fahaka strigosus* and *P. garouensis.* The haemolytic activity observed in crude extracts of *T. fahaka strigosus* and *P. garouensis* against cow, goat, chicken erythrocytes, although maximum haemolytic activity was observed in cow red blood cells for puffer innards extract. This effect has been attributed to presence of a membrane pore-forming toxins (cytolisin), which is present in all of the studied members of the Scorpaenidae family (Chen *et al*., 2015). It is probable that the haemolytic cytolysin would be also the toxin that induces cytotoxicity in endothelial and other cell types, even though other venom components could also participate in this effect. We found that crude extracts from puffer innards are potent in all types of erythrocytes. These results suggest important differences between the haemolytic mechanisms of these pore-forming agent. An anticancer effect in a member of the cytolysin has been reported (Chen *et al*., 2015). Thus, the haemolytic compounds might have the potential bioresources for future anticancer drugs.
In the present work, we have evaluated some of the most common enzymatic activities reported for animal venoms. The presence of Phospholipase A2 (PLA2) activity has been widely reported in crude extracts and venoms from several cnidarian species (García-Arredondo *et al*., 2016). These enzymes have also been detected in venoms of many diverse animals and display a broad spectrum of biological activities. Furthermore, PLA2s are considered as the major pharmacologically active components of snake venoms. It has been proposed that the presence of PLA2 enzymes in venoms plays an important role in defense against predators, and in the immobilization and digestion of prey (García-Arredondo *et al*., 2016). Moreover, some studies have related PLA2 activity to haemolysis. Serine proteases are other enzymes widely reported in animal venoms that not only have been associated with several physiological functions such as platelet aggregation, fibrinolytic activity, spreading activity of other toxins, but also may induce post-translational modifications of other toxins. The presence of serine protease and PLA2 in the crude venom from tentacles of the jellyfish *Olindias sambaquiensis* was experimentally confirmed, showing that the levels of activity of these enzymes were comparable to those observed in venoms of Bothrops snakes (Selistre and Giglio, 1987); which is also experimentally confirmed with PLA2 presence and protease activities in the crude extracts of *T. fahaka strigosus* and *P. garouensis*. The presence of protease and phospholipase activities were also observed in all crude extracts with higher activities demonstrated by extract from puffer innards for both *T. fahaka strigosus* and *P. garouensis.* The levels of PLA_2 activity of these extracts were lower to those previously reported for the hydrozoans: *M. complanata* and *M. alcicornis*, whereas the protease activity levels were similar to those observed in jellyfish and snakes (Knottel *et al*., 2016). These results suggest that PLA2 and serine proteases play an important role in the toxicity of the scleractinian corals.

In the determination of median lethal dose, it was found that the extracts from the two fishes were lethal to mice, with LD_{50} values varying from 31-90 mg/kg of body weight in mice. it was also detected that the skin of the puffer fish toxin showed more toxicities when compared to other organs under investigation. These results are opposed to those investigation by Ali (1996) who reported that the puffer fish toxins were concentrated in the ovaries and liver, with lesser amounts present in the digestive tract and skin. Mice treated with crude extracts from both fishes showed a slight decrease in body weight. The reduction in body weight is mainly attributed to the reduction in food intake of the treated rats compared with control. This result is in agreement with several studies observed in mice exposed to okadaic acid (a marine toxin) which induced a reduction in body weight after 5 days of treatment (Tubaro *et al*., 2003). During the experimental period, a decrease in consumption food associated with diarrhoea could be the cause of reduction in growth-rate of animals.
Writhing induced by acetic acid injection on animal is a method used to investigate the peripheral analgesic effect of natural products. The crude extracts from *T. fahaka strigosus* and *P. garouensis*, administered significantly inhibit acetic acid induced writhing in mice. The writhing is related to increase in the peritoneal level of prostaglandins and leukotrienes. This result could be suggesting that the analgesic effect was related to inhibition of endogenous mediators, such as substance P and prostaglandins ($PGE_{2\alpha}$, $PGF_{2\alpha}$). Acetic acid injection releases endogenous mediators including histamine, serotonin, bradykinin, substance P and prostaglandins ($PGE_{2\alpha}$, $PGF_{2\alpha}$), which are responsible of abdominal writhing (Sawadogo *et al.,* 2011).

Further processing of crude venomous fishes for potential utilization and management are very important in fish industry, where great economic, nutritional and environmental values can be obtained by the better uses of byproducts (Tongnuanchan *et al*., 2014). The use of antioxidants can reduce the risk of free radical exposure inside the human body. However, long term use of synthetic antioxidants can cause mutagenetic, carcinogenetic and pathogenic. Many animal sources have been found to possess alternative natural antioxidant to enhance the economic and ecological aspects such as red deer, sheep and cattle blood (Bah *et al*., 2016). The potential of natural antioxidant compounds from venomous fishes can play a role in replacing synthetic antioxidants in their use in food or drug ingredients.

Marine organisms are receiving more attention because of their special structure and living environment; notably, a number of studies have been conducted using fish protein hydrolysates as antioxidant peptides, like cod, tuna, salmon (Wang *et al*., 2017). Percent of inhibition is the ability of a substance to inhibit free radical activity. Antioxidant activity expressed in IC_{50} value that is the concentration of an antioxidant substance which can give fifty percent inhibition percentage. A compound can be powerful antioxidant if the IC_{50} value is less than 50 µg/mL, strong if the IC_{50} value is between 50-100 µg/mL, moderate if the IC_{50} values range between 100-150 µg/mL, and weak if the IC_{50} values range between 150-200 µg/mL. The IC_{50} value showed in Figure **2**, indicates that the two samples have strong antioxidant activity. The DPPH scavenging activity of different parts (innards and skin) of puffer fish and stingray as shown in Figure 2 gave IC_{50} values 0.16 mg/ml and 0.15 mg/ml and 0.20 mg/ml and 0.01 mg/ml respectively have significantly antioxidant activity when compared to IC_{50} value 1.56 mg/L for lionfish (Sommeng *et al*., 2019). These values are also better than for other marine animal extracts such as sea urchin extract *Diadema setosum* that has IC_{50} 2.823 mg/L and sea anemone *Stichodactyla gigantea* with 2.07 mg/L (Sommeng *et al*., 2019). However, the venom toxins from different parts of both fishes: puffer fish and stingray have very strong antioxidant activity when compared to other venoms, such as bee venom *Apis dorsata* with IC_{50} value 0.14 mg/L (Sommeng *et al*., 2019). Bioactive components from amino acid and non-amino acid groups such as phenolics that can act as antioxidant compounds. Phenolics are organic compounds known consisting of hydroxyl group (–OH) attached directly to a carbon atom that is a part of aromatic ring. The hydrogen atom of hydroxyl group can be donated to free radicals, thereby preventing other compounds to be oxidized (Nguyen *et al*. 2003). The antioxidant activity is generally related to the major active compounds in essential oils. However, the other compounds like gelatin exhibited antioxidant activities, in which peptide fraction containing particular amino acids such as glycine and proline had high activity as well as tuna-skin and bovine-hide gelatin have been reported to exhibited antioxidant activities (Tongnuanchan *et al*., 2014). Therefore, the extract of different parts of stingray and puffer fish exhibited the potential of natural antioxidant compounds with IC_{50} values 0.16 mg/ml and 0.15 mg/ml and 0.20 mg/ml and 0.01 mg/ml respectively. However, further studies are required to determine the protein concentration and Molecular Weight (MW) of each sample in order to increase the antioxidant activity levels using stingray and puffer fish venoms so that they could potentially provide economic benefits.

The FT-IR analyses of the crude venom extract of both T. *fahaka strigosus* and *P. garouensis* suggest wide spectral profile which confirms the presence of primary amine group, aromatic compound, halide group, aliphatic alkyl group, and polysaccharides. Therefore, further studies are needed in order to purify the active compounds to identify their chemical nature and to evaluate their potential as novel drug from the puffer fish toxin.

Conclusion

The crude extracts from skin and innards of the *Tetraodon fahaka strigosus* (Puffer fish) *Potamotrygon garouensis* (Stingray) composed of cytolysins agent capable of lysing cow, sheep, goat and chicken erythrocytes. The extract contains enzymes such as proteases and phospholipase that undoubtedly play an important role in the toxicity of these fishes. *In-vitro* antioxidant and analgesic activities against acetic acid induced writhing were also demonstrated in mice. The result further suggests that primary amine group, aromatic compound, aliphatic alkyl group indicative of the major biochemical constituents such as polysaccharides, lipids and proteins that can be easily evidenced by FTIR spectroscopy. Additionally, it is suggested that a bio-guided fractionation of the crude venom could lead to isolation of new compounds responsible for the observed biological and pharmacological activities and these potentials are good for further exploitation for possible therapeutic interventions.

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