Isolation, characterization, anticancer and antioxidant activities of 2-methoxy mucic acid from *Rhizophora apiculata*: An *in vitro* and *in silico* studies

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Abstract

In this present study, the sugar based bioactive molecule, 2-methoxy mucic acid (4) was isolated for the first time from the methanolic extract from the leaves of *Rhizophora apiculata* . The structure of compound was well characterized by different spectroscopic analysis, including FT-IR, ¹ H, ¹³ C NMR spectroscopy and HRMS. Anticancer activity of 2-methoxy mucic acid (4) was evaluated against HeLa and MDA-MB231 cancer cell lines and they displayed promising activity with the IC 50 values of 22.88283±0.72 µg/ml in HeLa and 2.91925±0.52 µg/ml in case of MDA-MB231, respectively. The antioxidant property of 2-methoxy mucic acid (4) was found to be (IC 50) 21.361±0.41 µg/ml. Apart from in vitro studies, we also performed extensive in silico studies (molecular docking and molecular dynamics simulation) on four key anti-apoptotic Bcl-2 family members (Bcl-2, Bcl-w, Bcl-xL and Bcl-B) towards 2-methoxy mucic acid (4) and the results revealed that this molecule showed higher binding affinity towards Bcl-B protein (ΔG = -5.8 kcal/mol) and the structural stability of Bcl-B protein was significantly improved upon binding of this molecule. The present study affords key insights about the importance of 2-methoxy mucic acid (4), and thus leads to open the therapeutic route for anticancer drug discovery process.

Introduction

Mangrove plants are rich sources of natural products of polyphenols, phenolic compounds, alkaloids, flavonoids, and tannins which are active secondary metabolites having wide applications in pharmacy and medicine (Kathiresan and Bingham, 2001; Sachithanandam et al., 2020). However, the mangrove plants from Andaman and Nicobar Islands (ANI), one of the most ecologically diverse places in the tropical region, have not been chemically and biologically studied in detail. The marine living and non-living resources of ANI are unique because of the volcanic and mid-oceanic characteristics (Indo-Pacific triangle), with ecologically rich marine habitats that provide a wide variety of marine goods and services. The *Rhizophora* genus consists of more than 10 species widely distributed all over the world (Sachithanandam et al., 2019). The *R. apiculata* in the family of Rhizophoraceae, has been exploited for numerous traditional medicines by people in Indo-Pacific region for treating various diseases and ailments, such as astringent, diarrhoea, chronic typhoid fever, septic wounds, nausea, pain, diabetes, bleeding in fresh wounds and inflammation (Loo et al., 2007; Kaliyamurthi et al., 2014). The tribal communities of ANI are a reservoir of vast knowledge. They have been using *R. apiculata* for treatment of various diseases such as Diarrhoea, Nausea, Vomiting, Typhoid, Hepatitis, Ulcers, an antiseptic, Insecticide, Amoebiasis, Stomach pain related problems and Bone fractures and Joint pain cure since ages (Sachithanandam et al., 2019; Lalitha et al., 2019). Research into the scientific validation of medicinal plants used by tribal communities in A&N to treat various diseases has not been attempted so far. Very few studies have been documented on this aspect (Sachithanandam et al., 2019; Sachithanandam et al., 2020; Sachithanandam et al., 2021). The present study attempted the scientific validation of *R. apiculata* mangroves plants by encouraging some studies on bioactive compounds extraction, compound purification, antibacterial activities used by tribal communities in A&N Islands.

Isolation, characterization, and bioprospecting of bioactive compounds from mangrove plants have always generated interest to many researchers (Wu et al., 2008; Sachithanandam et al., 2020). In addition, many bioactive compounds exhibited various biomedical properties such as anti-oxidant (Loo et al., 2008), anticancer, antimicrobial (Prabhu and Guruvayoorappan, 2012; Ramalingam and Rajaram, 2018) anti-nociceptive, antiviral (Premanathan et al., 1999), antihypoglycemic (Sachithanandam et al., 2019), and anti-cholinesterase activities (Suganthi et al., 2009). The mangrove plant contains various natural bioactive compounds such as, alkaloids, benzoquinone, campesterol, cinnamate, diterpene, flavonoid, lupeol, essential oils, polysaccharide, polyphenols, sitosterol, sterols, stigmasterol and triterpenoids (Premanathan et al., 1999; Sachithanandam et al., 2020). Novel taraxeryl triterpenoid, taraxeryl *cis-p*-hydroxycinnamate phenolic compounds (pyroligneous acid), and diterpenoid have been isolated from the leaves of *R. apiculata* (Kokpol et al., 1990; Loo et al., 2008; Gao et al., 2011). The nitrogen alkaloids identified from this plant act as agonist for PPAR γ receptor as reported by Selvaraj et al., (2015). *R. apiculata* leaves are the bioavailable source to develop biomedical application, which comprises more bioactive products when compared to bark and seedlings (Bhakuni et al., 1992). In our search for new bioactive phyto-chemical compounds from ANI mangroves, we studied leaves of *R. apiculata* to explore the possibilities of isolating novel biological compounds with promising antioxidant and anticancer activities. A breakthrough result was achieved when the *R. apiculata* leaves were subjected to methanol extraction followed by column chromatography, a new bio-active compound, namely, 2-methoxy mucic acid (**4**) (**Fig. 1**) was obtained with high purity (**Fig. 1**). Moreover, we also performed molecular docking studies on 2-methoxy mucic acid (**4**) towards four anti-apoptotic drug-target proteins (Bcl-2, Bcl-w, Bcl-xL and Bcl-B). Herein, we present the isolation, structure characterization, biological evaluation and *in silico* studies of 2-methoxy mucic acid from *R. apiculata*. In addition, the structural stability of 2-methoxy mucic acid molecule towards Bcl-B was studied through molecular dynamics (MD) simulation.

Mucic acid (**1**) (**Fig. 1**) is a tetrahydroxylated dicarboxylic organic acid, a naturally occurring sugar acid in putrefied blood and fresh juice from sugar beet (Stark et al., 1950). It was first isolated from full ripe peaches and pears (Anet and Reynolds, 1954). Limited research only has been carried out towards the isolation and identification of mucic acid and its derivatives (Thompson and Kies, 1965). Traditionally, this acid is used in skin care and cosmetic products in India, China, and Thailand (Yu and Van Scott, 1995); used as a chelator in pharma industries (Lewkowski, 2001) and as an intermediate chemical for the synthesis of polymer materials (Mehtiö et al., 2015). Earlier, the mucic acid gallate (**2**) and di-O-gallate (**3**) (**Fig. 1**) was isolated from *Phyllanthus emblica* medicinal plant and studied for antioxidant activity (Zhang et al., 2017; Olennikov et al., 2015). Naturally, the mucic acid could be produced by fungal enzyme *Trichoderma reesei* and *Escherichia coli* as biological sources and through extraction of pectin from dry citrus peel (Barth and Wiebe, 2017). The synthesis of mucic acid was made both by nitric oxidation of D-galactose and electrolytic oxidation of D-galacturonate (Kiely and Kirk, 2010). Because of the less solubility of mucic acid in water, it could be easily precipitated as solid in the culture broth (Zhang et al. 2016). Among the large variety of carbohydrate derivatives, the mucic acid (**1**) and its analogues (**2-3**) have evoked great interest in the industry as precursor of looking for several other derivatives. In this

regard, the mucic acid has been used as a versatile intermediate for the synthesis of various natural products as well as pharmaceutical drugs such as pyrones (Leonardi et al., 2020), pectin derived galacturonic acid (Purushothaman et al., 2018), furan and its polyester (Zhao et al., 2019), salts of mucic acid (Tian et al., 2000), adipic acid (Li et al., 2014), mucic acid 1,4-lactone methyl ester 3-O-ferulate (Sengoku et al., 2012) and mucic acid polymer products (Pan et al., 2015). In addition to mucic acid, its derivatives have exhibited various biological properties such as antioxidant (Luo et al., 2011; Olennikov et al., 2015), antimicrobial (Nasr and Mostafa, 2005), anticancer (Luo et al., 2011; Zhang et al., 2017) and antidiabetic activities (Patel and Goyal, 2011).

Materials And Methods

Plant material

Raw and fresh leaves of mangrove plant, *R. apiculata* (**Fig. 2**) were collected from the Sippighat mangrove area, South Andaman Island, India in September 2018. The leaves were washed thoroughly with deionized water and dried at room temperature and later pulverized into fine powder by using mixer grinder.

Chemicals, reagents, and Standards

All Chemicals, inorganic solvents, and reagents were purchased from Finar, Merck, Sigma, Himedia Pvt Ltd., India. Glass plates coated with silica gel (60–120 mesh SRL chemicals) were employed for thin layer chromatography (TLC).

Instruments

Nuclear Magnetic Resonance (NMR), ^1H -NMR (400 MHz), ^{13}C -NMR (100 MHz) and Distortionless Enhancement by Polarization Transfer (DEPT)-135 spectra were recorded for methanol- D_4 (CD_3OD) solutions on a Bruker Advance 400 spectrometer with tetramethyl silane (TMS) as internal standard; J values in Hz. ^1H NMR data are reported as follows: chemical shift, multiplicity (s = singlet, d = doublet, t = triplet, q = quartet and m = multiplet), coupling constant, integration. Other Instrumentation: The Ultraviolet–Visible spectroscopy (UV) spectra were obtained by Waters 490 spectrometer, the Fourier-Transform Infrared spectroscopy (FT-IR) spectra by Biorad FT-80 spectrophotometer and High-resolution mass spectra (HRMS) were recorded on an Agilent Q-TOF micro hybrid quadrupole mass spectrometer using electron spray ionization mode. All the reactions and chromatographic separations were observed by thin layer chromatography (Parthiban and Makkam, 2020; Parthiban et al., 2015; Rao and Parthiban, 2014).

Extraction

The extraction of bioactive component details described our recent study (Sachithanandam et al., 2020), obtained as fine powder (10 g) mix with high polarity organic solvent like methanol (300 ml), was done in

a conical flask and kept overnight with increasing polarity at ambient temperature (27 °C). It was incubated at room temperature for 48 hours at 150 rpm in an orbital shaker (Thermo Fisher Scientific and Floor table top). The extracts were filtered with Whatman No. 1 filter paper. Each supernatant of the extract was concentrated/dried *in vacuo* by using rotary evaporator (CYBER, Germany) under reduced pressure at 40 °C temperature to afford 2 g of crude components.

Purification of natural product molecule (4)

The methanolic crude extract was dissolved in about 0.5 ml of dichloromethane (DCM) and analytical TLC (50: 50, Chloroform: Methanol) was performed on aluminium sheets precoated with silica gel G/UV-254 of 0.2 mm thickness (Merck, Germany). Plates were observed under short wavelength UV light and they showed one active intensive spot with a few low intensive multiple spots. The value of Retention factor (R_f) of active fraction was found to be 0.57 using mobile phase chloroform: methanol (50:50, v/v). The Crude extracts (about 1g) was dissolved in 50 ml DCM solvent and mix with silica gel (100-200 mesh) which was evaporated through rotary evaporator (CYBER, Germany) under without pressure to get crude slurry. After obtained slurry, it was then subjected to column chromatography and packed with 100% chloroform solvent on a silica gel (100-200 mesh). Then crude extracts were eluted with increasing order of polarities, ranges from (10-100%) mixtures of chloroform: methanol to provide multiple fractions according to TLC. The active fraction ($R_f = 0.57$) was collected at 60 % solvent mixture (60% methanol: 40% chloroform) and these fractions were collected through test tube and concentrated / dried *in vacuo* by using rotary evaporator (CYBER, Germany) under reduced pressure to get orange pure product (4). The isolated compound collected from column chromatography was further purified by slow evaporation method using mixture of 4.5 ml of dichloromethane solvent (90% DCM) and 0.5 ml of hexane solvent (10% Hexane) and kept in vial at room temperature for one day settlement of crude product. For further ultra-purification, this obtained compound was subjected to recrystallization process by using ethanol solvent and heated to 40-50 °C and cooled it to room temperature and stored in refrigerator at 4 °C for two days. Further, it gives pure crystalline form of potassium-2-methoxy mucic acid (4) (orange colour) with a yield of 10% (100 mg). The remaining low intensity fractions were unable to be characterized due to low quantity, impurity, and decomposition factors. Further, the structure of the isolated pure 2-methoxy mucic acid (4) was characterized by spectral analysis such as FT-IR, ^1H NMR, ^{13}C NMR spectral data and HRMS analysis.

Antioxidant assay

Antioxidant activity of 2-methoxy mucic acid (4) was determined by free radical scavenging activity as defined by Olennikov et al., (2015); Zhang et al., 2017; Luo et al., (2011). Potassium-2-methoxy mucic acid (4), isolated from *R. apiculata*, with various concentrations (1, 10, 25, 50, 100, 150 and 200 µg/ml, respectively), were taken in test tubes. 3.9 ml of 2,2-diphenyl-1-picrylhydrazyl (DPPH) solution (0.1 Mm) was dissolved in methanol, added to each test tube, and then shaken vigorously (Chang et al., 2001; Kumar et al., 2014). All the test tubes with DPPH solution were shaken gently and then allowed to standpoint at 27 °C in dark for 45 mins. The blank (distilled water) and ascorbic acid as a positive control

were equipped in a similar way without pure compound (sample extract). After 30 min, the absorbance of the prepared samples was measured using UV spectroscopy at a wavelength of 517 nm. This experiment was replicated in three times. The percentage of DPPH scavenging effect was calculated by the following equation given below.

% Inhibition: Absorbance of control (A0) – Absorbance of test sample (A1) / Absorbance of control X 100

Whereas A0 is the absorbance of negative control (0.004% DPPH solution) and A is the absorbance in presence of extract. The results were reported as IC₅₀ values and ascorbic acid equivalents (AAE, mg/g) of mangrove extracts.

Cytotoxicity study by MTT assay

Cytotoxic effect of 2-methoxy mucic acid (**4**) was determined by employing the 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT) assay. Briefly, Cervical (HeLa) and Breast (MDA-MB231) cancer cells were seeded at a density of 0.2×10^5 cells in 96 well plate with a volume of 100 μ l cell per well and were incubated for 24 h (cancer cells were collective from NCCS, Pune). The cells were treated in triplicates with different concentrations of 2-methoxy mucic acid (**4**) (0.5, 5, 25, 50 and 100 μ g/ml) dissolved in dimethyl sulfoxide (DMSO) and incubated at 37°C for 24 h. DMEM (Dulbecco's Modified Eagle's *medium*) medium with cancer cells act as a control. At the end of the incubation period, 10 μ l of MTT (5 mg ml⁻¹) was added to each well and the plates were further incubated at 37°C for 4 h. The formazan crystals that formed due to the cleavage of tetrazolium salt was dissolved by the addition of 100 μ l of dimethyl sulfoxide (DMSO) per well. The soluble formazan produced was quantified by spectrophotometer using an Enzyme Linked Immunosorbent Assay (ELISA) plate reader at 590 nm (Perkin Elmer multimode plate reader). The effect of each treatment was calculated as percentage inhibition against their respective controls (Lalitha et al., 2016).

Molecular docking and molecular dynamics simulation studies

Molecular docking is a computational procedure to understand the binding orientation, mode of binding, key intermolecular interactions, key interacting residues and estimated free energy of binding, estimated inhibition constant of a ligand molecule towards chosen drug target protein. To our knowledge, there is no existing reports on 2-methoxy mucic acid (**4**) with reference to interaction studies towards drug target proteins by either *in silico* or *in vitro* studies. We have identified this vital research gap and to fill this, very first time, we have selected anti-apoptotic proteins as the suitable drug targets for this natural product. Initially, the 3D structure of Bcl-2, Bcl-w, Bcl-xL, and Bcl-B anti-apoptotic proteins were retrieved from Research Collaboratory Structural Bioinformatics - Protein Data Bank (RCSB – PDB) (www.rcsb.org) with the accession number of 1GJH, 2Y6W, 2YXJ and 4B4S, respectively. Then, the protein structures were subjected to protein preparation using Auto Dock Tools (<http://mgltools.scripps.edu/>) (Morris G.M et al. 2009) which includes addition of polar hydrogens, merging non-polar hydrogens, assigning each atom with kollman partial charges, and recorded into PDBQT (XYZ coordinates + partial charges + atom types) format. Similarly, the three-dimensional structure of 2-methoxy mucic acid (**4**) was also prepared with the

addition of polar hydrogens, merging of non-polar hydrogens, addition of gasteiger charges and recorded into PDBQT format by the same Auto Dock Tools. Once protein and ligand preparation steps were completed, the receptor grid map was generated for four key anti-apoptotic proteins based on their binding sites or binding clefts or binding groove (Bcl-2 protein: center_x = 9.71 Å, center_y = 18.45 Å, center_z = 8.48 Å, size_x = 28.71 Å, size_y = 18.95 Å, size_z = 22.33 Å, Bcl-w protein: center_x = -24.93 Å, center_y = 5.95 Å, center_z = -1.35 Å, size_x = 27.043 Å, size_y = 25.0 Å, size_z = 29.75 Å, Bcl-xL protein: center_x = -16.17 Å, center_y = -32.25 Å, center_z = -33.95 Å, size_x = 31.01 Å, size_y = 33.61 Å, size_z = 20.21 Å and Bcl-B protein: center_x = -10.08 Å, center_y = 27.17 Å, center_z = 9.09 Å, size_x = 19.64 Å, size_y = 35.92 Å, size_z = 19.82 Å). In this study, we have utilized site-specific or direct docking calculation for four anti-apoptotic proteins with the ligand molecule using Auto Dock Vina program (Trott and Olson, 2010). The best docking complex was selected based on estimated binding free energy (ΔG), estimated inhibition constant (K_i), and the higher number of docking orientations present at the binding site of anti-apoptotic proteins. Finally, the docking analysis were performed by using several programs namely PyMOL (The PyMOL Molecular Graphics System, Version 1.2r3pre, Schrödinger, LLC.), MGLTools (<http://mgltools.scripps.edu/>) and LigPlot (Wallace et al, 1995) programs, respectively.

Based on the results obtained from molecular docking calculation, we found that 2-methoxy mucic acid (**4**) showed more binding affinity towards Bcl-B protein. Therefore, apart from molecular docking calculation, to understand the structural stability of Bcl-B upon 2-methoxy mucic acid binding, in this study, additionally we have performed molecular dynamics (MD) simulation of free, and 2-methoxy mucic acid (**4**) bound Bcl-B structures with the period of 10 ns using Gromacs Version 2019 (Van Der Spoel, Lindahl et al. 2005). The MD simulation is a computational technique to mimic and understand the physiological condition of protein or other macromolecular complexes in the presence of an aqueous environment. The MD simulation starts with Gromacs formatted or GRO, topology (TOP) and positional restraint (POSRE) files from the input PDB file of Bcl-B protein. After that, the cubic box was generated around Bcl-B and Bcl-B: 2-methoxy mucic acid (**4**) complex. Then, the extended Single Point Charge (SPCE) water molecules were added to the cubic box. If the system did not attain the neutral charge, then the counterions (positive or negative charges) were added to neutralize the system. Once the system attained the neutral charges, energy minimization followed (using steepest descent approach) by equilibration steps were performed at two different phases (Constant temperature, constant volume - NVT and Constant temperature, constant pressure - NPT) with the time period of 100 ps and (vii) then finally production MD simulation was performed with the time period of 10 ns for both unbound Bcl-B and Bcl-B: 2-methoxy mucic acid (**4**) complex. For MD simulation of Bcl-B: 2-methoxy mucic acid (**4**) complex, firstly, the topology and GRO files of 2-methoxy mucic acid (**4**) was generated using PRODRG webserver (external web server) (Schüttelkopf and van Aalten 2004), after that, MD simulation steps were carried out on Bcl-B: 2-methoxy mucic acid (**4**) complex with the changes in the molecular dynamics parameters (MDP) files of energy minimization, NVT, NPT equilibration and production MD steps. Finally, the comparative MD simulation analyses were performed for both (a) unbound and (b) 2-methoxy mucic acid (**4**) acid bound Bcl-B structures. The following analysis were performed for both unbound and ligand bound Bcl-B structures with the aid of various Gromacs built-in functions (commands are mentioned in

brackets) namely Root Mean Square Deviation (gmx rms), Root Mean Square Fluctuation (gmx rmsf), Radius of Gyration (gmx gyrate), Solvent Accessible Surface Area (gmx SAS), Hbonds (gmx hbond), essential dynamics or essential motion (gmx covar and gmx anaeig) based on Principal Component Analysis. Apart from global and essential dynamics, the Molecular Mechanics with Poisson-Boltzmann and Surface Area Solvation (MM/PBSA) binding free energy analysis of Bcl-B:mucic acid complex was performed by using *g_mmpbsa* tool [53]. It is one of the most reliable end-point approaches for free energy calculations. The 10 ns of stable simulated trajectories were extracted, and snapshots were captured at a regular interval of 100 ps. In short, 100 structures from the 0-10 ns period were utilized for the MM/PBSA binding free energy calculations.

Results And Discussion

Characterisation of natural product 2-methoxy mucic acid (4)

The 2-methoxy mucic acid (**4**) was isolated by TLC which was found to possess a molecular formula of $C_7H_7K_5O_8$, and was characterized by FT-IR, 1H NMR, ^{13}C NMR spectral data and HRMS analysis, as described in a previous work (Leonardi et al., 2019; Purushothaman et al., 2018; Zhao et al., 2019). The IR absorption spectrum of 2-methoxy mucic acid (**4**) showing absorption peaks at 3272 cm^{-1} indicates the presence of potassium salt of OH stretching. The peak at 2931 cm^{-1} because of C-H stretching and the value at 1047 cm^{-1} due to carboxylate symmetric stretching were confirmed. Importantly, peak at 1607 cm^{-1} was due to asymmetric stretching of salt of carboxylate ion, ($C=O$, COO^-K^+) (**Supplementary Information, Fig. 1**). These IR values were matched with those of earlier literature (Tian et al., 2000). Results obtained from the NMR data analyses is given in Table 1. The 1H NMR (400 MHz) spectrum of 2-methoxy mucic acid (**4**) displayed a characteristic methoxy proton signal at 3.49 ppm at a singlet (**Supplementary Information, Fig. 2**). The four aliphatic methine C-H protons such as C2-H, C3-H, C4-H and C5-H were appeared at 3.36-3.27 ppm as multiplet, 4.03 ppm as singlet, 3.77 ppm as doublet and 3.99 – 3.73 ppm as doublet, respectively (**Supplementary Information, Fig. 2**). The OH and COOH protons did not appear due to interaction between four OH and two COOH protons with potassium ions (Tian et al., 2000). The ^{13}C NMR (100 MHz) spectrum of 2-methoxy mucic acid (**4**) displayed the predicted seven signals (**Supplementary Information, Fig. 3**). The methoxy carbon appeared at 57.25 ppm. The COOH carbonyl peaks were observed at δ 179.52 ppm (C-1) and 178.16 (C-6) ppm, respectively. The aliphatic CH carbons were observed at 82.52 (C-2), 69.09 (C-3), 70.60 (C-4) and 73.52 (C-5) ppm respectively. These types of carbons were further confirmed by ^{13}C Distortionless enhancement by polarization transfer (DEPT-135), an NMR method (**Supplementary Information, Fig. 4**). The mass of the 2-methoxy mucic acid (**4**) was determined as $C_7H_7K_5O_8$ with 413.2626 atomic mass units (amu) and found 413.2627 amu by Electrospray Ionisation method (ESI-HRMS) (**Supplementary Information, Fig. 5**). The purified compound were analysed by Flame photometer spectroscopy for the total mineral content to conform the potassium salts present in the molecule (Systronics, Type 128) according to (APHA). All instrumental parameters were corrected to the best flame condition. Then calibration of the Flame photometer was done using the suitable standard solution (Perkin Elmer) for potassium. After the calibration, samples

were run in triplicate to estimate the potassium concentration. Good recovery of 99% were obtained for the potassium standard solution, indicates the accuracy of the analytical methodology.

Characterization data of 2-methoxy mucic acid (4)

IR Data (v) 3272 (OH), 2931 (Ar C-H), 1607 (C=O), 1521, 1441, 1361, 1282, 1203, 1047, 866, 788 cm⁻¹: ¹H NMR (400 MHz, CD₃OD) δ 4.03 (s, 1H, C-3), 3.99 – 3.73 (d, *J* = 5.6 Hz, 1H, C-5), 3.77 (d, *J* = 4.5 Hz, 1H, C-4), 3.49 (s, 3H, OMe), 3.36 – 3.27 (m, 1H, C-2). ¹³C NMR (100 MHz, CD₃OD) δ 178.43 (C-6), 179.12 (C-1), 82.51 (C-2), 73.52 (C-5), 70.59 (C-4), 69.09 (C-3), 57.25 (OMe) ppm. HR ESI MS Calcd for C₇H₇K₅O₈ were 413.8300 amu and found 413.2627amu.

Table 1: Characterization of ¹H and ¹³C NMR chemical shift (δ) values for isolated 2-methoxy mucic acid (4).

Structure of compounds	Position	Characteristic Hydrogens δ in <i>J</i> (Hz)	Carbon numbers (δ ppm)	ESI-HRMS
	1	-	179.52	413.2627amu.
	2	3.36-3.27 (m, 1H)	82.52	
	3	4.03 (s, 1H)	69.09	
	4	3.77 (d, <i>J</i> = 4.5 Hz, 1H)	70.60	
	5	3.99 – 3.73 (d, <i>J</i> = 5.6 Hz, 1H)	73.52	
	6	-	178.16	
	OMe	3.49 (s, 3H, OMe)	57.25	

¹H and ¹³C NMR recorded in CD₃OD solvent; ppm-parts per million

Determination of antioxidant activity by DPPH assay

The antioxidant activity of 2-methoxy mucic acid (4) isolated from methanolic leaf extract of *R. apiculata*, was analysed with different doses, namely 1, 10, 25, 50, 100, 150 and 200 µg/ml, respectively. The results confirmed a dose dependent inhibitory activity of 2-methoxy mucic acid (4) and a free radical scavenging activity was found. The IC₅₀ values for DPPH antioxidant activity of 2-methoxy mucic (4) was 21.361±0.41 µg/ml; however, the Ascorbic acid used as positive control exhibited better radical

scavenging effect with IC₅₀ values of 5.5486±0.81 µg/ml (**Fig. 3**). In the literature review on antioxidant activity of mucic acid derivatives, it was found that the fruit of *Phyllanthus emblica* L. yielded mucic acid derivatives with good antioxidant activity and the extracts from this fruit produced many phytochemical compounds having IC₅₀ values: gallic acid 18.71 ± 0.77, ellagic acid 13.81 ± 0.59, mucic acid 1,4-lactone 3-O-gallate 23.72 ± 1.05, isocorilagin 8.12 ± 0.59, chebulanin 11.27 ± 0.67, chebulagic acid 4.14 ± 0.19, and Mallotusin 3.99 ± 0.11 µM, respectively (Luo et al., 2011). Another work also reported that mucic acid gallates isolated from *P. emblica* showed strong antioxidant activity with IC₅₀ values of 12.84 µM, by using DPPH method (Olennikov et al., 2015).

Cytotoxicity activity by MTT assay

In vitro inhibitory activity of 2-methoxy mucic acid (**4**), isolated from mangrove plant *R. apiculata*, was determined by MTT reduction assay on cervical (HeLa) and breast (MDA-MB231) cancer cell lines. The results confirmed a dose-dependent inhibitory activity of 2-methoxy mucic acid (**4**) with cytotoxicity activity against HeLa and MDA-MB231 cancer cell lines, with the IC₅₀ concentration of 22.88283±0.72 and 2.91925±0.52µg/ml, respectively (**Fig. 4**). Previous reports of anticancer activity of mucic acid derivatives, mucic acid gallate, isolated from *P. emblica* also revealed good anticancer activity against B16F10 (human gastric adenocarcinoma), HeLa (human uterine carcinoma) and B16F10 (murine melanoma) with IC₅₀ values of 15µg/ml for B16F10, 24µg/ml for HeLa and 41µg/ml for MK-1 (Zhang et al., 2016). Luo et al., (2011), reported that the bio-active compounds in fruit extract of *P. emblica* showed good anticancer activity against MCF-7 cancer cell lines. Fruit extract of *P. emblica* produced many compounds and IC₅₀ values of such compounds varied: Gallic acid -64.88 µg/ml and Mallotusin -50.92µg/ml. Other compounds, such as ellagic acid, mucic acid 1,4-lactone 3-O-gallate, isocorilagin, and chebulagic acid showed less than 29% inhibition activity (Luo et al., 2011). These results suggested that mucic acid and its derivatives displayed significant anticancer activities.

Molecular Docking and molecular dynamics simulation studies:

Molecular docking or *in silico* interaction studies was carried out between 2-methoxy mucic acid (**4**) and four members of anti-apoptotic proteins (Bcl-2, Bcl-w, Bcl-xL and Bcl-B) to understand the mode of binding, key interacting residues, type of intermolecular interactions, binding orientation of ligand towards binding site of the drug target proteins. The estimated free energy of binding (ΔG) and estimated inhibition constant (*K_i*) of 2-methoxy mucic acid (**4**) towards four drug target proteins was presented in **Table 2**.

Table 2: Estimated binding free energy and estimated inhibition constant of four anti-apoptotic Bcl-2 members towards 2-methoxy mucic acid (**4**)

S. No	Complex Structures	Estimated Free Energy of Binding (kcal/mol)	Estimated Inhibition Constant (μM)
1.	Bcl-2: Mucic acid	-4.5	502.8
2.	Bcl-w: Mucic acid	-4.4	593.5
3.	Bcl-xL: Mucic acid	-4.8	303
4.	Bcl-B: Mucic acid	-5.8	56

Of the four docking complexes, the 2-methoxy mucic acid (**4**) molecule showed higher binding affinity towards anti-apoptotic Bcl-B protein with the estimated binding free energy of -5.8 kcal/mol. The electrostatic potential surface calculation revealed that this ligand molecule mostly oriented in negatively charged portion of the drug target proteins (**Fig. 5**). Interaction analysis also explained that higher number intermolecular hydrogen bonding and hydrophobic interactions are observed between Bcl-B and 2-methoxy mucic acid (**4**). The intermolecular interaction results of four protein-ligand complexes are given in **Figs. 6 and 7**. Interaction pattern analysis of four complexes revealed that this natural molecule strongly binds to the BH3 binding groove or binding cleft of anti-apoptotic proteins. This region is very important for anti-apoptotic function as well as this site is responsible for interaction of their pro apoptotic interacting partners. The estimated binding free energy values (large negative) were corroborated with the results obtained from interaction pattern analysis (higher number of intermolecular interactions). Based on the results obtained from molecular docking calculation of 2-methoxy mucic acid (**4**) with four anti-apoptotic members, we have chosen Bcl-B as a suitable drug target, since the ligand molecule showed stronger binding affinity and higher number intermolecular interactions and therefore this Bcl-B: 2-methoxy mucic acid (**4**) complex subsequently used for further molecular dynamics simulation in order to understand the long term structural stability, structural integrity, structural compactness and folding properties. Moreover, we also tried to understand the behavior of 2-methoxy mucic acid (**4**) towards the structural stability of Bcl-B protein.

The main drawback of molecular docking studies is to consider ligand as completely flexible whereas protein or receptor as rigid entity (to some extent, few residues are treated as flexible not as a whole protein). To validate the results obtained from molecular docking calculation and understand whether the 2-methoxy mucic acid (**4**) molecule enhances the structural stability, structural integrity, structural compactness, and folding properties of Bcl-B, apart from molecular docking, we subsequently performed MD simulation in two different states such as unbound Bcl-B and 2-methoxy mucic acid (**4**) bound Bcl-B structures. Moreover, this study was also used to validate the results obtained from molecular docking calculation which is a preliminary study to understand the intermolecular interaction between protein and ligand molecule. The number of water molecules and counter ions are used in the molecular dynamics simulation of protein and protein-ligand complexes are given in **Table 3**. The molecular dynamics simulation results (**Figs. 7-8 and Table 3**) explained that upon binding of 2-methoxy mucic acid (**4**) to the binding site of Bcl-B protein which will significantly enhance the structural stability of Bcl-B with the

results obtained from essential dynamics analysis which covered the lesser region form (34.99 nm²) in conformational space in comparison with unbound form (56.82 nm²). Essential dynamics is very important analysis since it captures the essential motion from the global motions. The main application of Principal Component Analysis (PCA) is reduction of dimension, i.e., it can transform the high dimensional data (global dynamics or global motion) of protein dynamics or protein-ligand dynamics into low dimensional space (essential dynamics or essential motions) to derive a range of eigenvectors and eigenvalues. Generally, the first two principal components (PC1 and PC2) are highly responsible most of the dominant motions, therefore, we have considered first two principal components in the present study.

Table 3: Number of water molecules and counter ions used for the MD simulation

Protein and Complex	Number of water molecules	Number of Couter ions
Bcl-B	9678	1 CL
Bcl-B: 2-methoxy mucic acid	9660	1 NA

Regarding global dynamics, the root mean square deviation (RMSD) analysis explained that this 2-methoxy mucic acid molecule does not alter or deviate the structure of anti-apoptotic Bcl-B protein. Instead after binding of this ligand molecule to the binding site Bcl-B protein which significantly enhanced its structural stability. This will additionally be supported by other two important analysis namely Root Mean Square Fluctuation (RMSF) and Radius of Gyration (Rg) analyses. In comparison with unbound Bcl-B structure, the rmsd pattern of Bcl-B: 2-methoxy mucic is consistent. From the results of RMSF analysis, the flexibility of Bcl-B: 2-methoxy mucic complex is lesser than unbound Bcl-B structure. Moreover, Rg analysis also supported that this molecule significantly enhances the compactness of Bcl-B protein. Together the results obtained from both RMSF and Rg analyses suggested that this 2-methoxy mucic acid molecule decreased the flexibility of the Bcl-B protein and it enhances the compactness and integrity of Bcl-B structure. Very interestingly we have observed the maximum number of four intermolecular hydrogen bonds between Bcl-B and 2-methoxy mucic acid. This analysis again supported that this molecule binds strongly to the binding groove of Bcl-B protein. Finally, the Solvent Accessible Surface Area (SASA) pattern of Bcl-B was slightly changed due to influence of 2-methoxy mucic acid binding. Together the results obtained from global (RMSD, RMSF, Rg, SASA and Hbonds) and essential motion analysis (Principal Component Analysis) explained that this 2-methoxy mucic acid molecule could be the promising candidate for inhibiting the function of anti-apoptotic Bcl-B protein in cancerous cell. Time averaged structural properties obtained from molecular dynamics simulation of unbound and 2-methoxy mucic acid bound Bcl-B structure was given in **Table 4**. The average values obtained from various molecular dynamics simulation analysis of unbound and 2-methoxy mucic acid bound Bcl-B structure explained that this ligand molecule interestingly improve the stability of chosen drug target protein. Finally, the binding free energy calculations (**Fig. 10**) were carried out to quantify the binding

affinities of mucic acid towards anti-apoptotic Bcl-B protein. We have employed MM/PBSA approach for calculating binding free energy between Bcl-B and 2-methoxy mucic acid. The binding free energy of Bcl-B with Mucic acid complex -42.754+/-21.72 kJ/mol. This binding free value indicated that the ligand molecule binds strongly to the binding groove or binding cleft of anti-apoptotic Bcl-B protein. Although *in silico* results are very promising, further molecular level experimental validation studies against Bcl-B protein are required to validate the computational driven hypothesis.

Table 4: Time averaged structural properties obtained from anti-apoptotic Bcl-B with Mucic Acid

Protein and Complex	RMSD (nm)	RMSF (nm)	Rg (nm)	SASA (nm ²)	HBonds (nm)	Trace of Covariance matrix (nm ²)
Bcl-B	0.25	0.16	1.43	76.91	NA	56.82
Bcl-B:Mucic acid	0.26	0.12	1.44	75.38	4	34-99

Conclusion

In summary, a carbohydrate based natural biomolecule, namely 2-methoxy mucic acid (**4**) was isolated for the first time from the leaves of a mangrove species, *R. apiculata*, using methanolic extract with high purity. Remarkably, 2-methoxy mucic acid (**4**) has indicated a highly significant dual anticancer and antioxidant activity. Together the results of molecular docking and molecular dynamics simulation results suggested that 2-methoxy mucic acid (**4**) binds strongly to the anti-apoptotic Bcl-B protein. The *in vitro* and *in silico* results taken together indicate that the natural compound (**4**) is one of the most potent anti-cancer molecules. From the results of both *in vitro* anticancer and antioxidant studies, it has been concluded that these molecules possess the good apoptotic inducing activity, which may be used as promising leads for the development of more potent anti-cancer drugs. Further works on identification and biomedical application of more bioactive phyto-chemical products from other parts of *R. apiculata* as possible herbal source of medicines will be helpful in treatment of several critical diseases including cancer.

Declarations

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Ethics approval / statement

No need for ethics approval, since this work does not include research on identifiable human material or data.

Consent to Publish

I/We, the undersigned, give our consent for the publication of identifiable details, which can include photograph(s) and/or videos and/or case history and/or details within the text ("Material") to be published in the above Journal and Article.

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Authorship contribution statement:

V. Sachithanandam: Conceptualization, Formal analysis, Methodology, Project administration, Writing - original draft, Writing - review & editing.

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Jayaraman Muthukumaran: Molecular docking Study

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Ranjitha Misra: Cancer cell line analysis Data curation.

Sridhar R: Supervision

Purvaja R. Project administration

Ramesh, R. Funding acquisition

Availability of data and materials

The present study data's are available under the on request to the corresponding author.

Abbreviations

IC₅₀ : Half maximal inhibitory concentration

MTT: 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

MD: Molecular Dynamics

PCA: Principal Component Analysis

RMSD: Root Mean Square Deviation

RMSF: Root Mean Square Fluctuation

Rg: Radius of Gyration

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Figures

Figure 1

Reported structures of mucic acid analogues (1-3) and 2-methoxy mucic acid (4, Present work).

Figure 2

Leaves, Flower, and Bark of *R. apiculata* mangrove plant collected from ANI

Figure 3

Antioxidant activity for 2-methoxy mucic acid (4) isolated from methanolic leaf extract of *R. apiculata* in comparison with Ascorbic acid.

Figure 4

Cytotoxicity effect of 2-methoxy mucic acid (4) on HeLa and MDA-MB231 cancer cell lines. (Dose-response analysis of 2-methoxy mucic acid was done pertaining to inhibition of HeLa and MDA-MB231 cancer cell lines. 0.2×10^5 cells/ well were seeded in 96-well tissue culture plate, followed by treatment with indicated concentration (0.5, 5, 25, 50 and 100 µg/ml) of 2-methoxy mucic acid (4) for 24h. Cytotoxicity effect was determined by MTT assay after the specified incubation period).

Figure 5

Electrostatic surface potential map analysis of four docking complexes of (a) Bcl-2: 2-methoxy mucic acid (4), (b) Bcl-w: 2-methoxy mucic acid (4), (c) Bcl-xL: 2-methoxy mucic acid (4) and (d) Bcl-B: 2-methoxy mucic acid (4)

Figure 6

Schematic representation of intermolecular interaction between (a) Bcl-2: 2-methoxy mucic acid and (b) Bcl-w: 2-methoxy mucic acid

Figure 7

Schematic representation of intermolecular interaction between (a) Bcl-xL: 2-methoxy mucic acid and (b) Bcl-B: 2-methoxy mucic acid

Figure 8

Molecular dynamics simulation of Bcl-B and Bcl-B: 2-methoxy mucic acid complex, (a) Root Mean Square Deviation, (b) Root Mean Square Fluctuation, (c) Radius of Gyration and (d) Solvent Accessible Surface Area.

Figure 9

Intermolecular hydrogen bonds of Bcl-B: 2-methoxy mucic acid and principal component analysis of Bcl-B and Bcl-B: 2-methoxy mucic acid complex

Binding Energy

Figure 10

MM-PBSA binding free energy of Bcl-B: 2-methoxy mucic acid complex

Supplementary Files

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