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Article

Evaluation of the Metabolic Production from the Co-Culture of *Saccharicola* sp. and *Botryosphaeria parva*, an Endophytic Fungi Associated with *Eugenia jambolana* Lam.

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A new compound, (6*R*,7*S*,2*E*,4*E*)-6,7-dihydroxy-4,6-dimethylocta-2,4-dienoic acid (**1**), together with eight known compounds were isolated from the co-culture of *Saccharicola* sp. and *Botryosphaeria parva*, an endophytic fungi associated with *Eugenia jambolana* Lam. (Myrtaceae) plant species. The structures were elucidated by spectroscopic analysis of the one-dimensional (1D) and two-dimensional (2D) nuclear magnetic resonance (NMR) and mass spectrometry (MS) data as well as by comparison with literature data. The bioactivity (antioxidant and antifungal) of the crude EtOAc was evaluated. All crude extracts presented antioxidant activity and only the crude extract from the co-culture was active on the fungus *Cladosporium sphaerospermum*. This investigation contributed to the knowledge about the metabolic production of two endophytic fungi *Saccharicola* sp. and *Botryosphaeria parva* in co-culture, once, until the present date, there are no studies in the literature that report the understanding of the chemical interaction of both grown in the same environment.

Keywords: fungi, *Eugenia jambolana*, *Botryosphaeria parva*, *Saccharicola* sp., dienoic acid

Introduction

Endophytic fungi are microorganisms that colonize the internal tissues of plants without causing any disease or apparent immediate negative symptoms.[1,2] These endophytes are known for the biosynthesis of a range of secondary metabolites that act as a defense of the host plant species against external threats from superficial pathogen, disease resistance and stress tolerance. Most of these compounds, in addition to promoting host protection, contribute significantly to the advancement of medicine.[2] As a strategy for inducing new and different bioactive secondary metabolites through these microorganisms, a promising approach called co-culture is reported in the literature. Co-culture involves the cultivation of two or more species in the same confinement environment by promoting interaction through signaling or defense molecules and thereby activating the silent gene of these microorganisms by increasing metabolic production.[3-5]

In the present investigation, we presented the co-culture of the endophytic fungi *Botryosphaeria parva* and *Saccharicola* sp., associated with the *Eugenia jambolana* (Myrtaceae) plant species.

Chemical and biological previous studies of fungi of the genus *Botryosphaeria* and *Saccharicola* afforded several bioactive compounds of different classes. Derivatives of benzofuran, diterpenoids, lactones, naphthalenones, and polyketides are commonly biosynthesized by fungi of the genus *Botryosphaeria*. These compounds present relevant biological activities such as antibacterial, antiseptic, phytotoxic, and antimicrobial.[6] Chapla *et al.*[7] describe the potential of the endophyte *Saccharicola* sp. in the production of oxygenated cyclohexanoids. Cyclohexanols are known for present important antiviral, antifungal, antibacterial, and antitumor activities.[8]

In order to explore the metabolic production of the endophytes associated with the medicinal plant *E. jambolana*, the *Saccharicola* sp. and *Botryosphaeria parva* were co-cultured in Czapek liquid medium. This co-culture is described for the first time in the literature. One new compound (**1**) and eight known compounds were isolated from the ethyl acetate co-culture extract.[9] The structural elucidation of compounds was performed by one-dimensional (1D) and two-dimensional (2D) nuclear magnetic resonance

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(NMR) and high-resolution electrospray ionization mass spectrometry (HRESIMS) analysis.

Experimental

General experimental procedures

The 1D (^{1}H and ^{13}C) and 2D (^{1}H-^{1}H correlation spectroscopy (COSY), heteronuclear single quantum correlation (HSQC) and heteronuclear multiple bond correlation (HMBC)) nuclear magnetic resonance (NMR) experiments of the secondary metabolites were obtained on the Bruker Avance™ III 600 (14.1 T) (Rheinstetten, Germany) spectrometer at 600 MHz (^{1}H) and 151 MHz (^{13}C) using deuterated solvent (CD_3OD, 99.98% D) as internal standard for ^{1}H NMR and ^{13}C NMR chemical shifts. High-resolution mass spectra were recorded on a Bruker™ Maxis Impact ESI-QTOF-HRMS (electrospray ionization quadrupole time-of-flight high-resolution mass spectrometry) spectrometer with direct insertion device in the sample-injection analysis with continuous flow of 3.0 μL min^{-1}. The samples were solubilized in MeOH 100% and diluted in MeOH:H_2O (1:1, v/v, containing 0.1% formic acid) and were ionized by electrospray (ESI) in negative or positive mode.

Thin layer chromatography (TLC) analyses were performed using silica gel 60 (Whatman™, 20 × 20 cm × 0.2 mm). Spots on the TLC plates were visualized under ultraviolet (UV) light (λ = 254 and 365 nm) and by spraying with anisaldehyde-H_2SO_4 reagent followed by heating at 130 °C. Column chromatography was performed on a RP-18 adsorbent phase (Merck, 40-75 μm, 210 × 40 mm, internal diameter (i.d.) 28 mm), using ACN:H_2O gradient under reduced pressure.

High performance liquid chromatography (HPLC) was performed on a Shimadzu (Kyoto, Japan) system coupled to a UV diode array detector (DAD) SPD-M20A, containing two LC-20AT pumps, DGU-20A3 degasser, CBM-20A communicator, SIL-20A automatic injector, and CTO-20A oven. The analytical column used was Phenomenex Luna RP-18 (250.0 × 4.6 mm, 5 μm, 100 Å), and as eluent it was used a gradient of H_2O:ACN (95:05-0:100) in 50 min, flow rate of 1.0 mL min^{-1}, λ = 254 nm and 30 μL injection volume. Semi-preparative HPLC was performed on a Shimadzu (Kyoto, Japan) system coupled to a UV diode array detector (DAD) SPD-M20A, containing two LC-6AD pumps, CBM-20A communicator, SIL-10AF automatic injector, using a Phenomenex Luna RP-18 column (250.0 × 10.0 mm, 5 μm, 100 Å), at a flow rate of 4.0 mL min^{-1}. Data acquisition was performed using the software Shimadzu-LC solutions (LC Solution 2.1).

The isolation of compounds **5**-**8** was performed using a HPLC-SPE-TT (high performance liquid chromatography-solid phase extraction-transfer tube) equipped with an solid phase extractor (SPE) Bruker/Spark Prospekt II as an interface between an HPLC in the analytical mode, an Agilent 1260 infinity series HPLC (HP1260 infinity, Agilent, USA) with photodiode array ultraviolet detector (PDA) and an automatic NMR sampler and tracer (TT). The analytical column used was Phenomenex Luna RP-18 (250.0 × 4.6 mm, 5 μm, 100 Å), and as eluent it was used a gradient of H_2O:MeOH (80:20-0:100) in 30 min, flow rate of 0.8 mL min^{-1}, λ = 220 nm and 30 μL injection volume. The ultrapure water used was obtained from a Milli-Q equipment (Millipore, Darmstadt, Germany). HPLC grade solvents were LiChrosolv® from Merck (Darmstadt, Germany).

The circular dichroism (CD) curve of **1** was obtained using a Jasco™ LC-NetII/ADC liquid chromatograph (Tokyo, Japan), equipped with circular dichroism (CD) (2095 Plus) and photodiode array (MD-2018 Plus) detectors. Chiracel OD-RH column (Diacel Chemical Ind., 5 μm, 150 mm × 4.6 mm, flow rate of 0.5 mL min^{-1}) along with the protective guard column Chiracel OD-RH (Diacel Chemical Ind., 5 μm, 10 mm × 4.0 mm), in gradient mode (5-100% MeOH:H_2O for 40 min) were used for analytical analysis.

Isolation and identification of the endophytic fungi

Leaves and stems of *Eugenia jambolana* were collected in Araraquara city, São Paulo State, Brazil (21°48'22.7"S 48°11'31.9"W), in March 2008. The species was identified by Dra Maria Inês Cordeiro and a voucher specimen (SP 454124) was deposited in the Herbarium "Maria Eneida Kauffmann", of the Botanic Garden of São Paulo, Brazil. The activity of access to genetic heritage was registered by Sistema Nacional de Gestão do Patrimônio Genético e do Conhecimento Tradicional Associado (SisGen A91372A).

The endophytic fungi were isolated from healthy leaves and stems of *E. jambolana*, which were subjected to surface sterilization. The leaves and stems were first washed with water and soap and immersed in 1% aqueous sodium hypochlorite solution for 3 min and 70% aqueous EtOH for 1 min (2×). Finally, the plant material was immersed in sterile H_2O for 1 min (2 times). The sterilized material was cut into 2 × 2 cm pieces and deposited onto a Petri dish that contained potato dextrose agar (PDA) and gentamicin sulfate (100 μg mL^{-1}).[7] The pure fungal strains were obtained after serial transfers on PDA plates, stored in sterile water at 25 °C, and then deposited at the Núcleo de Bioensaios Biossíntese e Ecofisiologia de Produtos

Naturais (NuBBE) fungi collection in Araraquara, Brazil.[7]

Two of the isolated endophytes by Chapla *et al*.[7] were identified by molecular taxonomy as *Botryosphaeria parva* and *Saccharicola* sp. from the leaves and stems of *E. jambolana*, respectively.

Co-culture and extraction

The preserved endophytic fungi *Botryosphaeria parva* and *Saccharicola* sp. were inoculated separately into Petri dishes containing PDA and incubated for 5 and 10 days at 25 °C, respectively, to obtain micellar mass. The strains of both fungi were inoculated together into forty-four flasks (500 mL), each containing 300 mL of Czapek liquid medium. The medium was inoculated with the endophytes and incubated at 25 °C for 28 days in static mode.

The mycelia biomass accumulated in the flasks was separated from the aqueous medium by filtration, and the filtrate was subjected to a liquid partition with EtOAc (3 × 1/3 filtered volume). The organic layers were combined and washed with distilled H_2O (2 × 1/2 filtered volume). The solvent was removed under reduced pressure yielding the crude EtOAc extract (249.5 mg).

In order to evaluate and compare the antifungal potential and the capacity to scavenge 2,2-diphenyl-1-picryl-hydrazyl-hydrate (DPPH) radical of the EtOAc extracts obtained from co-culture and pure strains, endophytes *Botryosphaeria parva* and *Saccharicola* sp. were cultivated separately, according to the methodology previously described, resulting in EtOAc crude with yields of 90.0 and 100.5 mg, respectively.

Fractionation and isolation

The crude co-culture EtOAc extract (249.5 mg) was dissolved in 100% MeOH (10 mL) and fractionated by column chromatography (CC) over RP-18 silica gel using H_2O:ACN gradient (95:05→0:100), to yield six fractions (Fr.1-Fr.6; 95.0 mL each).

Fr.1 (95.0 mg) and Fr.4 (23.9 mg) were submitted to semi-preparative HPLC-DAD (C18, ACN/H_2O, 5→45% ACN in 40 min and 40→90% ACN in 40 min, respectively; λ 254 nm), to yield **1** + **3** (1.0 mg, t_R = 3.0 at 8.0 min), **2** (1.2 mg, t_R = 18.51 min) and **4** (3.1 mg, t_R = 29.03 min), and **9** (0.4 mg, t_R = 19.36 min), respectively. Fr.2 (32.2 mg) was subjected to HPLC-SPE-TT using a RP-18 column in the analytical mode, MeOH:H_2O (20→60%), flow rate 0.8 mL min^{-1}, λ 220 nm, to give **5** (1.0 mg, t_R = 11.78 min), **6** (1.0 mg, t_R = 13.21 min), **7** (1.0 mg, t_R = 15.35 min), and **8** (1.0 mg, t_R = 15.65 min).

Antifungal activity

The EtOAc crude extracts from co-culture and the isolated culture of *Botryosphaeria parva* and *Saccharicola* sp. were evaluated against the phytopathogenic fungi *Cladosporium cladosporioides* (Fresen) Vries SPC 140 and *Cladosporium sphaerospermum* (Perzig) SPC 491 using the TLC diffusion method.[10] The crude extracts (40 μg μL^{-1}) were dissolved in 100% MeOH and applied on silica gel TLC plates. Nystatin was used as a positive control at 5.0 μg. After eluting with $CHCl_3$:MeOH (8:2), the plates were sprayed with the fungi suspension (5×10^7 spores mL^{-1}), and incubated at 25 °C for 48 h in the absence of light. The antifungal activities were detected as a clear zone of inhibition on the fungi suspension and by UV light in 254 and 366 nm.

DPPH scavenging capacity assay

The radical scavenging capacity of the EtOAc crude extract from co-culture and the isolated culture were evaluated from their ability to reduce the radical 2,2-diphenyl-1-picrylhydrazyl (DPPH) by TLC bioautography assay according to the described procedure.[11] The crude extracts (2.0 mg mL^{-1}) were dissolved in 100% MeOH, applied on silica gel TLC plates, and eluted with $CHCl_3$:MeOH (8:2). Rutin was used as a positive control. After the elution of the extracts, the plates were nebulized with a methanolic solution of DPPH 0.2% (m/v). The chromatoplate was kept in the dark for 1 h. After that, it was observed under white light. The compounds with antiradical activity appeared as yellow spots against the purple-blue background.

Results and Discussion

The EtOAc crude extracts from co-culture and the isolated culture of *Saccharicola* sp. and *Botryosphaeria parva* were tested for their antifungal activity against two phytopathogenic fungi (*C. cladosporioides* and *C. sphaerospermum*) using the TLC diffusion method.[10] The co-culture extract (40 μg μL^{-1}) presented antifungal activity against both fungal strains, showing potent antifungal activity against *C. cladosporioides* and moderate activity against *C. sphaerospermum*, in the concentration 5×10^7 spores mL^{-1}. The isolated culture extracts showed antifungal activity only against *C. cladosporioides*. Furthermore, the crude extracts were evaluated for their capacity to scavenge DPPH radical. TLC bioautography assay was selected due to simplicity, reproducibility and efficiency.[11] The TLC bioautography profile of the crude

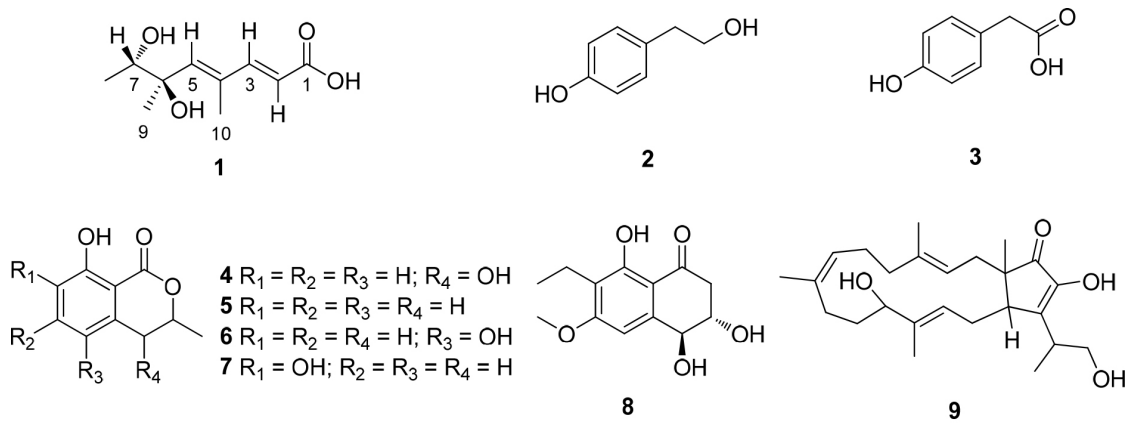

Figure 1. Chemical structures of compounds **1-9**, isolated from co-culture of *Botryosphaeria parva* and *Saccharicola* sp.

extracts from co-culture and the isolated culture showed strong yellow spots against the purple background, compared to the rutin standard, indicating the presence of compounds containing groups with a high capacity of reduction of radical DPPH.

The chemical investigation of the EtOAc crude extract obtained from the co-culture in Czapek medium of the endophytes resulted in the isolation of nine compounds (**1-9**, Figure 1), including a new compound named (6*R*,7*S*,2*E*,4*E*)-6,7-dihydroxy-4,6-dimethylocta-2,4-dienoic acid (**1**).[9]

Compound **1** was isolated in a mixture with **3**. Compound **1** showed a UV curve obtained in HPLC-DAD with maximum absorption at 265 nm, similar to the ester isolated previously and described in the literature by Borges *et al.*[12] (Figure 2).

HRMS analysis showed $[M - H]^-$ ion at *m/z* 199.0961 for deprotonated molecule, indicating the molecular formula $C_{10}H_{16}O_4$ for **1** (calcd. for $C_{10}H_{15}O_4$, 199.0970), suggesting the presence of three unsaturations. Compound **1** was

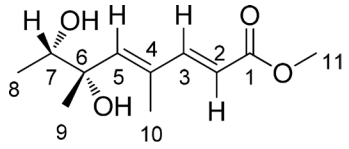

Figure 2. Compound named as methyl (6*S*,7*S*,2*E*,4*E*)-6,7-dihydroxy-4,6-dimethyl octanoate isolated by Borges *et al.*[12]

elucidated mainly by NMR spectroscopy, including ^{1}H, HSQC, HMBC and ^{1}H-^{1}H COSY. The ^{1}H NMR spectrum displayed three signals of olefinic hydrogens at δ_H 7.26 (d, 1H, *J* 15.5 Hz, H-3), δ_H 5.95 (s, 1H, H-5) and δ_H 5.87 (d, 1H, *J* 15.5 Hz, H-2). Other signals at δ_H 3.68 (q, 1H, *J* 6.4 Hz, H-7) and δ_H 2.07 (s, 3H, H-10), δ_H 1.33 (s, 3H, H-9) and δ_H 1.15 (d, 3H, *J* 6.4 Hz, H-8) indicated the presence of oxygenated methine proton and three methyl protons, respectively (Table 1). The ^{1}H-^{1}H COSY experiment indicated correlations between H-7 and H-8, H-2 and H-3, H-5 and H-10 (Figure 3).

The attribution of the carbons was carried out by the data obtained through the HMBC and HSQC experiments.

Table 1. NMR data obtained for compound **1** and comparison with similar ester from the literature[12]

Position	Compound **1**		Methyl (6*S*,7*S*,2*E*,4*E*)-6,7-dihydroxy-4,6-dimethyl octanoate[12]	
	δ_H / ppm	δ_C[a] / ppm	δ_H / ppm	δ_C / ppm
1	–	170.6	–	NO
2	5.87 (d, *J* 15.5)	117.5	5.89 (d, *J* 1.57)	116.6
3	7.26 (d, *J* 15.5)	150.2	7.32 (d, *J* 15.7)	152.6
4	–	134.9	–	136.2
5	5.95 (s)	143.1	6.00 (s)	145.7
6	–	74.3	–	76.9
7	3.68 (q, *J* 6.4)	73.5	3.66 (q, *J* 6.4)	75.1
8	1.15 (d, *J* 6.4)	16.3	1.15 (d, *J* 6.4)	17.7
9	1.33 (s)	23.3	1.33 (s)	24.7
10	2.07 (s)	11.9	2.07 (s)	13.3
11			3.77 (s)	52.1

[a] ^{13}C data obtained by HSQC and HMBC. ^{1}H NMR at 600 MHz and ^{13}C NMR at 150 MHz, *J* in Hz, CD_3OD. NO: not observed.

HMBC spectrum showed correlations to ^{2}J and ^{3}J of the H-2↔C-4, H-5↔C-3/C-6/C-9/C-10, H-7↔C-5/C-6/C-9, H-8↔C-7, H-9↔C-5/C-7, H-10↔C-3/C-4/C-5. Furthermore, an important correlation was observed among the hydrogen at δ_H 7.26 (H-3) with the carbons in δ_C 170.6 (C-1)/C-4/C-5/C10, suggesting the structure of a α,β-unsaturated carboxylic acid (Figure 3).

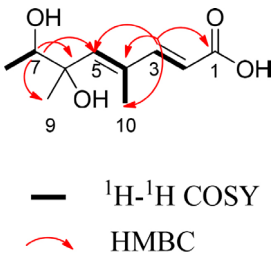

Figure 3. Selected HMBC correlation and ^{1}H-^{1}H COSY for compound **1**.

The *E* configuration from the double bond in C-2 of **1** was attributed due to the ^{1}H-^{1}H coupling constant value *J* 15.5 Hz of H-2 with H-3. The configuration of the C-6 and C-7 stereocenters was deduced based on the CD experiment obtained by CD coupled HPLC-DAD and by comparison with the model compound data described in the literature.[12] The circular dichroism curve of **1** showed negative and positive Cotton effects at 265 and 238 nm, respectively. These effects refer to π→π* electronic transitions of the diene group. By comparison with the calculated spectra by Borges *et al.*,[12] the experimental electronic circular dichroism (ECD) data of **1** showed similarity with the negative and positive Cottons effects at approximately 255 and 220 nm, respectively, referring to the centers with 6*R*,7*S* configuration.[12]

Thus, **1** was determined to be (6*R*,7*S*,2*E*,4*E*)-6,7-dihydroxy-4,6-dimethylocta-2,4-dienoic acid. All spectra are provided in the Supplementary Information section.

The structures of the known compounds (**2**-**9**) were determined by spectroscopic analysis of the ^{1}D and ^{2}D NMR, as well as by comparison with literature data. The known compounds were identified as tyrosol or 2-(4-hydroxyphenyl)ethanol(**2**),[13-15] 2-(4-hydroxyphenyl) acetic acid (**3**),[16] *cis*-4-hydroxymellein (**4**),[17,18] mellein (**5**),[17] 5-hydroxymellein (**6**),[18] 7-hydroxymellein (**7**),[18] *trans*-botryosphaerone D (**8**),[19] and 11-epiterpestacin (**9**)[20] (Figure 1).

In this study, the predominant class of isolated compounds was isocoumarin. The isocoumarins are secondary metabolites found in a wide variety of organisms, such as bacteria, lichens and fungi, being reported from different species and genus of endophytic fungi.[21] Structurally they are similar to coumarins, having as difference an inverted lactonic ring. This class of compounds can have several biological activities, such as protease inhibitor, antimicrobial, growth regulators, antiallergic, and antimalarial.[21,22]

Isocoumarins isomers **4**, **5**, **6** and **7** have already been isolated from endophytic fungi such as *Neofusicoccum parvum* associated with the plant species *Elaeocarpus serratus* (Elaeocarpaceae),[18] and *Penicillium* sp. associated to *Alibertia macrophylla* (Rubiaceae).[23,24] Tests with phytopathogenic fungi *C. cladosporioides* and *C. sphaerospermum* revealed that 4-hydroxymellein and 7-hydroxymellein have potent antifungal activity with a limit of detection of 5.0 and 10.0 µg, and 10.0 and 25.0 µg, respectively, comparable to nystatin, used as a standard.[23,24] These activities corroborate the activities of the crude extracts against the respective pathogenic fungi obtained in this study. In addition, 4-hydroxymellein showed moderate inhibitory activity of acetylcholinesterase (AChE).[23]

The compound **9** belongs to the terpene class and has a strong phytotoxic activity. In the literature, there are reports of its isolation from the endophyte *Botryosphaeria* sp. SCSIO KcF6 derived from the *Kandelia candel* mangrove plant,[6] and from the fungus *Bipolaris sorokiniana* NSDR-011.[20] Its planar structure is similar to that of terpestacin isolated from *Arthrinium* sp., which was isolated as an inhibitor of the syncytium formation of the human immunodeficiency virus (HIV).[20,25]

Conclusions

This study contributed to the knowledge about the metabolic production of two endophytic fungi *Saccharicola* sp. and *Botryosphaeria parva* in co-culture, once until the present date, there are no studies in the literature that report the understanding of the chemical interaction of both fungi growing in the same environment. Nine compounds were isolated, including carboxylic acids, isocoumarins and terpenes. Compound **1**, (6*R*,7*S*,2*E*,4*E*)-6,7-dihydroxy-4,6-dimethylocta-2,4-dienoic acid, is being described for the first time in the literature.

Supplementary Information

Supplementary information is available free of charge at http://jbcs.sbq.org.br as PDF file.

Acknowledgments

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Author Contributions

Angela R. Araujo and Vanderlan S. Bolzani granted the project, guided and designed the experiments; Mayra F. Costa and Maiara S. Borges isolated the compounds and determined the chemical structures; Vanessa M. Chapla and Carolina R. Biasetto performed the isolation of the fungi and helped in writing the manuscript; Isabele R. Nascimento assisted in the structural determination of the compounds and in writing of the manuscript.

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