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RESEARCH

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UHPLC assessment of embelin in specialized mangrove plant *Aegiceras corniculatum* (L) Blanco

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Abstract

Background: Embelin is one of the biologically charming natural benzoquinones with wide medicament use in therapeutics that is pertained to its unique biochemical structure. Mostly, the plants belonging to Myrsinaceae family especially the fruits of genus *Embelia* are rich source of embelin. However, the colossal use of these plants as an active ingredient in several drug formulations has led toward their decrease population number increasing the threat status. This major issue requires a well-versed scientific approach to search for suitable alternative substitute that can be used as an active embelin source. On this panorama, *Aegiceras corniculatum* (L.) Blanco can be used as alternate source of embelin. On this aspect, all the plant parts (root, stem bark, leaf and fruit) are being estimated for embelin content both qualitatively and quantitatively using spectrophotometric and ultra-high-performance liquid chromatographic (UHPLC) methods and also asserted through histochemical analysis. All the aforesaid plant parts are extracted through Soxhlet and water bath methods separately and analyzed for embelin content after isolation through column chromatography and thin layer chromatography.

Results: From results, it was evaluated that all plant parts showed the presence of embelin in a range of 0.17–1.95% dry wt. through UHPLC method, while the highest content was found in fruit followed by root, bark and leaves. The retention time for embelin was found to be 2.7 min.

Conclusions: From the above experimentation, both vegetative and reproductive parts of *A. corniculatum* have shown the presence of embelin that can be utilized in ample amount so as to reduce the threat status of its primary source.

Keywords: *Aegiceras corniculatum*, Embelin, Histochemical, UHPLC analysis

Background

Aegiceras corniculatum (L) Blanco, commonly known as River/black Mangrove, is a specialized Mangrove plant in the Myrsinaceae family category. Mangroves are intertidal productive forested wetlands, encumbered to the specific tropical and subtropical estuarine zones (Kumar 2000) and constitute a vital component of marine flora having significant ecological and socioeconomic value. *A. corniculatum* is a small evergreen mangrove tree commonly found along the eastern and western coasts of India (Clarke 1995). It is widely reported to contain several secondary metabolites, namely benzoquinones, tannins, coumarins, flavonoids, polyphenols, saponins, triterpenes, etc. (Bandaranayake 2002), with potent pharmacological activities, viz. antifungal, piscicidal activity (Aseer et al. 2009), anti-inflammatory, antioxidant (Banerjee et al. 2008; Roome et al. 2008), hepato-protective (Roome et al. 2011), antidiabetic (Gurudeeban et al. 2012) and anticancer (Rahman and Khan 2013). It is being scatteredly reported to have

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embelin (Bandaranayake 2002; Alongi 2009; Rahman and Khan 2013; Thota et al. 2016; Swami et al. 2017), yet no validated study has till now been carried out to explore embelin quantity from this less known but special group of plant. Though structural elucidation through crystallography study was seen (Thota et al. 2016), no report on the UHPLC study is grounded from each and every plant part. Moreover, several studies have been carried out for determination of many unknown compounds through UHPLC-based technique utilization (McCalley 2012) even used as biomarkers for disease (Denoroya et al. 2013). It is an efficient method for rapid identification of compounds. Hence, the identification of embelin through UHPLC method would validate its content in all the plant parts of *A. corniculatum.* Due to the lack of concrete evidence regarding the presence of embelin in this special group of plants, the present piece of work has been taken forward to validate the presence of embelin in each and every *A. corniculatum* plant parts and to quantity its quantity.

Methods

Plant materials

Aegiceras corniculatum (L) Blanco (Myrsinaceae) different plant parts (minimum number of preferential specimens) were collected during the month of July from Bhitarkanika mangrove forest areas (procuring permission and approval of local and government administration), Odisha coast, India (20°18′–20°32′N and 86°41′–86°48′E). The species is identified by one of the authors Dr. Uday C. Basak, from the Regional plant Resource Center, India. The species was deposited in the herbarium of Regional plant Resource Center, India, for the purpose of verification (bearing voucher specimen no 4618) and also verified through the reference book "The Flora of Odisha" (Saxena and Brahmam 1995).

Standard preparation

For comparison and validation with the unknown extracted plant samples, standard embelin solution was prepared taking synthetic embelin (Sigma-Aldrich, Germany) in HPLC Grade methanol (1 mg/ml) (Spectrochem, Bangalore, India) and kept at 4 °C temperature for further use. The purity of the synthetic embelin was verified by measuring its λmax and HPLC chromatogram.

Pre-treatment and preparation of sample extract

All shed dried samples were pulverized, and 10 g of each was extracted through Soxhlet apparatus using methanol and chloroform as solvent system (Spectrochem, Bangalore, India) termed as Extraction-1 (Rastogi et al. 2014). Similarly, 5 g of each was extracted through water bath using same methanol and chloroform as solvent system (Spectrochem), termed as Extraction-2 (Ganesan et al. 2010). The extracted samples were crystallized using ice-cold absolute ethanol (Spectrochem, Bangalore, India) (Radhakrishnan et al. 2011; Gupta et al. 2012) for further use.

Embelin isolation (column chromatography method)

For isolation of embelin, the crude extracts were subjected to column chromatography procedure using petroleum ether/benzene as column solvent with a ratio of 2:3 and silica gel (60–120 mesh) as packing material (Gupta et al. 2013). The chromatographically isolated compound was re-crystallized as shiny orange compound with ethanol, and its purity was confirmed through UHPLC method (Rastogi et al. 2014).

Embelin identification and isolation (TLC method)

After column chromatography, eluted samples were further isolated using thin layer chromatography (TLC) method. For the isolation process, TLC was performed on TLC plates (Kukkar et al. 2010). Various mixtures of solvent systems were used to develop the optimal suitable mobile phase for isolation of embelin. Among all, the mobile phase selected for TLC process was a mixture of n-propanol/n-butanol/ammonia (SRL, India) in a ratio of 7:1:2. For viewing the plates, 1% vanillin sulfuric acid (SRL, India) solution was used as chromatogenic reagent. After TLC, the retention factor (Rf) value of the test samples was determined against the standard embelin solution (Vandana and Arora 2010).

Embelin estimation (spectrophotometric method)

Isolated embelin compound from each extract was dissolved in methanol and chloroform; the solution was scanned within the range of 200–800 nm on UV–visible spectrophotometer in a 1-cm quartz cell get the maximum wavelength at 298 nm. Now known amount of sample extracts were measured through UV spectrophotometer at 298 nm wavelength for embelin content (Sudani and Vidyasagar 2012; Belete et al. 2014).

Embelin estimation (UHPLC method)

UHPLC instrumentation

The analysis was done in UHPLC system (Elizabeth and Jashna 2021), Dionex Summit (Dionex Corp., Sunnyvale, CA, USA) equipped with a P680 quaternary low-pressure gradient pump unit, TCC-100 Thermostatted column compartment and DAD UV 340U UV detector. C18 column (4.6 × 150 mm column, 5 µm diameter pore size) was used for the separation process. The instrument was loaded with Chromeleon Chromatography Management System (version 6.7) for data collection and acquisition. Processing was done using HPLC Syringe of 25 µL

capacity (Model-Hamilton 1702 RNR, Sigma-Aldrich, Germany).

Mobile phase optimization

Mobile phase optimization was done based on the level of resolution of the analyte that was separated along with the peak properties and applicability of the method involved. For the assessment, methanol (Spectrochem) along with 0.1% TFA (SRL, India) in water with a ratio 65:35 was selected as a suitable solvent system for the separation process (Rastogi et al. 2014).

Chromatographic conditions

Chromatographic separation was performed on a Dionex make UHPLC system (Rastogi et al. 2014). The detailed chromatographic conditions are given in Table 1.

Method validation

The purposed UHPLC method was validated by defining linearity, peak purity, correlation coefficient, limit of quantification, limit of detection, relative standard deviation, accuracy, peak purity, recovery, sensitivity and precision.

Reproducibility and precision

The reproducibility and precision of embelin were verified by carrying out replicate measurements of the same samples. Ten replicate injections of standard and three replicate injections of extracted samples were used for this purpose. For determining the intra-day accuracy and precision, five replicates of each sample were analyzed thrice on the same day. The inter-day accuracy and precision were assessed by analysis of five replicates of samples on three different days.

Linearity

The calibration curve was developed by the least-square regression for peak area and concentration of the analyte. A linear calibration curve was developed for the concentration range of 0–1000 µg/ml. The relative standard deviation (% RSD) values did not exceed 0.01 for any of the concentrations. After linear regression analysis, the slope (± SD of mean) for the calibration curve of embelin was found to be 15,691 (±0.17) with a regression coefficient (r2) value of 0.997 (Table 2).

Limit of detection and limit of quantitation

Limit of quantification and limit of detection were determined from the standard deviation and slope of the calibration curve. Limit of detection (LOD) is the lowest concentration of analyte that can be detected in an injected sample extract under optimized conditions. The limit of quantification (LOQ) is the lowest concentration of the sample that can be quantified with accuracy and precision. These were determined based on the signal-to-noise ratio as per International Council on Harmonisation (ICH) guidelines.

Accuracy and extraction recovery

To check the accuracy of the developed method, a recovery experiment was carried out by standard addition method. A known amount of sample was taken. To each tube known amount of embelin was added. Each sample was analyzed by the developed UHPLC method, and the amount of embelin recovered for each level was calculated. Percent recovery of embelin from all the plant parts of *A. corniculatum* was 97.89%. Low % RSD (relative standard deviation) values established extraction efficiency robustness of the selected method (Table 3).

Histochemical localization

As the major non-harmful plant part is leaf, the presence of deposition of embelin in the leaf parts is a matter of search and this was achieved by the ammonia solution method (Belete et al. 2014). The deposition of embelin in the mesophyll cells is observed by taking the *A. corniculatum* matured leaf TS sections. Microphotographs of

Table 1 Chromatographic condition for assessment of embelin

Sl No	Conditions	Selected measures
1	Mobile phase	Methanol: 0.1% TFA (65:35)
2	Detection wavelength	298 nm
3	Column temp	Room temperature
4	Injection volume	10 µl
5	Flow rate	1 ml/min
6	Retention time	2.7 min
7	Mode of operation	Isocratic

Table 2 Statistical validation of UHPLC method

Sl No	Parameters	Values
1	Absorption maxima	298 nm
2	Correlation coefficient (r2)	0.997
3	Regression equation	$Y = 15691x + 0$
4	Intercept (c)	0
5	Slope (b)	15,691
6	Retention time	2.7
7	Precision (% RSD)	0.0095
8	Accuracy (%)	97.884
9	LOD mg/ml	105.42
10	LOQ mg/ml	319.46

Table 3 Accuracy and extraction recovery studies for embelin analysis

Sl No	In Sample (mg/ml)	Added (mg/ml)	Estimated (mg/ml)	% RSD	% of Recovery
1	50	100	146.37 ± 0.007	0.0048	97.58
2	100	150	247.72 ± 0.061	0.025	99.1
3	150	200	347.65 ± 0.032	0.009	99.33
4	200	250	439.81 ± 0.017	0.0038	97.73
5	250	300	526.24 ± 0.028	0.005	95.68

NB: Data are expressed as mean ± SD, (where $n = 5$)

Table 4 Crude extract yield (% dry wt.) in *A. corniculatum*

Plant Parts	Extraction method	Crude yield (% dry wt.)
Fruit	Soxhlet—Methanol	1.82 ± 0.043
	Soxhlet—Chloroform	0.67 ± 0.0458
	Water bath—Methanol	2.74 ± 0.04
	Water bath—Chloroform	0.91 ± 0.04
Leaf	Soxhlet—Methanol	0.56 ± 0.072
	Soxhlet—Chloroform	0.12 ± 0.062
	Water bath—Methanol	0.68 ± 0.08
	Water bath—Chloroform	0.18 ± 0.07
Stem bark	Soxhlet—Methanol	0.75 ± 0.061
	Soxhlet—Chloroform	0.23 ± 0.036
	Water bath—Methanol	0.87 ± 0.05
	Water bath—Chloroform	0.34 ± 0.046
Root	Soxhlet—Methanol	0.77 ± 0.056
	Soxhlet—Chloroform	0.43 ± 0.01
	Water bath—Methanol	0.84 ± 0.04
	Water bath—Chloroform	0.62 ± 0.056

NB-Data are expressed as mean ± SD, (where $n = 3$)

sections were analyzed using Nikon Eclipse Microscope (Japan, Model 50i) equipped with Nikon Y-IDT. Images were concomitantly viewed and analyzed for pharmacognostic characteristics, and quantitative measurements were taken using Nikon Digital Sight DSL Camera (Model No.-215,492).

Statistical analysis

All the results were validated by using two-way RM ANOVA (repetitive measures) using GraphPad Prism software (Version 6.05) at 99.9% significant level. All the data were expressed as mean ± SD.

Results

Crude Yield

The crude yield of embelin in case of *A. corniculatum* was found to be in a range of 0.12–2.74% dry wt. (Table 4). The fruit part showed the highest yield of crude embelin followed by the root parts followed by stem bark and then finally by the leaf parts. When the solvent systems were considered, the methanol solvent system was prevailed to be eminent to that of chloroform solvent system. All data were analyzed statistically at 99.9% confidence interval of difference through one-way RM ANOVA along with Holm–Sidak's multiple comparisons test. In the multiple comparison analysis, the row factors, i.e., the plant parts extracted through different processes with various solvent systems, were found to be significant at P value = 0.0243 and column factors, i.e., the crude yield of embelin, were found to be significant at P value = 0.5260.

Embelin content (% dry wt.) through spectrophotometric method

In the case of *A. corniculatum* samples, embelin content in the crude extracts was found to be in a range of 0.23–1.95% dry wt. Among all the selected plant parts, crude embelin content was found to be highest in fruit (1.95% dry wt.), when extracted with Soxhlet using chloroform solvent, while in the case of leaves the least embelin content was found in water bath using methanol solvent extracts (0.23% dry wt.) (Table 5). All data were analyzed statistically at 99.9% confidence interval of difference through two-way RM ANOVA along with Sidak's multiple comparisons test. In the multiple comparison analysis, the row factors, i.e., the plant parts extracted through different processes with various solvent systems, were found to be significant at P value < 0.0001 and column factors, i.e., the crude and purified isolates, were found to be significant at P value = 0.0030.

Identification and isolation of pure Embelin

The Rf value of the synthetic embelin standard was 0.35. However, the crude extracted samples during the identification process for the presence of embelin showed Rf in a range of 0.345–0.356 in case of *A. corniculatum*. Fruit samples, extracted with Soxhlet—chloroform, showed highest Rf value (0.356) and leaf samples, extracted with water bath—methanol, showed least Rf value (0.345). The isolated compound from samples of *A. corniculatum* showed Rf in a range of 0.342–0357. Fruit samples, extracted with Soxhlet—chloroform, showed highest

Table 5 Embelin content (% dry wt.) in extracts and isolates of *A. corniculatum* through spectrophotometric method

Plant Parts	Extraction method	Embelin Content (% Dry wt.)	
		Crude	Pure
Fruit	Soxhlet—Methanol	1.85 ± 0.021	1.45 ± 0.038
	Soxhlet—Chloroform	1.95 ± 0.015	1.65 ± 0.015
	Water bath—Methanol	1.57 ± 0.023	1.39 ± 0.036
	Water bath—Chloroform	1.73 ± 0.011	1.58 ± 0.031
Leaf	Soxhlet—Methanol	0.37 ± 0.011	0.30 ± 0.021
	Soxhlet—Chloroform	0.44 ± 0.026	0.39 ± 0.026
	Water bath—Methanol	0.23 ± 0.021	0.19 ± 0.011
	Water bath—Chloroform	0.28 ± 0.025	0.23 ± 0.026
Stem Bark	Soxhlet—Methanol	0.73 ± 0.034	0.64 ± 0.011
	Soxhlet—Chloroform	0.84 ± 0.015	0.71 ± 0.021
	Water bath—Methanol	0.53 ± 0.026	0.48 ± 0.036
	Water bath—Chloroform	0.63 ± 0.021	0.56 ± 0.026
Root	Soxhlet—Methanol	1.37 ± 0.021	1.25 ± 0.021
	Soxhlet—Chloroform	1.41 ± 0.035	1.33 ± 0.026
	Water bath—Methanol	1.1 ± 0.036	0.9 ± 0.073
	Water bath—Chloroform	1.21 ± 0.035	1.17 ± 0.011

NB-Data are expressed as mean ± SD, (where $n = 3$)

Rf value (0.357) and leaf samples, extracted with water bath—methanol, showed least Rf value (0.342) (Table 6).

TLC sheets confirming the presence of embelin in the crude extracts and the isolates are given in Fig. 1. All data

Table 6 Rf values of crude and purified isolates of *A. corniculatum*

Plant Parts	Extraction method	Rf Value	
		Crude	Pure
Fruit	Soxhlet—Methanol	0.347 ± 0.006	0.346 ± 0.003
	Soxhlet—Chloroform	0.356 ± 0.006	0.357 ± 0.006
	Water bath—Methanol	0.35 ± 0.005	0.355 ± 0.006
	Water bath—Chloroform	0.349 ± 0.001	0.351 ± 0.007
Leaf	Soxhlet—Methanol	0.354 ± 0.006	0.35 ± 0.004
	Soxhlet—Chloroform	0.348 ± 0.003	0.349 ± 0.003
	Water bath—Methanol	0.346 ± 0.005	0.342 ± 0.006
	Water bath—Chloroform	0.347 ± 0.003	0.354 ± 0.005
Stem bark	Soxhlet—Methanol	0.349 ± 0.004	0.352 ± 0.006
	Soxhlet—Chloroform	0.353 ± 0.007	0.349 ± 0.005
	Water bath—Methanol	0.352 ± 0.003	0.351 ± 0.006
	Water bath—Chloroform	0.352 ± 0.002	0.346 ± 0.003
Root	Soxhlet—Methanol	0.353 ± 0.005	0.348 ± 0.003
	Soxhlet—Chloroform	0.35 ± 0.001	0.351 ± 0.006
	Water bath—Methanol	0.355 ± 0.001	0.353 ± 0.005
	Water bath—Chloroform	0.347 ± 0.003	0.351 ± 0.006

NB-Data are expressed as mean ± SD, (where $n = 3$)

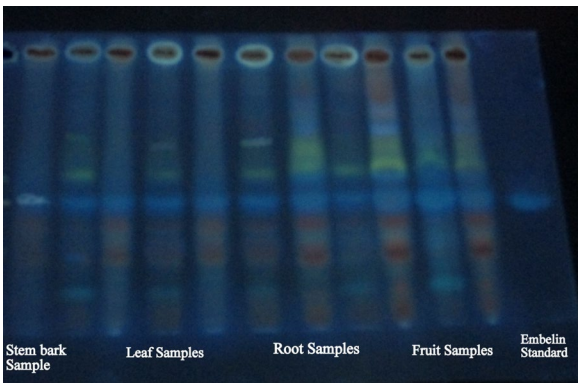


Fig. 1 TLC sheets with sample extracts from different plant parts of *A. corniculatum*

were analyzed statistically for 99.9% confidence interval through two-way RM ANOVA along with Sidak's multiple comparisons test. In the multiple comparison analysis, both the row factors, i.e., the plant parts extracted through different processes with various solvent systems, and column factors, i.e., the isolates, were found to be significant at P value < 0.0001. The difference in Rf for all the isolates was due to the different types of solvents used along with the time.

Embelin content (% dry wt.) through UHPLC method

In the case of *A. corniculatum* samples, embelin content in the crude extracts was found to be in a range of 0.17–1.64% dry wt. Among all the plant parts, embelin content was found to be highest in fruit (1.64% dry wt.), when extracted with Soxhlet method using chloroform as solvent, while in the case of leaves the least embelin content was found in water bath method using methanol solvent extracts (0.17% dry wt.). Embelin content in isolates of *A. corniculatum* was found to be in a range of 0.028–1.60% dry wt. The highest embelin content was found in fruit (1.60% dry wt.) extracted with chloroform solvent, while in the case of leaves, the least embelin content was found in methanol extracts from water bath method (0.028% dry wt.) (Table 7). The chromatogram peak value of eluted samples is shown in Fig. 2. All data were analyzed statistically at 99.9% confidence interval (CI) of difference through two-way RM ANOVA along with Sidak's multiple comparisons test. In the multiple comparison analysis, the row factors, i.e., the plant parts extracted through different processes with various solvent systems, were found to be significant at P value < 0.0001 and column factors, i.e., crude and purified isolates, were found to be significant at P value = 0.0004.

However, the embelin content available in the crude extracts and purified samples are correlated with their

Table 7 Embelin content (% dry wt.) in extracts and isolates of *A. corniculatum* through UHPLC method

Plant Parts	Extraction method	Embelin Content (% Dry wt.)	
		Crude	Pure
Fruit	Soxhlet—Methanol	1.61 ± 0.011	1.23 ± 0.021
	Soxhlet—Chloroform	1.64 ± 0.062	1.60 ± 0.015
	Water bath—Methanol	1.48 ± 0.031	1.29 ± 0.015
	Water bath—Chloroform	1.56 ± 0.025	1.34 ± 0.017
Leaf	Soxhlet—Methanol	0.32 ± 0.021	0.19 ± 0.021
	Soxhlet—Chloroform	0.37 ± 0.031	0.47 ± 0.017
	Water bath—Methanol	0.17 ± 0.041	0.028 ± 0.003
	Water bath—Chloroform	0.23 ± 0.017	0.037 ± 0.005
Stem Bark	Soxhlet—Methanol	0.71 ± 0.035	0.21 ± 0.015
	Soxhlet—Chloroform	0.82 ± 0.031	0.51 ± 0.015
	Water bath—Methanol	0.51 ± 0.025	0.04 ± 0.002
	Water bath—Chloroform	0.61 ± 0.072	0.06 ± 0.011
Root	Soxhlet—Methanol	1.22 ± 0.026	0.40 ± 0.011
	Soxhlet—Chloroform	1.38 ± 0.031	0.59 ± 0.021
	Water bath—Methanol	0.94 ± 0.021	0.083 ± 0.003
	Water bath—Chloroform	1.16 ± 0.031	0.087 ± 0.001

NB-Data are expressed as mean ± SD, (where $n = 3$)

corresponding Rf values for embelin and the correlation between its content and Rf value is depicted in Fig. 3.

Microscopic identification of Embelin

The microscopic figures have revealed the presence of embelin in specialized vacuoles mainly below the epidermal layer (both upper and lower) and in mesophyll cells. After ammonia treatment, the deposition of embelin in specialized vacuoles was visible as brownish red-colored patches (Fig. 4). The deposition of embelin in case of mesophyll cells was found to be more prominent as compared to the deposition in the epidermal layer.

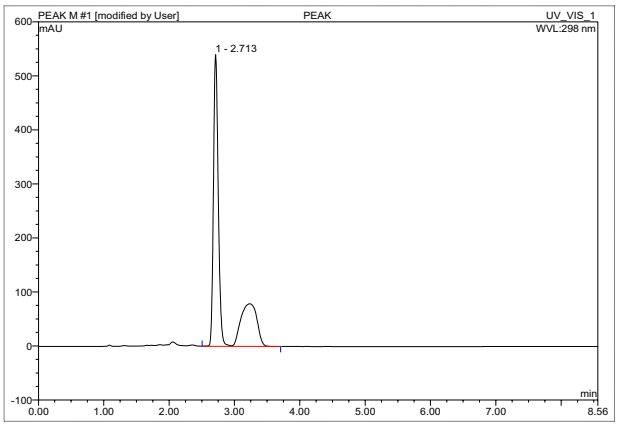
Discussion

Embelin is one of the biologically active benzoquinone derivatives that act as the active principle compound in the fruits of *Embelia ribes*, a well-known yet very rare and endangered medicinal plant of the Myrsinaceae family (Ferreria and Laddha 2013). Due to the rare, endangered and threatened (RET) status, heavy consumption of fruits, for embelin extraction, led to a disturbance in the production of new plants through the seed germination process. To mitigate the difficulty in regulating and elevating their minuscule population and to meet the market demand of embelin, possible and suitable alternative substitutes are considered for extraction of embelin. To this aspect, allied plants in the same Myrsinaceae family, viz. *E. tsjeriam-cottam, Ardisia solanacea, A. japonica, Aegiceras corniculatum* and many more, can enlighten the path (Bandaranayake 2002; Podolak and Strzałka 2008; Alongi 2009; Stasiuk and Kozubek 2011; Poojari 2011; Rahman and Khan 2013). However, *A. corniculatum* act as an interesting substitute, available in Mangrove category that can act as a prominent embelin source and has been scatteredly reported to contain embelin (Bandaranayake 2002; Alongi 2009). The main intention of this research work is to find out suitable alternative substitutes besides the traditionally used *E. ribes* as a source of embelin to minify the pressure on the later plant, which will lead to its least exploitation. *A. corniculatum* is the least concerned mangrove flora that could be utilized as an alternative substitute for the yield of embelin.

The crude extracted embelin was yielded in crystallized manner to get orange yellowish needle-like structures. For the yield of crude extracts of embelin, methanol and chloroform solvent systems were used separately. The structural complexity of embelin with the presence of long alkyl chain leads to its solubility in both the solvent systems (Rahman and Khan 2013). In our study, the crude embelin yield was found to be in a range of 0.12–2.74% dry wt., which corroborated to other findings with embelin yield in a range of 0.23–0.28% dry wt. (Swami et al. 2017). Though structural elucidation of embelin in *A. corniculatum* had been described previously (Thota et al. 2016) along with its quantification in various plant parts (Swami et al. 2017), its detailed quantitative analysis yet has not been done. In the study of Thota et al. (2016), *Aegiceras corniculatum* leaf and stem parts were taken from Godavari estuary, Andhra Pradesh, and were extracted through subsequent extraction system taking chloroform/methanol (1:1) followed by ethyl acetate. All the column chromatographed and TLC eluted samples were identified through ^{1}H and ^{13}C NMR and mass spectral study. However, in our study all of the *Aegiceras corniculatum* samples (fruit, leaf, stem bark and root) were collected from Bhitarkanika Mangrove forest, Odisha, followed by the sample extraction through Soxhlet and water bath method taking both methanol and chloroform solvents. All column chromatographed followed by TLC eluted samples is evaluated for embelin content through spectrophotometric and UHPLC method of analysis. The major novelty in the current study in response to the study carried out by Thota et al. 2016 is the estimation of embelin content in all the vegetative and reproductive plant parts of *Aegiceras corniculatum* (fruit, leaf, stem bark and root) samples. The prime focus in this study was being given only to isolate and estimate embelin content from various plant parts. In the study of Thota et al. (2016), only compound identification was done where as in our study both identification and quantification of

1 RPRC, Bhubaneshwar

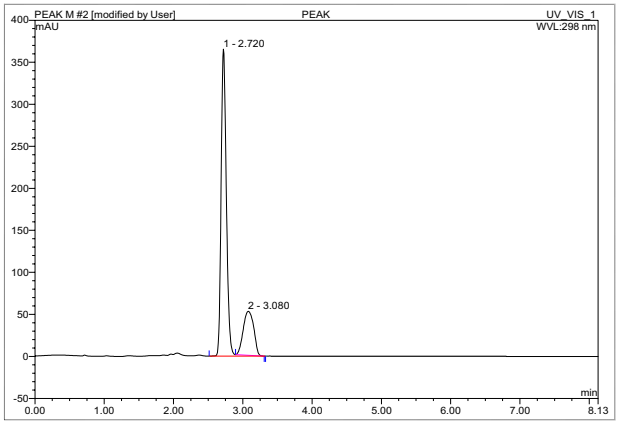
Sampl ɜ Name:	**PEAK**	*Injection V ɔlume:*	**10.0**
Vial Number:	**1**	*Channel:*	**UV_VIS_1**
Sample Type:	**unknown**	*Wavelength:*	**298**
Control Program:	**PEAK M**	*Bandwidth:*	**n.a.**
Quantif. Method:	**PEAK M**	*Dilution Factor:*	**1.0000**
Recording Time:	**7/22/2017 11:59**	*Sample Weight:*	**1.0000**
Run Time (min):	**8.56**	*Sample Amount:*	**1.0000**



No.	Ret.Time min	Peak Name	Height mAU	Area mAU*min	Rel.Area %	Amount	Type
1	2.71	n.a.	539.819	68.583	100.00	n.a.	BMB*
Total:			539.819	68.583	100.00	0.000	

2 RPRC, Bhubaneshwar

Sampl ɜ Name:	**PEAK**	*Injection V ɔlume:*	**10.0**
Vial Number:	**2**	*Channel:*	**UV_VIS_1**
Sample Type:	**unknown**	*Wavelength:*	**298**
Control Program:	**PEAK M**	*Bandwidth:*	**n.a.**
Quantif. Method:	**PEAK M**	*Dilution Factor:*	**1.0000**
Recording Time:	**7/22/2017 12:10**	*Sample Weight:*	**1.0000**
Run Time (min):	**8.13**	*Sample Amount:*	**1.0000**

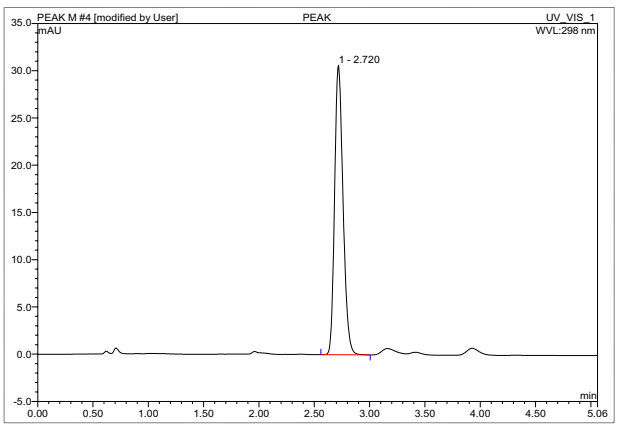


No.	Ret.Time min	Peak Name	Height mAU	Area mAU*min	Rel.Area %	Amount	Type
1	2.72	n.a.	365.467	32.210	77.78	n.a.	BMB*
2	3.08	n.a.	52.340	9.201	22.22	n.a.	Rd
Total:			417.807	41.411	100.00	0.000	

Fig. 2 Embelin chromatograms of different samples of *A. corniculatum*

4 RPRC, Bhubaneshwar

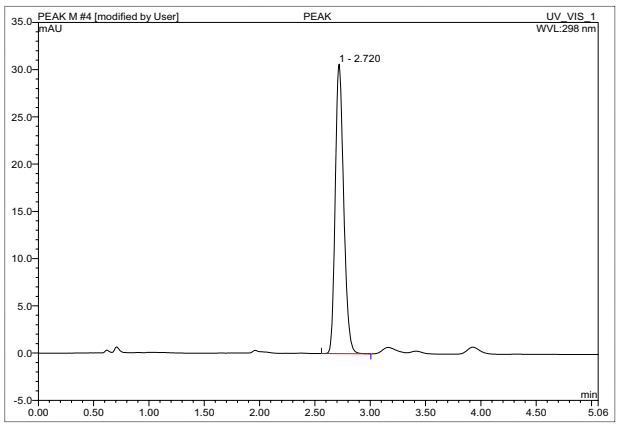
Sampl ɜ Name:	**PEAK**	*Injection V ɔlume:*	**10.0**
Vial Number:	**4**	*Channel:*	**UV_VIS_1**
Sample Type:	**unknown**	*Wavelength:*	**298**
Control Program:	**PEAK M**	*Bandwidth:*	**n.a.**
Quantif. Method:	**PEAK M**	*Dilution Factor:*	**1.0000**
Recording Time:	**7/22/2017 12:24**	*Sample Weight:*	**1.0000**
Run Time (min):	**5.06**	*Sample Amount:*	**1.0000**



No.	Ret.Time min	Peak Name	Height mAU	Area mAU*min	Rel.Area %	Amount	Type
1	2.72	n.a.	30.661	2.714	100.00	n.a.	BMB
Total:			30.661	2.714	100.00	0.000	

4 RPRC, Bhubaneshwar

Sampl ɜ Name:	**PEAK**	*Injection V ɔlume:*	**10.0**
Vial Number:	**4**	*Channel:*	**UV_VIS_1**
Sample Type:	**unknown**	*Wavelength:*	**298**
Control Program:	**PEAK M**	*Bandwidth:*	**n.a.**
Quantif. Method:	**PEAK M**	*Dilution Factor:*	**1.0000**
Recording Time:	**7/22/2017 12:24**	*Sample Weight:*	**1.0000**
Run Time (min):	**5.06**	*Sample Amount:*	**1.0000**

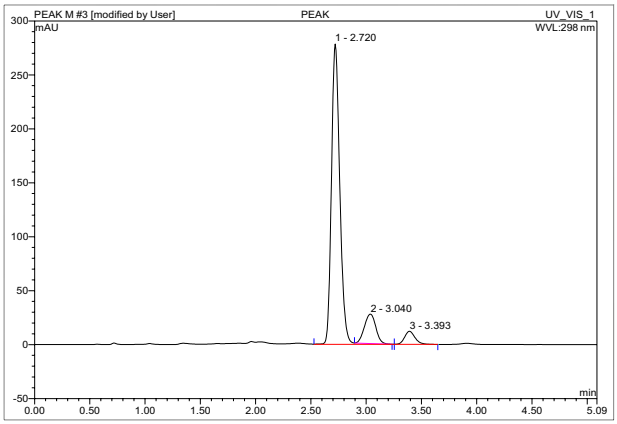


No.	Ret.Time min	Peak Name	Height mAU	Area mAU*min	Rel.Area %	Amount	Type
1	2.72	n.a.	30.661	2.714	100.00	n.a.	BMB
Total:			30.661	2.714	100.00	0.000	

Fig. 2 continued

3 RPRC, Bhubaneshwar

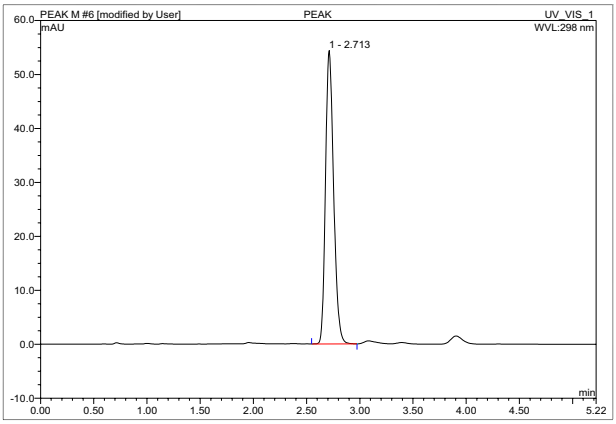
Sampl ɜ Name:	**PEAK**	*Injection V ɔlume:*	**10.0**
Vial Number:	**3**	*Channel:*	**UV_VIS_1**
Sample Type:	**unknown**	*Wavelength:*	**298**
Control Program:	**PEAK M**	*Bandwidth:*	**n.a.**
Quantif. Method:	**PEAK M**	*Dilution Factor:*	**1.0000**
Recording Time:	**7/22/2017 12:19**	*Sample Weight:*	**1.0000**
Run Time (min):	**5.09**	*Sample Amount:*	**1.0000**



No.	Ret.Time min	Peak Name	Height mAU	Area mAU*min	Rel.Area %	Amount	Type
1	2.72	n.a.	278.314	24.347	84.26	n.a.	BMb*
2	3.04	n.a.	27.213	3.247	11.24	n.a.	Rd
3	3.39	n.a.	12.059	1.302	4.51	n.a.	bMB
Total:			317.586	28.896	100.00	0.000	

6 RPRC, Bhubaneshwar

Sampl ɜ Name:	**PEAK**	*Injection V ɔlume:*	**10.0**
Vial Number:	**6**	*Channel:*	**UV_VIS_1**
Sample Type:	**unknown**	*Wavelength:*	**298**
Control Program:	**PEAK M**	*Bandwidth:*	**n.a.**
Quantif. Method:	**PEAK M**	*Dilution Factor:*	**1.0000**
Recording Time:	**7/22/2017 12:38**	*Sample Weight:*	**1.0000**
Run Time (min):	**5.22**	*Sample Amount:*	**1.0000**

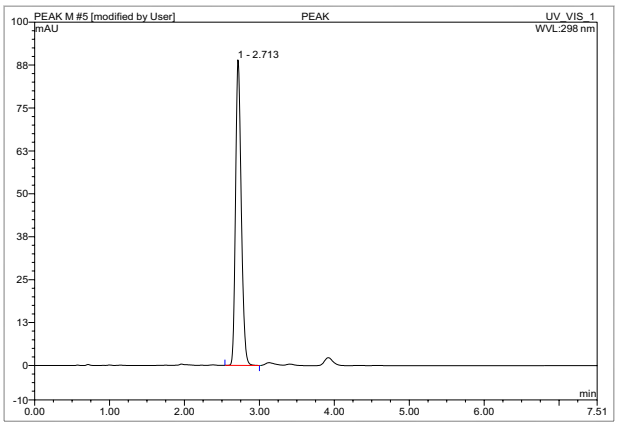


No.	Ret.Time min	Peak Name	Height mAU	Area mAU*min	Rel.Area %	Amount	Type
1	2.71	n.a.	54.393	4.814	100.00	n.a.	BMB*
Total:			54.393	4.814	100.00	0.000	

Fig. 2 continued

5 RPRC, Bhubaneshwar

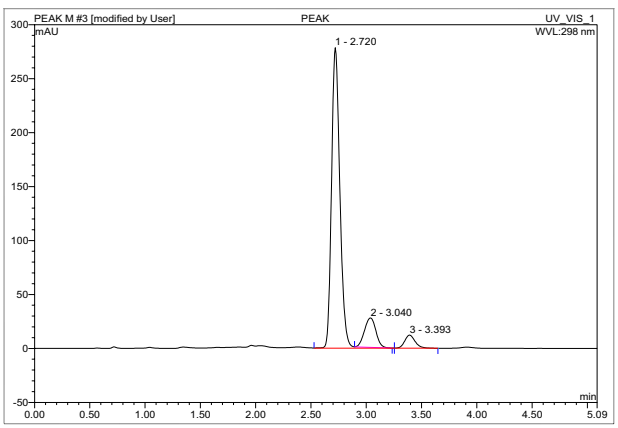
Sampl ɜ Name:	**PEAK**	*Injection V ɔlume:*	**10.0**
Vial Number:	**5**	*Channel:*	**UV_VIS_1**
Sample Type:	**unknown**	*Wavelength:*	**298**
Control Program:	**PEAK M**	*Bandwidth:*	**n.a.**
Quantif. Method:	**PEAK M**	*Dilution Factor:*	**1.0000**
Recording Time:	**7/22/2017 12:30**	*Sample Weight:*	**1.0000**
Run Time (min):	**7.51**	*Sample Amount:*	**1.0000**



No.	Ret.Time min	Peak Name	Height mAU	Area mAU*min	Rel.Area %	Amount	Type
1	2.71	n.a.	89.003	7.894	100.00	n.a.	BMB
Total:			89.003	7.894	100.00	0.000	

3 RPRC, Bhubaneshwar

Sampl ɜ Name:	**PEAK**	*Injection V ɔlume:*	**10.0**
Vial Number:	**3**	*Channel:*	**UV_VIS_1**
Sample Type:	**unknown**	*Wavelength:*	**298**
Control Program:	**PEAK M**	*Bandwidth:*	**n.a.**
Quantif. Method:	**PEAK M**	*Dilution Factor:*	**1.0000**
Recording Time:	**7/22/2017 12:19**	*Sample Weight:*	**1.0000**
Run Time (min):	**5.09**	*Sample Amount:*	**1.0000**



No.	Ret.Time min	Peak Name	Height mAU	Area mAU*min	Rel.Area %	Amount	Type
1	2.72	n.a.	278.314	24.347	84.26	n.a.	BMb*
2	3.04	n.a.	27.213	3.247	11.24	n.a.	Rd
3	3.39	n.a.	12.059	1.302	4.51	n.a.	bMB
Total:			317.586	28.896	100.00	0.000	

Fig. 2 continued

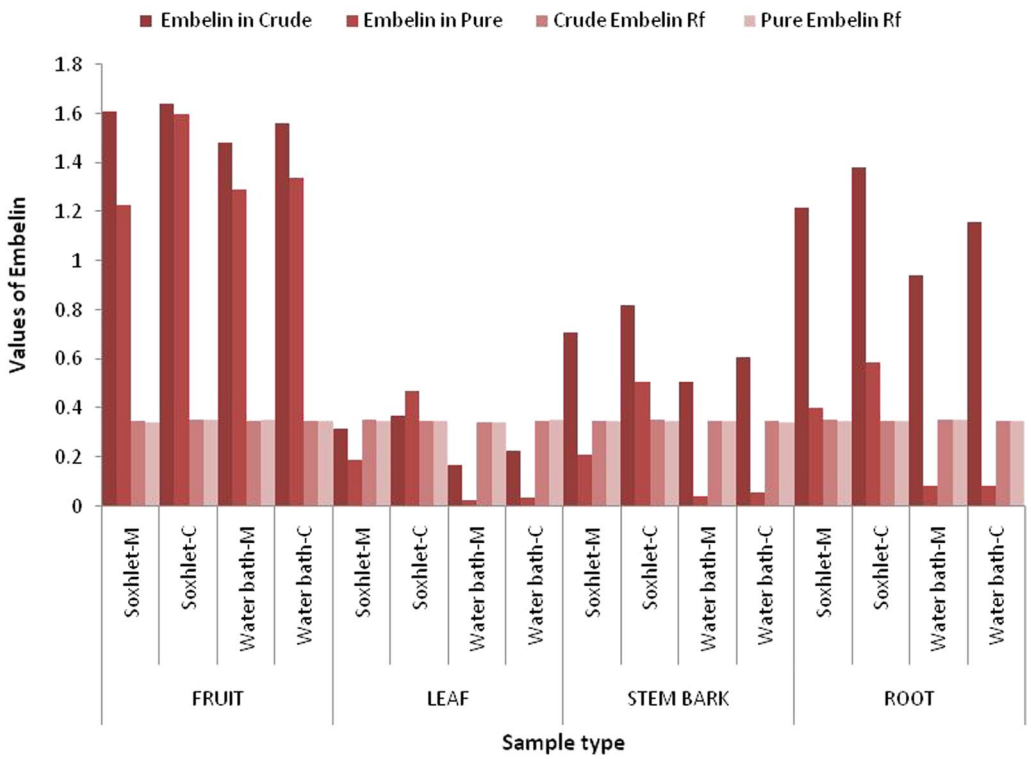


Fig. 3 Comparative graph between embelin content and its Rf value

each plant part were done for embelin. This is the first attempt made for the estimation of embelin in all the plant parts of *A. corniculatum*, collected from Odisha, through both spectrophotometric and UHPLC method of analysis. For isolation and separation of compounds, mobile phase optimization was done based on the solubility and polarity index of the desired compounds along with the mobile phase and compound's affinity with the stationary phase. As all the extracts are being isolated using different solvents, this would definitely have a direct impact during desires compounds separation process (Bele and Khale 2011). Similarly, all the TLC plate's varying stationary phase thickness and unequal time interval used for TLC plate development also affect the change in Rf values of the result (Elizabeth and Jashna 2021). Several studies have reported embelin content in most of the *Embelia tsjeriam-cottam* plant to be in range of 0.3–0.35 (Vandana and Arora 2010). Even in case of *A. corniculatum*, similar kind of Rf value was found that validated our study, of getting Rf values in a range of 0.345–0.356 (Thota et al. 2016). Embelin is widely used in several Ayurvedic and Pharmaceutical industries, which increases the extensive use of the fruit parts, thereby increasing its threat status. Hence, several efforts have been made to localize this bioactive benzoquinone in different plant parts of particularly *E. ribes* (Nakve et al. 2011; Ferreria and Laddha 2013; Sudhakaran 2015), thereby reducing the difficulties of the extraction procedure. In all their studies, they have localized the presence of embelin in seed and pericarp portion of the fruits. As leaf samples prevailed to contain embelin within it and are the non-harmful source of the concerned plants in terms of yield of embelin, the histochemical localization of embelin within the leaf samples was done. However, anatomical study regarding the localization of embelin in *A. corniculatum* has yet not been studied so far.

Conclusions

Hence, it could be opined that all plant parts of *A. corniculatum* do contain embelin and it can act as an alternate substitute source of embelin besides *E. ribes* and *E. tsjeriam-cottam*. However, as leaf parts though contain $1/10^{th}$ of embelin compared to the fruit parts, it can be used as a suitable alternate nondestructive source of embelin. Leaves are abundantly available, and using leaves for medicinal formulations will also not be a threat to the concerned plant species. Thus, the outcome of this research work will facilitate conservation, domestication and sustainable utilization of potential wild medicinal plant resources.

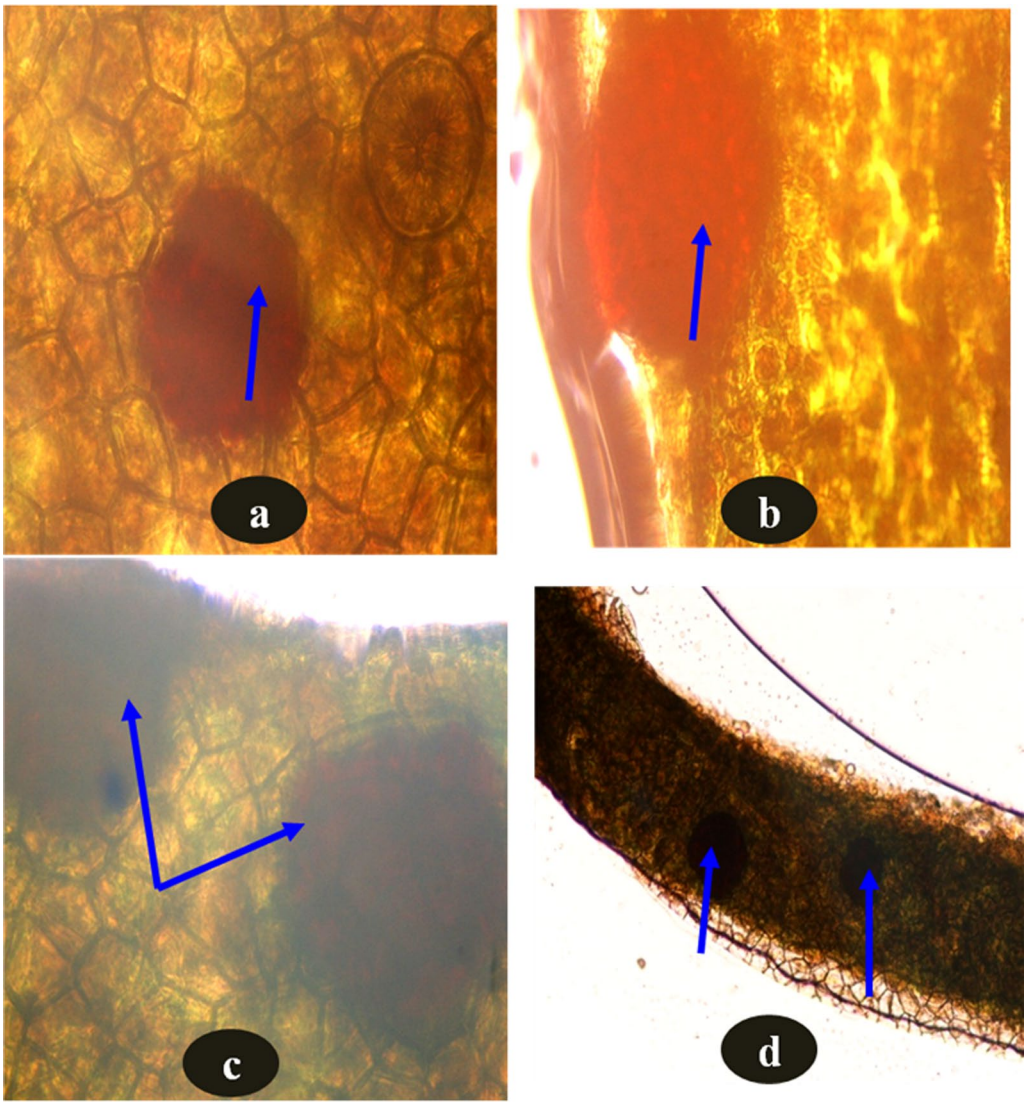


Fig. 4 TS of leaf samples of *A. corniculatum* showing localization of embelin in **a** mesophyll cells, **b** below the epidermal layer (arrowhead), **c** presence of two-embelin deposition near epidermal layer (arrowhead) (All three in 40× Magnification), **d** presence of two-embelin deposition (arrowhead) (10× Magnification)

Abbreviations
LOD: Limit of detection; LOQ: Limit of quantitation; RSD: Relative standard deviation; PTLC: Preparative thin layer chromatography; UHPLC: Ultra-high-performance liquid chromatography; RET: Rare, endangered and threatened.

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Author contributions
UCB and MM contributed to experimental design and supervision and manuscript writing and review; MM was involved in experimental implementation. Bothauthors read and approved the final manuscript.

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Availability of data and materials
The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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