

Toxicity of plant extracts containing pyrrolizidine alkaloids using alternative invertebrate models

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Abstract. Pyrrolizidine alkaloids (PAs) are a widespread class of hepatotoxic heterocyclic organic compounds found in approximately 3% of world flora. Some PAs have been shown to have genotoxic and carcinogenic effects. The present study focuses on the toxicity effects of four dry extracts obtained from medicinal plants (*Senecio vernalis*, *Symphytum officinale*, *Petasites hybridus* and *Tussilago farfara*), on two aquatic organisms, *Artemia salina* and *Daphnia magna*, and the correlation with their PAs content. A new GC-MS method, using a retention time (TR)-5MS type capillary column was developed. PAs Kovats retention indices, for this type of column were computed for the first time. The lethal dose 50% (LC₅₀) values for the two invertebrate models were correlated (Pearson's coefficient, >0.9) and the toxicity was PA concentration-dependent, for three of the four extracts. All

tested extracts were found to be toxic in both aquatic organism models. The results can be used to develop a GC-MS validated method for the assay of PAs in medicinal plants with a further potential application in the risk assessment study of PAs toxicity in humans.

Introduction

Over the last few decades, there has been increasing interest in the use of natural compounds, often from traditional medicine, as adjuvants and even as replacement of allopathic treatment (1). While prescription medicines have well-defined chemical composition and are supported by evidence-based published studies, in terms of their efficacy and toxicity, this has not been the case with traditional medicines. Indeed, the popular misconception that all 'natural' products are safe has tended to discourage investigation into their potential toxicity (2). Such problems arise even in the case of certain therapeutic teas which have even special monographs in Pharmacopoeias (3).

Pyrrolizidine alkaloids (PAs) are heterocyclic organic compounds, which are found in more than 6,000 plant species (approximately 3% of world flora) as secondary metabolites (4). Approximately 95% of PAs are found in plants from Senecioneae and Eupatorieae tribes (Asteraceae), in several genera of the Boraginaceae family, in the genus *Crotalaria* (Fabaceae), and in some genera of the Orchidaceae family (5). PAs are esters of heterocyclic amino alcohols termed necines (Fig. 1), with aliphatic monocarbonic or dicarbonic acids (necine acids). These alkaloids can be found, in plants, both as a free base and as the corresponding pyrrolizidine alkaloids N-oxides (PANOs) (4).

The toxicity of PAs is structure-dependent as the presence of a double bond in the necine base, often referred to as 1,2-unsaturated PAs or dehydroPAs, has been associated with greater toxicity in comparison with the saturated necine bases (6). PAs are essentially stored in the plant as protoxins, in the benign N-oxide form, whereas in the gastrointestinal

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Abbreviations: PAs, pyrrolizidine alkaloids; PANOs, pyrrolizidine alkaloids N-oxides; SEN, *Senecio vernalis* extract; SYM, *Symphytum officinale* extract; PET, *Petasites hybridus* extract; TUSS, *Tussilago farfara* extract; RI, Kovats retention indices; T_R, retention time; dw, dry material; LC₅₀, lethal dose 50%; CI 95%, 95% confidence interval

Key words: pyrrolizidine alkaloids, *Artemia salina*, *Daphnia magna*, acute toxicity, medicinal plants, GC-MS analysis

tract of animals they are reduced to the corresponding amine becoming toxic (7).

Several cases of poisoning, some fatal, due to the use of medicinal plants containing PAs have been reported. In addition, the consumption of cereals and bakery products contaminated with seeds of species containing PAs has been involved in mass poisonings in rural areas of Afghanistan, India, South Africa and the former USSR (8). Poisoning can manifest as acute or subacute veno-occlusive disease with specific symptoms such as persistent hepatomegaly, which in most cases, progresses to cirrhosis (9). Some PAs have been shown to have genotoxic, mutagenic, teratogenic and carcinogenic effects (10). Thus, research into the presence, identification and quantification of PAs as well as their toxicity is important regarding human consumption of food from plant origin in general and medicinal plants particularly (11). It is thus important that commercially available beverages (infusions) of plants should be tested for their qualitative and quantitative levels of PAs.

Petasites hybridus (butterbur), *Tussilago farfara* (coltsfoot) and *Symphytum officinale* (comfrey) are species traditionally used in phytotherapy and are commonly found in specialized shops for tea beverages. *Petasites hybridus* root has been used in the treatment of migraine, dysmenorrhea, asthma and allergic rhinitis (12). *Tussilago farfara* leaves are mainly used to relieve dry cough and other respiratory disease symptoms (13). Findings have shown that a methanolic extract obtained from the leaves and stems of this species could be used in anti-cancer therapy as a TNF-related apoptosis-inducing ligand sensitizer (14). *Symphytum officinale* root is used in cases of gastro-intestinal and respiratory tract diseases (4), whilst *Senecio vernalis* (spring groundsel) is not as commonly used in phytotherapy. However, the aerial parts of *Senecio* species, under the generic name of *Senecionis herba*, have been used for their antidiarrheal, diuretic, emmenagogue, galactagogue and expectorant properties (15).

Daphnia magna (Straus) is a freshwater zooplankton of the order Cladocera that has been routinely used as a standard test species in ecotoxicology (16) due to their ease of handling in the laboratory, and also to its relatively high sensitivity to a large number of toxicants. *Daphnia magna* are sexually parthenogenic and their clonal reproduction offers the supreme advantage of genetic uniformity (17). *Artemia salina* is a species of brine shrimp from the Anostraca order, extensively used in ecotoxicology, due to the reliability, feasibility and cost-effectiveness of the tests (18).

Most toxicity studies of natural products containing PAs have been carried out on laboratory animals using isolated alkaloids including lasiocarpine, senkirkine, retrorsine, seneciphylline, and riddelliine (19). The toxicity of different plant extracts obtained from the four studied plant species was investigated on cell cultures, bacteria or animals (20-23). To the best of our knowledge, the effect of these species on aquatic species has not been studied so far.

The present study focuses on the PAs content in some extracts obtained from the aforementioned plants. To test their toxicity upon the two *in vivo* invertebrate models, *Artemia salina* and *Daphnia magna*, and the PAs content was correlated with the toxic effect.

Materials and methods

Dry plant extracts. Coltsfoot leaves, common butterbur roots teas (produced by Stef Mar, Ltd., Ramnicu Valcea, Romania) and comfrey roots tea (produced by Fares, Orastie, Romania) were purchased from retail stores. *Senecio vernalis* aerial part was harvested in May 2013, from Craiova Botanical Garden (Craiova, Romania), naturally dried and conserved in laboratory conditions. The morphological characters of the vegetal material were compared with the ones quoted by literature. A voucher specimen is available in the collection of the Department of Botany and Cell Biology, 'Carol Davila' University of Medicine and Pharmacy (Bucharest, Romania), and at 'Dimitrie Brandza' Botanical Garden (Bucharest, Romania; no. 405789).

The dry extracts were obtained as previously described (24). Briefly, the dried plants were ground (Tyler mesh 48), and 20 g of each plant material were refluxed twice for 2 h with 1,000 ml of 50% methanol acidified with citric acid to pH 2.0-3.0. The combined extracts were evaporated, under reduced pressure with a rotary evaporator system (Buchi, Flawil, Switzerland) to about 300 ml and atomized with a Mini Spray Dryer B-290 (Buchi). The extracts were coded as follows: SEN (*Senecio vernalis*), SYM (*Symphytum officinale*), PET (*Petasites hybridus*) and TUSS (*Tussilago farfara*).

GC-MS analysis. For the PAs content assay, 2 g of SEN and SYM and 4 g of PET and TUSS were dissolved in 30 ml of methanol acidified with 50% citric acid to pH 2.0-3.0. After complete dissolution, zinc powder (Merck KGaA, Darmstadt, Germany) was added in excess and the solutions were stirred for 3 h using a magnetic stirrer (HI 190M; Hanna Instruments, Woonsocket, RI, USA) to convert PANOs to PAs. After filtration, the solutions were purified by liquid-liquid extraction twice with chloroform and twice with diethyl ether. The aqueous solutions were alkalized with 25% aqueous ammonia (pH 9-10) and the PAs were extracted three times with 30 ml of chloroform. The chloroform solutions were dried under nitrogen flow at room temperature, the residue was dissolved in 2 ml methanol and filtered through a 0.2 μ m syringe filter (Pall Life Science, Port Washington, NY, USA), and analyzed by GC-MS.

Standard solutions in methanol (HPLC isocratic grade; Merck KGaA) were prepared from a stock solution of senecionine (200 mg/ml, GC \geq 95%; Carl Roth GmbH & Co. KG, Karlsruhe, Germany). The GC-MS analysis was carried out on a Focus gas chromatograph (Thermo Fisher Scientific, Inc., Waltham, MA, USA) equipped with retention time (TR)-5MS (5% phenyl polysilphenylene-siloxane) capillary column (30 m x 0.25 mm x 0.25 μ m; Thermo Fisher Scientific, Inc.). The capillary column was directly coupled to a quadrupole mass spectrometer (DSQII; Thermo Fisher Scientific, Inc.).

Chromatographic conditions were as follows: Samples (1 μ l) were injected in the split mode using helium as carrier gas (flow rate of 1.0 ml/min); the injector temperature was 225°C and column temperature program: 100°C for 1 min; ramp to 200°C at 20°C/min; ramp to 300°C at 10°C/min; analysis performed at 300°C, the ionization by electron impact at 70 eV; detection in full scan mode within the range m/z 60-650. In order to obtain the Kovats retention indices (RI), to be further used

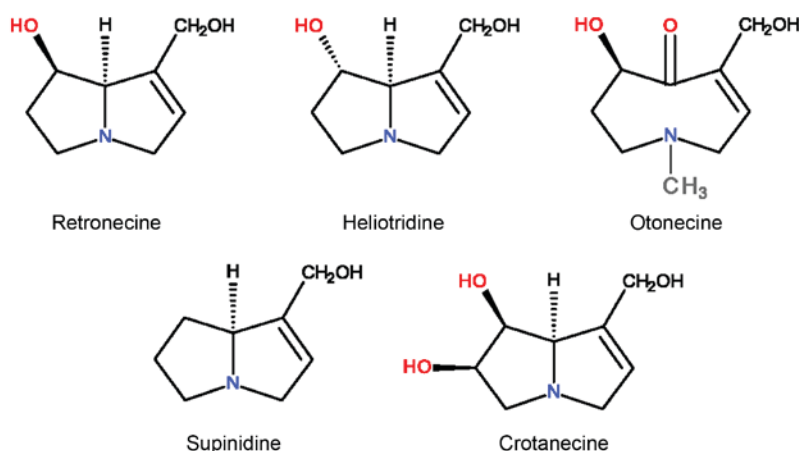


Figure 1. The structures of major 1,2-unsaturated necines.

to compare the retention times obtained on the TR-5MS with those obtained on the DB-5 type column (25,26), a mixture of n-alkanes (C21-C40) was analyzed at 300°C and maintained up to 40 min (26,27).

Identification of the PAs was carried out by means of the Kovats RI and the comparison of their mass spectra with those found in the NIST 0.2 database when available, and data from the literature (25-28). Due to the characteristic mass fragmentation pattern of PAs, a selected ion monitoring (SIM) method was used for the determination of retronecine/heliotridine type (m/z 93, 120 and 136) and otonecine type (m/z 110, 151 and 168).

For the quantitative determination, senecionine was used as a standard for the calibration curve. The area obtained for each PA was interpolated on this curve and the results were expressed in milligrams senecionine equivalents per 100 g of dry material (dw).

The total amount of PAs obtained was compared to the results obtained for the same extracts with those obtained by a modified Ehrlich spectrophotometric method (24).

All the measurements were performed in triplicate. The results are expressed as means \pm standard deviation (SD).

Toxicity assessment

Artemia salina assay. Brine shrimp eggs were obtained from a local aquarium shop (Bucharest, Romania) and hatched for 48 h in breakers containing artificial sea water (36 g/l salinity) at 25 \pm 1°C under continuous aeration, in a plant growth chamber (Sanyo MLR-351 H; Sanyo, San Diego, CA, USA), using a 16 h photoperiod per day. The newly hatched nauplii were separated from the shells and transferred to fresh artificial sea water with a micropipette. The assay was performed in the growth chamber, under the conditions described above, using different concentrations of extracts (1.0-1,000.0 μ g/ml) in artificial sea water with 1% DMSO, on 50 larvae in 500 μ l final volume of each dilution of the extracts. Concentrations varied for each extract and were chosen based on results obtained from preliminary studies. Artificial sea water with 1% DMSO was used as the negative control. After 24 h, the number of surviving nauplii was counted and recorded. Larvae were considered dead only if they did not move their appendages for 10 sec during observation (29-31).

Daphnia magna assay. The daphnids, maintained parthenogenetically in 'Carol Davila' University, Department of Pharmaceutical Botany and Cell Biology, were selected according to their size and kept in fresh synthetic water (32,33) under continuous aeration, 24 h prior to the experiment. The assay was performed for the extracts in fresh synthetic water with 1% DMSO (10-1,000 μ g/ml) in 15 ml glass tubes, with 10 daphnids/tube. Concentrations for each extract were settled after several preliminary tests. Fresh synthetic with 1% DMSO was used as the control. Lethality was recorded after 24, 72 and 120 h. The daphnids were considered dead only if they did not move their appendages for 30 sec during observations (34). During the experiment the daphnids were kept at 25 \pm 1°C under continuous aeration, in a plant growth chamber (Sanyo MLR-351 H), using a 16 h photoperiod per day (35).

Statistical analysis. The toxicity tests were performed in duplicate. The lethality percentage was calculated and plotted against the logarithm of concentrations and the lethality-concentration curves were calculated using the least squares fit method. The lethal concentrations (LC₅₀) which produce a lethality value of 50, were determined by interpolation on lethality-concentration curves. The upper and lower limits of the 95% confidence interval (95% CI) were calculated. The correlation between the LC₅₀ values for the two invertebrate models was determined by the use of Pearson coefficient. All calculations were performed using GraphPad Prism version 5.01 software (GraphPad Software, Inc., La Jolla, CA, USA).

Results

GC-MS analysis. Both the qualitative and quantitative analysis of the extracts were validated. Thus, the RI values were determined on a TR-5MS column. The consulted literature did not report any RI values for PAs using this type of column, but there were reports on a DB-5 type column (25,26). Fletcher *et al* (27) analyzed the PAs from *Crotalaria* sp. by GS-MS using a TR-5MS column and observed that the same retention order for the compounds, although RI values were all 1.02-fold higher than those obtained on DB-5 columns. Similarly, our results showed that for the TR-5MS type column

Table I. Pyrrolizidine alkaloid content and composition of SEN, PET, TUSS and SYM.

Extract	PA (type)	T _R (min)	RI _{DB-5}	RI _{TR-5MS}	PA content ^a
SEN	Senecivernine (R)	13.10	2,330	2,395	3.78
	Senecionine (R)	13.23	2,341	2,412	346.14
	Seneciphylline (R)	13.45	2,360	2,439	40.40
	Integerrimine (R)	13.77	2,402	2,481	26.26
	Senkirkine (O)	14.71	2,530	2,608	78.28
SYM	Intermedine (R)	11.97	2,188	2,253	7.48
	Symphytine (R)	12.45	2,244	2,312	117.92
	Lasiocarpine (H)	12.55	2,257	2,325	31.64
	Symveridine (R)	13.97	2,325	2,406	0.52
TUSS	Senecionine (R)	13.23	2,341	2,412	0.74
	Senkirkine (O)	14.66	2,530	2,604	2.44
PET	Senecionine (R)	13.24	2,341	2,413	1.84
	Integerrimine (R)	13.78	2,402	2,482	0.47
	Senkirkine (O)	14.69	2,530	2,605	0.86

^aExpressed in mg senecionine/100 g dry weight extract. SEN, *Senecio vernalis* extract; PET, *Petasites hybridus* extract; TUSS, *Tussilago farfara* extract; SYM, *Symphytum officinale* extract; PA, pyrrolizidine alkaloid; T_R, retention time; RI_{DB-5}, DB-5 (as in refs. 25,26); RI_{TR-5MS}, TR-5MS obtained RIs; R, retronecine type; O, otonecine type; H, heliotridine type.

Table II. Comparison of the quantitative analysis results for the extracts.

Extract	Total PAs (mg senecionine/100 g dry weight extract)	
	GC-MS	Spectrophotometric ^a
SEN	424.92	416.58
SYM	150.24	157.56
PET	2.11	2.31
TUSS	0.97	0.74

PAs, pyrrolizidine alkaloids; SEN, *Senecio vernalis* extract; SYM, *Symphytum officinale* extract; PET, *Petasites hybridus* extract; TUSS, *Tussilago farfara* extract. ^aAs in ref. (24)

the elution order of the PAs from the 4 extracts coincided with that reported in the literature for a DB-5 type column and the RI values were 1.03-fold higher (Table I). Therefore, we can assume that the retention times were correctly assigned.

For the quantitative analysis, the total amount of PAs obtained by the GC method was compared to the results of a spectrophotometric quantitative analysis which also quantified PAs, expressed as mg senecionine/100 g dry weight of each extract. Results are presented in Table II.

Toxicity assessment. Preliminary tests showed, in case of *Artemia salina*, that a lethality >90% was caused by SEN (over 250 µg/ml), TUSS (over 500 µg/ml), PET (over 400 µg/ml) and SYM (over 750 µg/ml) (Table III). Lethality vs. the logarithm of concentration regression curves were

afterwards plotted, and the LC₅₀ and 95% CIs (α=0.05) could be calculated (Table III).

In case of *Daphnia magna* SEN, TUSS and PET induced lethality of >90% in the concentration range of 500-1,000 µg/ml and SYM at 1,000 µg/ml. After logarithm of the concentration vs. lethality regression curves were plotted, LC₅₀ could not be calculated for SYM at 24 h, therefore it was considered >2,000 µg/ml. In all cases tested, the viability of the controls was 100%.

Discussion

Even though the identification of PAs by GC-MS was first carried out in the early 1990s (25), their assay, mainly in plants used as therapeutic teas, remains of interest due to their potential toxicity (36-39). This report presents four plant extracts, which were analyzed for their qualitative and quantitative content in PAs: *Senecio vernalis*, *Petasites hybridus*, *Tussilago farfara* and *Symphytum officinale*. While the last three are commonly used in phytoterapy, *Senecio vernalis* was analyzed mainly for its already known high content of PAs (40), in an attempt to emphasize the risk of using PAs in therapy, if any. Moreover, while the extracts from the last three plants (PET, TUSS and SYM) were obtained from commercial teas (dry plant material), SEN extract was obtained from plants harvested and prepared similarly to teas (aerial parts were dried in the conditions in which common teas are dried) mainly as it contained a significant amount of senecionine.

A novel GC-MS method for the qualitative and quantitative determination of certain PAs from dry vegetable extracts of the four plants was developed using a TR-5MS capillary column. In order to compare our results to those already described in the literature, which used a DB-5 column (25,26), the Kovats

Table III. Acute toxicity of the extracts on *Artemia salina* and *Daphnia magna*.

Extract	Determination time (h)	AS		DM	
		LC ₅₀ (μg/ml)	95% CI (μg/ml)	LC ₅₀ (μg/ml)	95% CI (μg/ml)
SEN	24	131.22	109.14-148.59	95.67	95.58-95.75
	72	nt	nt	83.31	74.73-92.51
	120	nt	nt	5.28	a
SYM	24	707.95	698.23-726.11	b	a
	72	nt	nt	801.0	690.42-876.48
	120	nt	nt	412.3	237.18-565.89
PET	24	296.48	269.15-343.56	339.8	256.9-449.4
	72	nt	nt	178.6	51.42-620.3
	120	nt	nt	43.52	a
TUSS	24	222.33	181.13-270.40	509.04	a
	72	nt	nt	189.97	105.02-459.30
	120	nt	nt	37.40	5.84-314.20

a, 95% CI could not be determined, the variance being too large; b, LC₅₀ could not be calculated due to the lethality (%) values; it was considered >2,000 μg/ml. AS, *Artemia salina*; DM, *Daphnia magna*; 95% CI, 95% confidence interval; LC₅₀, lethal concentration 50; SEN, *Senecio vernalis* extract; SYM, *Symphytum officinale* extract; PET, *Petasites hybridus* extract; TUSS, *Tussilago farfara* extract.

RI was computed for senecivernine, senecionine, seneciphylline, integerrimine, senkirkine, intermedine, symphytine, lasiocarpine, and symveridine. Both the order of elution for these compounds and the ratio between the retention times obtained in this study and those reported on a TR-5 column, of 1.03, were the same as in the case of DB-5ms and DB-5 columns (27).

The largest amount of PAs was found in SEN (494.86 mg/100 g dw), the predominant alkaloid being senecionine (346.14 mg/100 g dw). For TUSS and PET the total PAs concentration was similar, 3.17 mg/100 g dw and 3.18 mg/100 g dw, respectively. The main PA for PET was senecionine (1.84 mg/100 g dw) and for TUSS the otonecine-type PA senkirkine (2.44 mg/100 g dw). For SYM the total PAs concentration was 157.56 mg/100 g dw with symphytine as the main alkaloid (117.92 mg/100 g dw). There is currently no international standard for the maximum allowable level of PAs in foods. However, in the Netherlands, the total PA content (including PANOs) in herbal preparations or herbal extracts, must not exceed 1 μg/kg (41). These limits are far exceeded by the tested extracts.

The toxicity of the extracts was determined using two experimental models of crustaceans - *Artemia salina* and *Daphnia magna*. These models can be predictive for the toxicity of extracts containing PAs in animals and humans because numerous studies have demonstrated the presence of cytochrome P450 in these organisms, enzymes needed for the biotransformation of PAs to the toxic pyrrole derivatives (42-45). The assays consisted in the exposure of the invertebrates at increasing concentrations of test substances and determination of the lethal concentrations 50% (LC₅₀) at different time intervals (24-120 h). Substances with LC₅₀ <1,000 μg/ml are considered toxic (46).

In case of *Artemia salina*, the low LC₅₀ obtained for SEN (131.22 μg/ml) indicates that this extract is the most toxic of those studied. TUSS and PET are approximately 1.5-fold and 2.3-fold less toxic and SYM is about 5.4-fold less toxic. The CIs (P<0.05) could be calculated for all extracts, and they presented very high limits for PET and TUSS, but were narrow for SEN (approximately 50 μg/ml) and SYM (approximately 30 μg/ml). It should be noted that all tested extracts were found to be toxic in the case of this species.

Concerning *Daphnia magna*, it was observed that the extracts were generally toxic in the following order: SEN > PET > TUSS > SYM. Thus, SEN has the highest toxicity, TUSS and PET exhibit similar toxicity, with LC₅₀ values being very similar, particularly on the 3rd and 5th day. SYM is substantially non-toxic at 24 h, has low toxicity after 72 h and moderate after 120 h of exposure.

In order to determine whether there is a correlation between the LC₅₀ values obtained from *Artemia salina* and *Daphnia magna* (at 24, 72 and 120 h) assays for each extract, Pearson coefficient was calculated for the pairs *Artemia salina* (at 24 h) and *Daphnia magna* at 24, 48 and 72 h. The values obtained were greater than 0.97, which indicates a strong positive Pearson correlation. SEN, the extract with the highest concentration of PAs, exhibits the highest toxicity and the LC₅₀ obtained for PET and TUSS, extracts that have similar PAs concentrations, had similar values. SYM, the extract with the second PAs concentration 157.56 mg/100 g dw, presented in both cases the lowest toxicity. This result could be explained by the fact that *Symphytum officinale* contained senecionine under the limit of detection, if any. Alternately, other components present in the extract could reduce the effect of existing PAs in SYM. Further studies are to focus on both hypotheses.

The LC₅₀ computed for each extract corresponded to an LC₅₀ of total PAs of 2.15-1,063 µg/l for the *Artemia salina* assay and 0.36-1,203 µg/l for the *Daphnia magna* assay. Mulder *et al.* (47) determined the occurrence of PAs in herbal teas from different European countries. For the teas containing no PA-producing plants (n=166), in the majority of samples (91%), one or more PAs were detected, with a mean total value of 6.13 µg/l. For the tea infusion obtained from PA-producing plants (n=12), the PAs levels were 2.4-414 µg/l. It can be seen that PAs levels in these infusions are in the same range as the LC₅₀ for the two aquatic organisms.

In conclusion, all four extracts containing PAs were shown to be toxic in the model tests on aquatic organisms. Toxicity increased with the PAs concentration, except for the extract from *Symphytum officinale*. PAs LC₅₀ was in the same range as PAs levels found in some herbal tea infusion from Europe.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

OCS, OTO, MI and DMM defined the research theme. OCS, OTO, CMG and GMN designed the methods and experiments and performed the laboratory experiments. SN, CEZ and CNP analyzed and interpreted the data. OCS and OTO wrote the manuscript. MI, DAS, MDC, AMT and DMM critically revised the paper for important intellectual content. AMT and DMM gave the final approval of the version to be published. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

Demetrios A. Spandidos is the Editor-in-Chief for the journal, but had no personal involvement in the reviewing process, or any influence in terms of adjudicating on the final decision, for this article.

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