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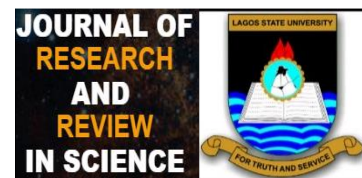

Isolation of Betulinic acid from the stem bark and root of *Uapaca guineensis*

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Abstract:
Aims: To investigate the stem bark and root of *Uapaca guineensis* extracts, with a view of isolating betulinic acid across the various part of the plant based on its reported biological activities.
Study design: The design included extraction of *U. guineensis* stem bark and root with ethanol and subsequent partitioning, isolation and characterization of the possible isolated compounds.
Place and Duration of Study: Chemistry Department (Organic Unit), Faculty of Science, University of Ibadan, Nigeria and H.E.J. Research Institute of Chemistry, ICCBS, University of Karachi, Pakistan between March 2012 and April 2013.
Methodology: The air-dried and pulverized stem bark and root of *U. guineensis* (0.5 kg each) were separately extracted with aqueous ethanol (80%) for 72 hour at room temperature, filtered, concentrated on rotatory evaporator at 37 °C and then stored in a desiccator. The ethanol extracts of the stem bark was partitioned successively with n-hexane, dichloromethane and methanol. The dichloromethane, methanol and ethanol extracts (root) were then chromatographed and the isolated compounds subjected to various spectroscopic analysis.
Results: The air-dried and pulverized stem bark and root of *U. guineensis* (0.5 kg each) extracted with ethanol and then partitioned yielded dichloromethane (6.9 g), methanol (17.9) and the ethanol (12.9) extracts. The chromatography of the various extracts results in the formation of whitish powder that are UV inactive and also gave single spot each with cerium sulphate coded as DBG-D_{13} (25.0 mg), MBG-Q_3 (6.1 mg) and ERG-E_7 (20.0 mg) for dichloromethane, Methanol and Ethanol extracts respectively. The structures were unequivocally established to be 3α-hydroxy-20(29)-lupen-28-oic acid and 3β-hydroxy-20(29)-lupen-28-oic acid by ^{1}HNMR, ^{13}CNMR, 2D-NMR, IR, UV and MS spectra analysis as well as by comparison of the spectra data with previously reported values.
Keywords: *Uapaca guineensis*, Euphorbiaceae, Betulinic acid

All co-authors agreed to have their names listed as authors.

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1. INTRODUCTION

Uapaca belong to a genus of the family Euphorbiaceae [1, 2] and is in the sub-tribe *Uapacinae.* It comprises approximately 105 species, of which 49 are confined to continental Africa. The species described so far in the genus are trees of 20 to 25 m high with about 60 cm in diameter [3, 4].

Phytochemical studies on extract of the genus revealed the presence of terpenoids, saponins, alkaloids and sterols [5]. The presence of phenolic compounds including tannins, gallotannins and flavanols from fruits of *Uapaca kirkiana* was also reported [6]. Betulinic acid and antidesmone as constituents of *Uapaca nitida* and *Uapaca robynsii* respectively were also reported [7, 8].

Uapaca guineensis Mull. Arg. (Euphorbiaceae) commonly called false mahogany and “Ewe akun” in the western part of Nigeria grows in humid localities, mixed evergreen forest, bush land on steep slopes and sea-level up to 1100 m altitude. They are widely distributed from West Africa to Democratic Republic of Congo. It is a tree of about 30 m high, rarely more, bore to 4 m girth by 13 m long, usually much less, more or less fluted with large stilt-roots up to 3 m high, dense low-branching crown and leaves may reach 30 cm long by 15 cm wide. The wood is hard, durable and moderately heavy, and when quarter-sawn is attractively figured with a silver grain [9, 10].

The isolation of substantial amounts of betulinic acid as almost the sole component from *Uapaca* species as well as the *in vitro* antitrypanosomal activity have been reported [4, 18]
The pharmacological activities of this species in Western and Eastern Africa folk medicine include anti-inflammation, treatment of fever, pain, sexual dysfunction and bacterial diseases [16, 17 and 8].

In Nigeria, the pulp is made into a refreshing drink; leaves are used to wrap kola nuts and the roots to treat male impotence. The wood is used locally in carpentry and to make planks, railway sleepers, beams, furniture, beds and kitchen utensils. It also produces good firewood and charcoal [7]. The stilt-rooted habit of this riverside species has raised the suggestion that it is able to stabilise river-banks, curb was and break the flood movement of water in dry-forest Zone Rivers [12].

Considering the report on the biological activities of betulinic acid from this family, the study was therefore aimed at investigating the stem bark and root of *U. guineensis* extracts, with a view of isolating betulinic acid across the various part of the plant.

2. MATERIAL AND METHODS

Plant materials

Uapaca guineensis plant was collected at Epe, Lagos, Nigeria. It was authenticated at Federal Research Institute of Nigeria (FRIN), with FHI number 108457 and a voucher specimen of the specie was deposited at the herbarium.

Extraction and fractionation of Plant material

Plant material were separated into its selected parts; 500 g each of stem bark and root were air dried, pulverized, exhaustively percolated separately with aqueous ethanol (80%) and intermittently agitated in ultrasonic water bath for 72 hour at room temperature. The extracts were filtered using whatman No. 1 filter paper and separately concentrated on rotatory evaporator at 37 ^{o}C, stored in labeled, sterile, screw-capped bottles and allowed for complete dryness by putting them inside desiccators. Successive partitioned was done in the order of n-Hexane, dichloromethane and methanol for the stem bark extract while the root ethanol extract was chromatographed directly.

Column Chromatography of dichloromethane extract of *U. guineensis* stem bark

The dichloromethane extract (6.9 g) of *U. guineensis* (stem bark) was preadsorbed on silica gel (6.9 g) to form a homogenous mixture which was subjected to column chromatography (weight of silica gel 200 g, length = 70 cm , internal diameter (id) = 4 cm) using gradient of hexane, dichloromethane (DCM), ethyl acetate (EtOAc) and methanol (MeOH) in order of hexane: (100%, 200 mL); hexane: DCM (9:1, 200 mL); (4:1, 100 mL); (7:3, 80 mL); (3:2, 620 mL); (1:1, 280 mL); (2:3, 460 mL); (3:7, 260 mL); (1:4, 300 mL); (1:9, 220 mL); DCM (100%, 660 mL); DCM: EtOAc: (9:1, 100 mL); (4:1, 320 mL); (7:3, 380 mL); (3:2, 260 mL); (1:1, 180 mL); (2:3, 160 mL); (3:7, 160 mL); (1:4, 180 mL); (1:9, 260 mL); EtOAc (100%, 360 mL); EtOAc: MeOH; (9:1, 620 mL) and (4:1, 200 mL) separately. A total of 328 fractions (20 mL each) were collected and pooled to 16 sub-fractions (D_{1-16}) based on TLC analysis. The sub-fraction D_{13} eluted with DCM: EtOAc, 4:1 was developed in a TLC tank with solvent ratio of 9.5:0.5 (DCM: MeOH). The isolate is UV inactive and gave a single spot when sprayed with cerium sulphate (R_f = 0.51). The whitish powder was re-crystallised in methanol to give a colourless needles powder coded DBG-D_{13} (25.0 mg).

Column Chromatography of Methanol fraction of *U. guineensis* stem bark

Methanol extract (17.9 g) of *U. guineensis* (stem bark) was mixed with equal weight of silica gel and chromatographed (silica gel weight = 350 g, length = 80 cm, id = 6 cm). Elution was carried out using gradient of hexane, ethyl acetate and methanol in order of hexane: (100%, 500 mL); hexane: ethyl acetate: (1:1, 700 mL); (2:3, 600 mL); (3:7, 500 mL); (1:4, 500 mL); (1:9, 500 mL); ethyl acetate: (100%, 500 mL); ethyl acetate: methanol: (9:1, 300 mL); (4:1, 300 mL) and (7:3, 700 mL). A total of 51 fractions of 100 mL each were collected and pooled to 4 sub-fractions

(P-S) based on their TLC profile. Fraction Q (2.5 g) eluted between 50-70% EtOAc in Hexane was further subjected to a silica gel column chromatography (weight of silica gel = 70 g, length = 60 cm, id = 2.5 cm) using isocratic elution of hexane: EtOAc (3:7). Fourteen fractions were collected at 30 mL each and pooled to 5 sub-fractions (Q_{1-5}) based on their TLC profile. Fraction Q_3 was analyzed on TLC with Hexane: EtOAc system (3:7) to give a single spot on spraying with cerium sulphate (R_f = 0.53). It gave a dirty whitish powder which was re-crystallised in dichloromethane to give a clean white powder coded MBG-Q_3 (6.1 mg).

Column Chromatography of ethanol extract of *U. guineensis* (root)

The ethanol extract (12.9 g) of *U. guineensis* was homogenised with equal weight of silica gel and subjected to column chromatography using silica gel (weight of silica gel = 370 g, length = 80 cm, id = 6 cm). Elution was carried out using gradient of hexane, dichloromethane, ethyl acetate and methanol in order of hexane; (100%, 2800 mL), hexane: dichloromethane; (9:1, 1600 mL); (4:1, 400 mL); (7:3, 600 mL); (3:2, 600 mL); (1:1, 600 mL); (2:3, 400 mL); (3:7, 400 mL); (1:4, 400 mL), (1:9, 400 mL), dichloromethane; (100%, 400 mL), dichloromethane: ethyl acetate; (9:1, 900 mL); (4:1, 900 mL); (7:3, 500 mL); (3:2, 400 mL); (1:1, 300 mL); (2:3, 600 mL); (3:7, 400 mL); (1:4, 600 mL), (1:9, 400 mL), ethyl acetate; (100%, 600 mL), ethyl acetate: methanol; (9:1, 1000 mL); (4:1, 400 mL); (7:3, 2100 mL); separately to give 177 fractions at 100 mL which were pooled to 9 sub-fractions (E_{1-9}) using TLC analysis. The sub-fraction E_6 (340.0 mg) eluted with 10% ethyl acetate: dichloromethane was further purified while sub-fraction E_7 eluted between 20-50% ethyl acetate: dichloromethane was developed in TLC tank with solvent ratio 3:7 (EtOAc: Hexane) to give a brown single spot on spraying with cerium sulphate (R_f = 0.52). The sample was a dirty white crystalline powder which was re-crystallised in methanol to give clean white powder coded ERG-E_7 (20.0 mg)

3. RESULTS AND DISCUSSION

Compound **1** was obtained as colourless needles powder with yield (25.0 mg) and melting point 279-280 ^oC. The IR spectrum of DBG-D_{13} showed diagnostic absorption bands for O-H, C-$H_{str.}$, C=O, C=C and C-H_{bend} at 3447, 2942/2870, 1688, 1648 and 1457 cm^{-1} respectively. The UV spectrum showed a single band at 207 nm. The ^1H NMR showed pattern characteristic of lupane type skeleton. The methylene protons on C-16 and C-22 resonated as triplet of triplet at δ 2.23/1.44 (2H, tt, *J* = 3.0/12.5 Hz, H-16) and 1.53/1.17 (2H, tt, *J* = 3.0/13.0 Hz, H-22) respectively. The spectrum also showed a one proton broad singlet at δ 3.30 and 10.5 which are characteristic of a hydroxyl and a carboxylic acid signals respectively. The methine protons on C-19 and C-13 also showed signals for two doublet of doublet of doublet (triplet of doublet) at δ 3.04 (1H, ddd, *J* = 5.0/11.0 Hz, H-19) and 2.33 (1H, ddd, *J* = 2.5/11.5 Hz, H-13). The triplet at δ 3.10 (1H, t, *J* = 5.0 Hz, H-3) was assigned to the proton at C-3. There is exomethylene group signal at δ 4.71 and 4.58 (1H, each dd, *J* = 2.5/2.0 Hz, H-29) which together with an allylic methyl at δ 1.68 (3H, s, H-30) indicated an isopropenyl moiety. The other five methyl groups resonated at δ 1.01, 0.95, 0.94, 0.84 and 0.74. The ^{13}C NMR spectrum displayed thirty carbon resonances which were sorted using Distortionless Enhancement by Polarisation Transfer (DEPT) experiment as six methyl carbons {δc 28.5 (C-23), 16.1 (C-24), 16.5 (C-25), 16.6 (C-26), 15.0 (C-27) and 19.5 (C-30)}, eleven methylene carbons {δc 109.9 (C-29), 39.5 (C-1), 30.4 (C-21), 35.2 (C-7), 32.8 (C-16), 31.3 (C-15), 37.5 (C-22), 28.3 (C-2), 26.4 (C-12), 21.7 (C-11) and 19.1 (C-6)}, six methine carbons {78.5 (C-3), 56.3 (C-5), 51.4 (C-9), 49.9 (C-18), 47.9 (C-19) and 39.0 (C-13)}, six quaternary carbons {151.6 (C-20), 56.8 (C-17), 43.0 (C-14), 41.5 (C-8), 39.6 (C-4) and 37.9 (C-10)} and one carbonyl carbon {177.5 (C-28)}. The assignment of chemical shifts of the protonated carbons was achieved by a Heteronuclear Multiple Quantum Correlation (HSQC) experiment. The ^1H-^1H Correlation Spectroscopy experiment (COSY) showed cross peaks for coupled protons at position 2 and 3, 7a and 7b, 12 and 13, 13 and 18, 19 and 21, 22 and 21, 23 and 24 for DBG-D_{13}. The Nuclear Overhauser Effect Spectroscopy (NOESY) experiment showed non coupling of protons at position 23 and 24 with its neighbouring protons at 3 suggesting β orientation of the hydroxyl group at C-3. The entire long ranged {^2J (CH) and ^3J (CH)} connections between the protons and carbons were observed in the Heteronuclear Multiple Bond Correlation (HMBC) spectrum. The EI-MS showed m/z 456.3 calculated for a molecular formula $C_{30}H_{48}O_3$ with HRMS of 456.3598. Comparison of DBG-D_{13} spectroscopic data with that of betulinic acid reported in literature [11] showed similarity. The structure of DBG-D_{13} was elucidated to be 3β-hydroxy-20(29)-lupen-28-oic acid.

Compound **2** was obtained as a whitish powder with molecular ion peak at m/z 456.0 in EI-MS (positive ion mode) which matched with high-resolution mass spectrometry (HRMS) $C_{30}H_{48}O_3$ (456.3598). The IR spectrum of MBG-Q_3 showed a broad peak of hydroxyl group at 3454 cm^{-1}, prominent carbonyl absorption at 1687 cm^{-1}, double bonded peaks at 1641 cm^{-1} and other diagnostic bands like 2942/2871 (C-$H_{str.}$), 3075 (=C-H) and 1452 (C-H_{bend}) cm^{-1}. The UV spectrum showed a single band at 206 nm. The ^1H NMR spectrum of MBG-Q_3 showed pattern characteristic of a lupane type carbon skeleton. The compound indicated six singlet methyl proton signals at δ 0.74 (s, H-25), 0.85 (s, H-24), 0.94 (s, H-26), 0.96 (s, H-27), 0.99 (s, H-23) and 1.69 (s, H-30). It also contains two proton signals corresponding to olefinic methylene proton at δ 4.70 (s, H-29a) and 4.58 (s, H-29b), which correlated with the ^{13}CNMR signal at δ 19.6 (C-30).The doublet of doublet at δ 3.11 (1H, dd, *J* = 4.5/11.0 Hz) could be assigned to H-3 with the vicinyl methylene protons suggested β orientation of the hydroxyl group at C-3. The methylene proton at C-16 and C-22 showed triplet of triplet at δ 2.21/1.40 (2H, tt, *J* = 3.5/13.0 Hz, H-16) and 1.18/1.51 (2H, tt, *J* = 3.0/13.5

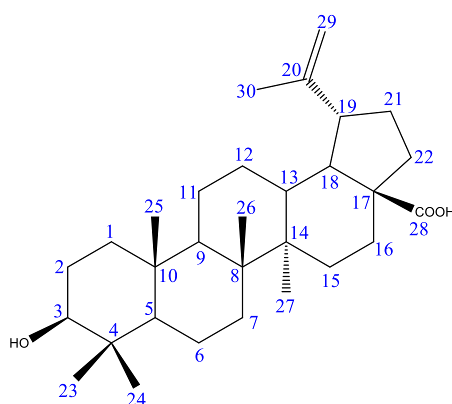
Hz, H-22). The spectrum also showed triplet of doublets (doublet of doublet of doublet) at δ 2.33 (1H, ddd, J = 2.5/11.5 Hz, H-13) and δ 3.02 (1H, ddd, J = 5.0/11.0 Hz, H-19) for the methine proton at C-13 and C-19 respectively. A total of thirty carbons resonances where observed in ^{13}C NMR spectrum which were sorted using DEPT experiment into six methyl carbons, eleven methylene carbons, six methine carbons, six quaternary carbons and one carbonyl carbon. Among the signals are olefinic methylene carbon signal at δ 110.1 (C-29) and carboxylic group carbon signal at δ 180.5 (C-28). Further information about the compound was obtained from the typical EI-MS related to the fragmentation pattern of lupine type triterpenes through the presence of m/z 438 (M-H_2O), 411 (M-COOH), 248 ($C_{16}H_{24}O_2$), 220 ($C_{15}H_{24}O$), 203 (248-COOH), 207 ($C_{14}H_{23}O$) and 189 (207-H_2O) which are characteristics peaks for betullinic acid [13]. Seven degree of unsaturation corresponding to five rings, a double bond and one acidic carbonyl in the molecule. The mass fragmentation pattern also confirms the molecular ion. The assignment of chemical shifts of the protonated carbons was achieved by a Heteronuclear Multiple Quantum Correlation (HSQC) experiment. The ^1H-^1H Correlation Spectroscopy experiment (COSY) showed cross peaks for coupled protons at position 2 and 3, 5 and 6, 12 and 13, 15 and 16, 13 and 18, 18 and 19, 21 and 22, 23 and 24 for MBG-Q_{13}. The Nuclear Overhauser Effect Spectroscopy (NOESY) experiment confirms α orientation of the hydroxyl group at C-3. This is due to the coupling of protons at position 23 and 24 with its neighbouring protons at position 3 based on orientations. The HMBC showed connectivity between the proton in position 18 and the C=O (180.5) in position 28 confirming the presence of a carboxylic group not shown in the ^1H NMR spectrum. Comparison of MBG-Q_3 spectroscopic data with that of betulinic acid reported in literature [11] showed similarity. The structure of MBG-Q_3 was elucidated to be 3α-hydroxy-20(29)-lupen-28-oic acid.The compound **3** showed diagnostic IR absorption bands for O-H, C-$H_{str.}$, C=O, and C-H_{bend} at 3307, 2941/2869, 1687 and 1456 cm^{-1} respectively. The UV spectrum showed a single band at 207 nm which indicated n→π* transition of carbonyl group of carboxylic acid. The ^1H NMR spectrum of ERG-E_7 showed pattern characteristic of a lupane-type carbon skeleton. It displayed signals attributed to exomethylene group at δ 4.71 and 4.58 (1H, each dd, J = 2.0/2.4 Hz, H-29) which together with an allylic methyl at δ 1.69 which indicated an isopropenyl moiety. The double doublet at δ 3.11 (1H, dd, J = 11.0/11.5 Hz) was assigned to H-3 with the vicinyl methylene protons suggested a β orientation of the hydroxyl group at C-3. The spectrum also showed a triplet of doublet at δ 2.33 (1H, ddd, J = 3.6/12.4 Hz, H-13) and δ 3.05 (1H, ddd, J = 5.2/10.8 Hz, H-19) for the methine proton at C-13 and C-19 while the triplet of triplet at δ 2.21/1.36 (2H, tt, J = 3.6/12.4 Hz, H-16) and δ 1.19/1.51 (2H, tt, J = 2.8/13.2 Hz, H-22) accounted for the methylene proton at C-16 and C-22. The other five methyl groups resonated at δ 1.01, 0.95, 0.94, 0.84 and 0.74. There also exist signals at δ 3.31 (1H, br. s) for alcoholic OH and δ 10.6 (1H, br. s) for carboxylic OH [19]. The EI-MS showed m/z 456.1 which was confirmed with High Resolution Mass Spectroscopy (HRMS) for a molecular formula $C_{30}H_{48}O_3$ (456.3598). The structure of ERG-E_7 was elucidated to be 3α-hydroxy-20(29)-lupen-28-oic acid. Comparison of MBG-Q_3, DBG-D_{13} and ERG-E_7 with betulinic acid showed similarity in spectroscopic data [14, 15] as shown in Table 1. They are all betullinic acid except that the hydroxyl group at C-3 is α-oriented for MBG-Q_3 and β-oriented for DBG-D_{13} and ERG-E_7. This was also confirmed through NOESY experiment. The structures were elucidated to be 3α-hydroxy-20(29)-lupen-28-oic acid and 3β-hydroxy-20(29)-lupen-28-oic acid as shown in Fig. 1.

Table 1. ^{13}C NMR Data for DBG-D_{13}, MBG-Q_3 and ERG-E_7 compared to Betulinic Acid

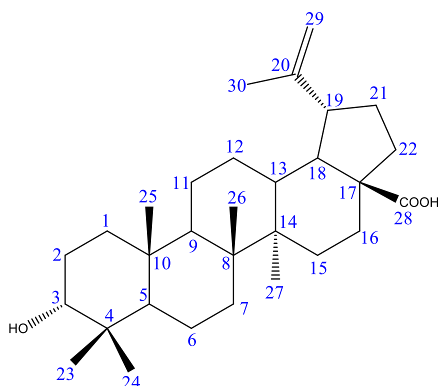
Assignment	DBG-D_{13}	MBG-Q_3	ERG-E_7	Betulinic acid	DEPT
1.	39.5	39.9	40.1	39.77s	CH_2
2.	28.3	28.1	28.1	28.79t	CH_2
3.	78.5	79.7	79.7	78.60d	CH
4.	39.6	40.1	38.3	39.99s	C
5.	56.3	56.9	56.9	56.41d	CH
6.	19.1	19.5	19.5	19.26t	CH_2
7.	35.2	35.6	35.6	35.32t	CH_2
8.	41.5	41.9	41.9	41.60s	C
9.	51.4	52.0	52.0	51.44d	CH
10.	37.9	38.3	39.9	38.00s	C
11.	21.7	22.1	22.1	21.69t	CH_2
12.	26.4	26.9	26.9	26.60t	CH_2
13.	39.0	39.7	39.7	39.10d	CH
14.	43.0	43.6	43.6	43.33s	C
15.	31.3	31.7	31.7	31.70t	CH_2
16.	32.8	33.4	33.4	33.36t	CH_2
17.	56.8	57.5	57.5	57.11s	C
18.	49.9	50.5	50.5	50.26d	CH
19.	47.9	49.6	49.6	48.25d	CH
20.	151.6	152.1	152.1	151.80s	C
21.	30.4	30.9	30.9	30.76t	CH_2
22.	37.5	38.2	38.2	38.06t	CH_2
23.	28.5	28.6	28.6	29.14q	CH_3
24.	16.1	16.1	16.1	16.80q	CH_3

25.	16.5	16.6	16.7	16.90q	CH_3
26.	16.6	16.7	16.7	16.90q	CH_3
27.	15.0	15.1	15.1	15.38q	CH_3
28.	177.5	180.5	180.5	179.32s	C=O
29.	109.9	110.1	110.1	110.41t	CH_2
30.	19.5	19.6	19.6	19.95q	CH_3

Ref. [14, 15]



Compounds 1 and 3
(3-beta-hydroxy-20(29)-lupen-28-oic acid)



Compound 2
(3-alpha-hydroxy-20(29)-lupen-28-oic acid)

Fig. 1. Structures of Compounds 1, 2 and 3

4. CONCLUSION

In conclusion, the present work confirms the isolation of large amount of betulinic acid from dichloromethane, methanol, (stem bark) and ethanol extract (root) of *Uapaca guineensis* with α and β orientation. Similarly, the values of ^{1}HNMR, IR, UV, EI-MS, HREI-MS, ^{13}CNMR and 2D-NMR obtained were in agreement with the literature values [15].

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

AUTHORS' CONTRIBUTIONS

This work was carried out in collaboration between all Authors. 'Author IO' designed the study, performed the statistical analysis and wrote the protocol. 'Author AO' managed the analyses of the study, wrote the first and final draft of the manuscript. 'Author PO and AO' managed the literature searches. 'Author IC and IO' monitored the analyses and provided major equipments used. All authors read and approved the final manuscript."

CONSENT

It is not applicable.

ETHICAL APPROVAL

It is not applicable.

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