

# Identification of 2,4-Di-tert-butylphenol (2,4-DTBP) as the Major Contributor of Anti-colon cancer Activity of Active Chromatographic Fraction of *Parkia javanica* (Lamk.) Merr. Bark Extract

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<https://dx.doi.org/10.13005/bpj/2609>

(Received: 21 April 2022; accepted: 19 October 2022)

*Parkia javanica* (Lamk.) Merr. is an ethnomedicinal leguminous plant species from northeastern India with a long history of medicinal use among various tribes of this region to treat cholera, dysentery, stomach aches, diarrhea and food poisoning, having antibacterial, wound-healing, anticancer and anti-inflammatory properties. Therefore, in this current study, the methanolic bark extract was carried out and fractionated by using flash chromatography, examined the cytotoxicity of the respective fractions on colon cancer cell lines, and evaluated the major phytochemical compounds present in the fractions using Gas Chromatography-Mass Spectrometry (GC-MS) chemical profiling. Chemical profiling of the fractions by GC-MS revealed in fraction-1 and -2, 2,4-Di-tert-butylphenol was the major compound (50.740% in fraction-1, 21.277% in fraction-2, and 7.859% in fraction-3) having reported anticancer activity. The gradation of the presence of this compound in the fractions was corroborated by the gradation of anti-colon cancer activity of the respective fractions on both the colon carcinoma cell lines, HCT116 and SW480. However, the presence of D-Allose in a substantial amount (20.870%) in only fraction-3 could not increase the anticancer activity of fraction-3 over the other two fractions. An in vitro cytotoxic assay guided evaluation of three flash chromatographic fractions (fraction-1, -2, and -3) of methanolic extract of *Parkia javanica* bark showed significant anticancer properties on two human colon carcinoma cell lines (HCT116 and SW480). The order of efficacy of the fractions was fraction-1 > fraction-2 > fraction-3. In a time and dose-dependent experiment, fraction-1, being the most active one, showed an IC<sub>50</sub> value of 16.25 µgml<sup>-1</sup> (24 hrs), 9.94 µgml<sup>-1</sup> (48 hrs), and 9.38 µgml<sup>-1</sup> (72 hrs) on HCT116 and 35 µgml<sup>-1</sup> (24 hrs), 20.14 µgml<sup>-1</sup> (48 hrs), and 19.71 µgml<sup>-1</sup> (72 hrs) on the SW480 colon cancer cell line. *Parkia javanica* bark extract is bestowed with the potential of anti-colon cancer property and upon chemical profiling of different chromatographic fractions of the extract, 2,4-Di-tert-butylphenol has been identified as the primary anticancer component of the extract.

**Keywords:** *Parkia javanica*, Bark extract, Anti-Colon cancer, Flash chromatography, GC-MS, 2,4-Di-tert-butylphenol.

According to GLOBOCAN 2020 data, colon cancer ranks third among all newly diagnosed cancers and second among all cancer deaths globally, irrespective of gender or age<sup>1</sup>. It's the second-most prevalent female cancer across the globe, and the third most common cancer reported in males<sup>2,3</sup>.

Numerous studies have revealed associations between colorectal cancer and eating choices, induced inflammation, and other factors, including diabetes mellitus, obesity, alcohol consumption, cigarette smoking, and genetic mutations<sup>4-10</sup>. 5-fluorouracil (5-FU) in conjunction with irinotecan, oxaliplatin, or levamisole is usually the conventional treatment<sup>11</sup>. Antibodies have also been created against the receptors of epidermal and vascular EGF (endothelial growth factor), which have improved the prognosis of colorectal cancer patients<sup>12</sup>.

Despite the fast advancements in the field of diagnosis and treatment of colon cancer in recent years, it remains a significant clinical concern globally due to its high prevalence<sup>13</sup>. Due to the toxicity and side effects of cytotoxic medications, as well as innate therapeutic resistance mechanisms in CRC tumor cells<sup>14</sup>, modern CRC therapy is still unsatisfactory. So, effective pure compounds must be introduced into the therapy of colon cancer that can help to reduce side effects and drug-related toxicity in humans.

*Parkia javanica* is one of the ethnomedicinal leguminous plants that have been traditionally used to cure a variety of ailments by several tribes in northeastern India<sup>15-17</sup>, among the other medicinal plants of this region<sup>18,19</sup>. Previously, we discovered that the crude bark extract of this plant had anticancer potential on colon cancer cell lines<sup>20</sup>. The current study aimed to explore the key phytochemical compound(s) contributing to the anti-colon cancer property of the extract of *Parkia javanica* bark using different chromatographic subfractions of the extract.

## MATERIAL AND METHODS

### Identification of Plant

*Parkia javanica* barks were obtained from Ambassa, Dhalai District of Tripura, India. The plant was identified by the renowned taxonomist of the Department of Botany, Tripura University,

Prof. Badal Kumar Dutta. The corresponding specimen, identified as BD-01/06, was also sent to the Herbarium.

### Preparation of the Plant Extract

Around 200 gm of shaded dried *P. javanica* bark powder was mixed in 600 ml of solvent (methanol) for 48 hrs (with periodic shaking) at normal room temperature. After 48 hrs, the filtration process of the solvent was repeated until the solvent appeared clear. The solvent was concentrated at 60°C using a rotary evaporator before being lyophilized (at 4°C) to remove all traces of the solvent from the extract.

### Conditions of Flash Chromatography

To fractionate the whole extract, a flash chromatographic technique (Teledyne ISCO Combi Flash Rf 150 model) was used. Column grade silica gel served as the stationary phase (mesh 60-120). Chloroform and methanol were the mobile phases. During the isolation procedure, the non-polar solvent was eluted initially, and then the polarity was gradually increased. The samples (12 ml) were automatically collected in test tubes using gradient elution. TLC was done again for each fraction using the same solvent system as in flash chromatography, and the fractions containing the equal Rf value pooled and evaporated to dryness, after which they crystallized to provide fine powdered crystals.

### Gas Chromatography-Mass Spectrometry (GC-MS) Analysis

To assess each fraction's chemical profile, a liquid auto-sampler GC-MS (Clarus 680 GC & Clarus 600C MS, Perkin Elmer, made in USA) was used. TurboMass 5.4.2 was the software utilized in the system. The peaks were examined using the NIST-2008 data analysis software. An 'Elite-5MS' column was utilized in this instrument. 5% diphenyl and 95% dimethyl polysiloxane were used as the stationary phase. As the mobile phase, 99.99% helium gas (flow rate: 1 ml/minute) was employed. In splitless mode, the total injection volume was 2 µl. The injector (with injection volume of 2 µl) temperature was set at 280°C and the ion source at 180°C. The oven's temperature: 60°C for 1 minute and after that increased the temperature at a speed of 7°C per minute up to 200°C (3 minutes holding time), then increased the temperature at a speed of 10°C per minute to 300°C (5 minutes holding time).

### Reagents and Cell Lines Used

Cancer as well as normal cell lines (HCT116, SW480: human colon cancer cells; GM00637: normal human fibroblast cells) were used in this study. DMEM (HiMedia) was used as culture media for HCT116 and fibroblast cell lines. RPMI 1640 medium (HiMedia) was used for the SW480 cell line. 10% FBS, 2% penicillin-streptomycin (HiMedia) were supplemented with culture media.

### MTT Assay

HCT116, SW480 and fibroblast cells (GM00637) were dispersed at 104 cells per well in 96 well microplates and kept in a CO<sub>2</sub> incubator. After attachment, the cells were treated with or without (vehicle-control) the flash chromatography-derived fractions and the positive control 5-fluorouracil (in depleted media) with a range of concentrations (the range was 200 µgml<sup>-1</sup> to 1.56 µgml<sup>-1</sup>) and placed in a CO<sub>2</sub> incubator for 24, 48, and 72 hrs. After incubation, the treated media was discarded and 100 µl of a working MTT solution (with PBS) of 0.5 mgml<sup>-1</sup> was added to every single well of the 96-well plate, followed by 3 hrs of incubation. After the incubation, each well's MTT medium was replaced by 100 µl of DMSO. Using a microplate reader, the absorbance was obtained by measuring it at 570 nm<sup>21</sup>.

### Cell Migration Assay

Culture plates of 35 mm in diameter were used to seed colon cancer cells. After reaching optimal confluency, each culture plate was manually scratched with a microtip to generate a scratch of around 0.5 mm in width. The cells were then treated (at their respective IC<sub>50</sub> concentrations) with or without (vehicle-control) the flash chromatography-derived active fraction. Microscopic images were acquired at various time periods (0 hr, 24 hrs, 48 hrs) after treatment. The chromatography-derived active fraction's activity was determined by comparing the proportion of the closed area of the untreated (vehicle-controlled) scratch to that of the treated scratch<sup>22, 23</sup>.

### Bright Field Microscopy Imaging

In 35 mm culture plates, colon cancer cells were seeded and, after attachment, treated with or without (vehicle-control) the respective IC<sub>50</sub> concentrations and incubated for 24, 48, and 72

hrs. Following incubation, the morphology of the treated cells was compared to the corresponding vehicle-controlled cells. An inverted microscope (Dewinter-Victory plus, Dewinter optical Inc., India) was used to capture each image.

### Apoptotic Study by Acridine Orange (AO) Ethidium Bromide (EB) Staining

Apoptosis is linked to modifications in the shape of the nucleus, which were studied using an AO/EB fluorescent imaging method<sup>24, 25</sup>. 35 mm culture plates were used to seed the colon cancer cells. Following attachment, both the colon cancer cells were treated with or without (vehicle control) the flash chromatography-derived active fraction for 48 hrs at their respective IC<sub>50</sub> concentrations. After being trypsinized, the cells were resuspended in cold PBS and stained with AO and EB (the concentration of both stains is 100 µgml<sup>-1</sup>). Each time, 9 µl of cell suspension (vehicle-controlled or treated) mixed with 1 µl of AO-EB (AO:EB =1:1) stain was viewed under the fluorescence microscope (Zeiss, Germany).

### Morphological Evaluation of the Nucleus Using DAPI Staining

The procedure described by Kntayya *et al*<sup>26</sup> was followed to stain the cells with 4,6-diamidino-2-phenylindole (DAPI) with little modification. HCT116 and SW480 cells were grown on coverslips placed in culture plates and treated with or without the IC<sub>50</sub> concentration of fraction-1 (the active fraction). The treated and untreated (vehicle- controlled) cells were then incubated for 48 hrs in the CO<sub>2</sub> incubator. Following incubation, the cells were fixed with paraformaldehyde (4% in PBS), permeabilised with 0.1% Triton X-100 (in PBS) and stained with DAPI (2.5 µgml<sup>-1</sup>, in PBS). Changes in nuclear morphology were observed under the fluorescence microscope (Zeiss, Germany).

### Statistical Analysis

All tests were done three times, and the findings reported in this study as the mean ±SD of three data sets. Paired t-test was used to calculate the p-value, which reflects the significance of differences between distinct sets of experimental data. P<0.05, P<0.01, and P<0.001 are denoted by \*, \*\*, and \*\*\*, respectively.

## RESULTS

### Analysis of Phytochemicals Present in the Chromatographic Fractions of MEPJB

The powdered crude methanolic extract of *Parkia javanica* bark (MEPJB) was fractionated using flash chromatography. This procedure produced three fractions from the crude methanolic extract, such as fraction-1, fraction-2, and fraction-3. The phytochemical composition of the three fractions was evaluated using a gas chromatography-mass spectrometry (GC-MS) analysis. The major peak in the primary GC-MS chromatogram of fraction-1 had a retention time of 22.17 (Fig. 2A) with an area percentage of 50.740 (Table 1). The major peaks found in the primary GC-MS chromatogram of fraction-2 and fraction-3 had a retention time of 22.189 (Area% =21.277) and 26.215 (Area% =20.870) respectively, with some minor peaks (Fig. 2 B, C). The major phytochemical compound identified by mass spectrometry in fraction-1 and 2 was a phenolic compound named 2,4-Di-tert-butylphenol (2,4-DTBP) (Fig. 3) and in fraction-3 was a stereoisomer



**Fig. 1.** *Parkia javanica* (Lamk.) Merr. plant (Ambassa, Tripura, India)

of D-glucose named D-Allose, according to area percentage. However, 2,4-DTBP was also found to be the second highest phytochemical present in the fraction-3. Aside from these two compounds, a further 13 compounds were also identified in small amounts among the three fractions of MEPJB (Table 1).

### Cytotoxicity of Fractions on Colon Cancer cells

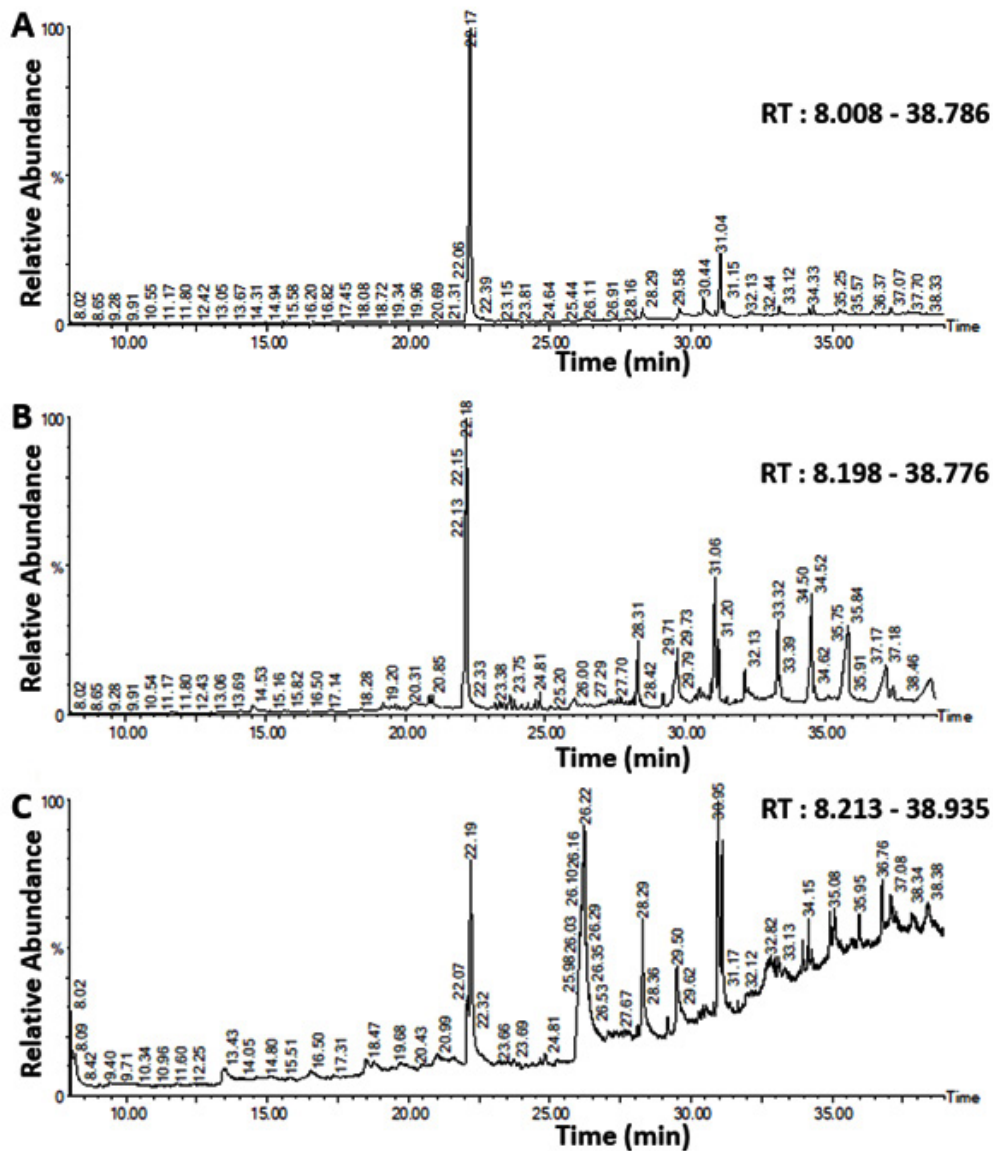
The cytotoxic activities of three flash chromatographic fractions (fraction-1, -2, and -3) on the two cancer cell lines (colon adenocarcinoma: HCT116 and SW480) and the normal human fibroblast cell line (GM00637) were determined by treating all the cell lines with a range of concentrations (the range was 200  $\mu\text{gml}^{-1}$  to 1.56  $\mu\text{gml}^{-1}$ ) of each fraction for 24, 48, and 72 hrs. On the HCT116 cells, fraction-1 exhibited half-maximal inhibitory concentrations (IC<sub>50</sub>) of 16.25  $\mu\text{gml}^{-1}$  (24 hrs), 9.94  $\mu\text{gml}^{-1}$  (48 hrs), and 9.38  $\mu\text{gml}^{-1}$  (72 hrs), whereas fraction-2 had IC<sub>50</sub> values of 43.75  $\mu\text{gml}^{-1}$  (24 hrs), 35.71  $\mu\text{gml}^{-1}$  (48 hrs), and 34.38  $\mu\text{gml}^{-1}$  (72 hrs), but fraction-3 showed a substantially higher IC<sub>50</sub> (>200  $\mu\text{gml}^{-1}$  for 24 and 48 hrs; 166.67  $\mu\text{gml}^{-1}$  for 72 hrs) than the other two fractions (Table 2). Similarly, on the SW480 cell line, fraction-1 showed an IC<sub>50</sub> of 35  $\mu\text{gml}^{-1}$  (24 hrs), 20.14  $\mu\text{gml}^{-1}$  (48 hrs) and 19.71  $\mu\text{gml}^{-1}$  (72 hrs), whereas, fraction-2 exhibited IC<sub>50</sub> values of 67.86  $\mu\text{gml}^{-1}$  (24 hrs), 36.02  $\mu\text{gml}^{-1}$  (48 hrs) and 21.63  $\mu\text{gml}^{-1}$  (72 hrs), but again, fraction-3 showed a much higher IC<sub>50</sub> (>200  $\mu\text{gml}^{-1}$  for 24 and 48 h; 155.26  $\mu\text{gml}^{-1}$  for 72 hrs) than the other two fractions (Table 2). Here, fraction-1 emerged to be the most active fraction as it showed the lowest IC<sub>50</sub> values for both colon cancer cell lines after 24, 48, and 72 hrs of incubation (Fig. 4 A-F). However, the abovementioned IC<sub>50</sub> concentrations (24, 48, and 72 hrs) of fraction-1 were almost nontoxic (more than 90% of the cells were alive) to normal human fibroblast cell line (Fig. 4 G-I). The IC<sub>50</sub> concentrations of the positive control (5-FU) for the colon cancer cell lines were found to be higher than the IC<sub>50</sub> concentrations of fraction-1 (Fig. 4 A-F). Moreover, the IC<sub>50</sub> concentrations of 5-FU for both the colon cancer cell lines showed toxicity to normal human fibroblast cell line (Fig. 4 G-I).

### Fraction-1 Alters the Migration of Colon Cancer Cells

As the cytotoxicity assay showed

fraction-1 to be the most active one among the three-flash chromatography-derived fractions of MEPJB, its effect on cell migration was tested for both the colon cancer cell lines (HCT116 and SW480) using a scratch assay. The cells were treated with or without the respective IC50 doses (treated with the respective IC50 concentrations of 24 hrs) of the active fraction-1 after creating a scratch on a uniform monolayer of cancer cells with the help of a microtip, and the scratched

region was microscopically imaged between 0 and 48 hours. The overall cell density and motility of the vehicle-controlled and treated wounds were compared, revealing that fraction-1 inhibits cell migration, suggesting a potential anti-metastatic agent<sup>27</sup>. The gap sizes of the untreated (without fraction-1; vehicle-controlled) wounds of both the cancer cells were reduced after 24 and 48 hrs of incubation. (Fig. 5 A and B).



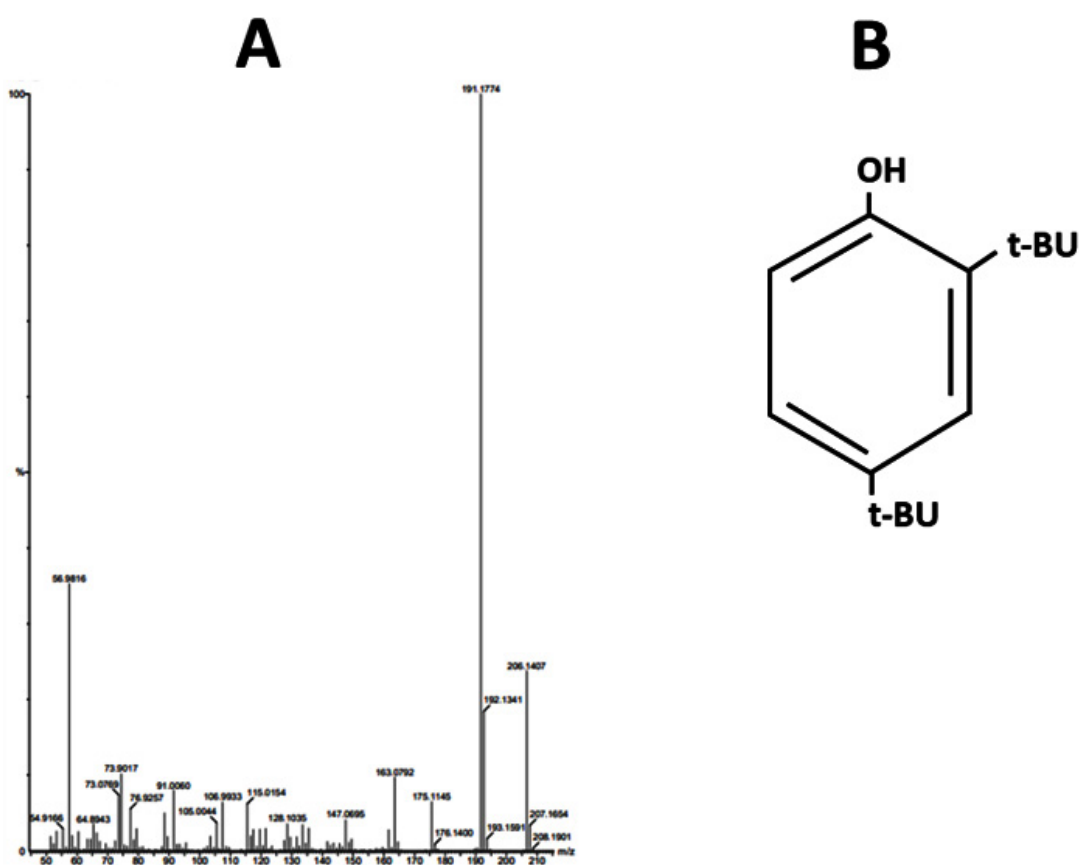
**Fig.2.** GC-MS total ion chromatogram of flash chromatography-derived fractions of methanolic extract of *Parkia javanica* bark (MEPJB). A: fraction-1; B: fraction-2; C: fraction 3; RT: retention time

### Morphological Changes in Colon Cancer cells Exposed to Fraction-1

In the presence of respective IC<sub>50</sub> doses (treated with the respective IC<sub>50</sub> concentrations of 24 hrs) of fraction-1, colon cancer cells (HCT116 and SW480) displayed considerable morphological changes as compared to the control (vehicle control). After 24 hrs of incubation, the colon cancer cells started changing their morphology and detaching from the surface. After 48 and 72 hrs, cells became totally rounded up, with a large number of cells that detached from the surface. Cell density also decreased after 48 and 72 hrs of incubation (Fig. 6 A and B). An inverted microscope (Dewinter-Victory plus, Dewinter optical Inc., India) was used to capture images.

### Apoptosis Induction by Fraction-1 on Colon Cancer cells (AO/EB Dual Staining)

It was discovered that fraction-1 induced time-dependent morphological alterations that might be linked to apoptosis<sup>27</sup>. To look into cell death and see if any apoptosis-related changes in the cell membrane took place in the presence of fraction-1, the AO/EB dual staining method (1:1) was used, and findings were obtained after 48 hrs of incubation. Ethidium bromide can penetrate nonviable cell membranes and attach to DNA. Acridine orange can easily penetrate through the viable cell membrane and attach to DNA. Nonviable cells glow red under fluorescence microscopy after AO/EB staining, whereas viable cells glow green. Most of the cells in the control group (vehicle-control) fluoresced light green after 48 hrs of incubation (Fig. 7 A),



**Fig. 3.** (A) Mass spectrum of the major compound Phenol,2,4-bis(1,1-dimethylethyl)- or 2,4-Di-tert-butylphenol (2,4-DTBP); m/z ratio: 191.1774 and (B) Chemical structure of 2,4-DTBP

whereas cells in the experimental group (treated with the respective IC50 concentrations of fraction-1) fluoresced yellow-green or orange-green, representing the early and late apoptotic phases, respectively. Membrane blebbing as well as chromatin condensation were seen in the experimental group of cells, which are indicators of early apoptosis. Condensation of the nucleus (ring and necklace), granular nucleus, as well as apoptotic bodies in the experimental group of cells indicated late apoptosis (Fig. 7 A and B).

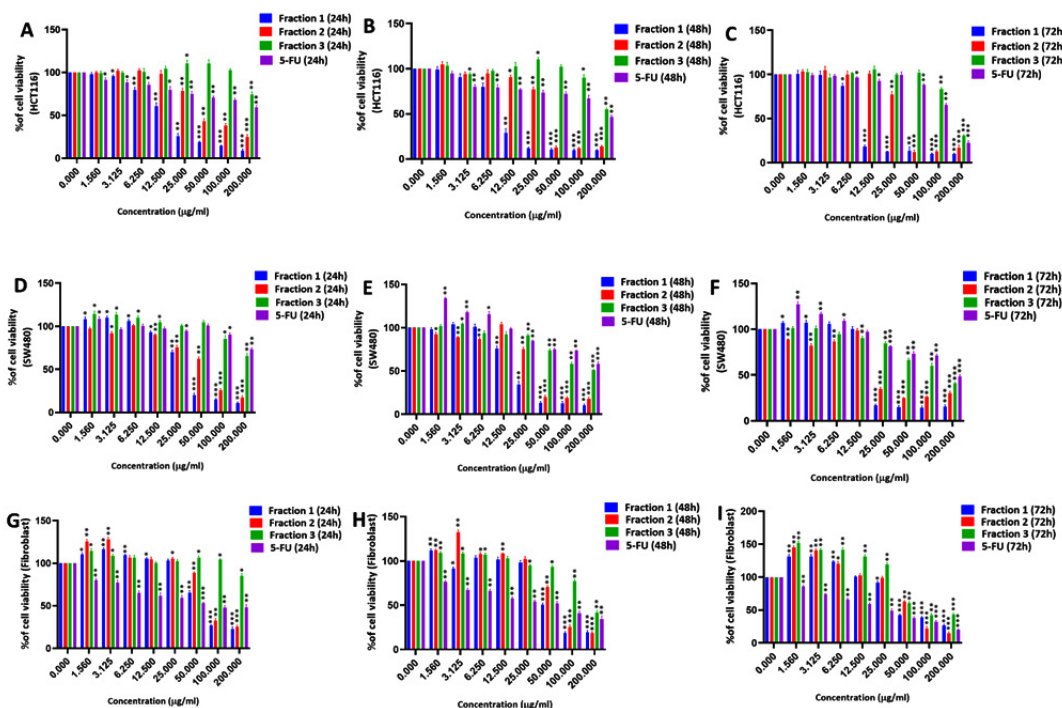
### Evaluation of Nuclear Morphology Pre- and Post-Treatment, Using DAPI Staining

In response to fraction-1 (treated with the respective IC50 concentrations of fraction-1), DAPI staining was used to assess the nuclear alterations in HCT116 and SW480 cells, particularly DNA fragmentation and chromatin condensation. After

48 hrs of treatment, staining displayed DNA fragmentation and chromatin condensation in fraction-1-treated cells (Fig. 8-B and D), but DNA fragmentation and chromatin condensation were not observed in untreated (vehicle controlled) cells (Fig. 8-A and C).

## DISCUSSION

The Indo-Burma biodiversity hotspot, which includes northeast India, is one of the 25 global biodiversity hotspots, and as a result, this region of India is the richest reservoir of flora and fauna in the country<sup>28,29</sup>. The state of Tripura, being geographically located in this biodiversity hotspot, harbors a great diversity of medicinal plants, some of which have a long ethnomedicinal history<sup>15-19</sup>. *Parkia javanica*, a plant belonging to the fabaceae



**Fig. 4.** MTT Assay: Effect of fraction-1, 2, 3, and 5-FU on HCT116 (A, B, C) and SW480 (D, E, F) colon cancer cell lines, and normal human fibroblast cell line (G, H, I). Each data point averages three experiments ( $n = 3$ ). The error bar demonstrates mean  $\pm$  SD. \* ( $P < 0.05$ ), \*\* ( $P < 0.01$ ), and \*\*\* ( $P < 0.001$ ) vs vehicle control, which was determined by two tailed paired t-test.

family, is widely distributed across the northeastern region of India, including the state of Tripura, and is used by various tribal populations of this region to cure a variety of ailments like stomach aches, cholera, diarrhea, dysentery, and food poisoning<sup>15-17</sup>. Many scientific studies have shown that this plant is antibacterial, anti-inflammatory, helps heal wounds, and fights leukaemia<sup>30-35</sup>. However, very few reports have been published regarding its anti-colon cancer property. Recently, anti-colon cancer activity of crude bark extract of *Parkia javanica* has been reported from our lab<sup>20</sup>.

The current research work has been designed in the quest to identify the active compound(s) conferring the anti-colon cancer property of the methanolic extract. To achieve this, the whole extract was further fractionated through flash chromatography, and activity-guided chemical analysis of all the fractions was performed.

A time-dependent and dose-dependent *in vitro* cytotoxic assay of the three-flash chromatography-derived fractions of the methanolic extract of *Parkia javanica* bark and 5-fluorouracil (positive control) on two colon

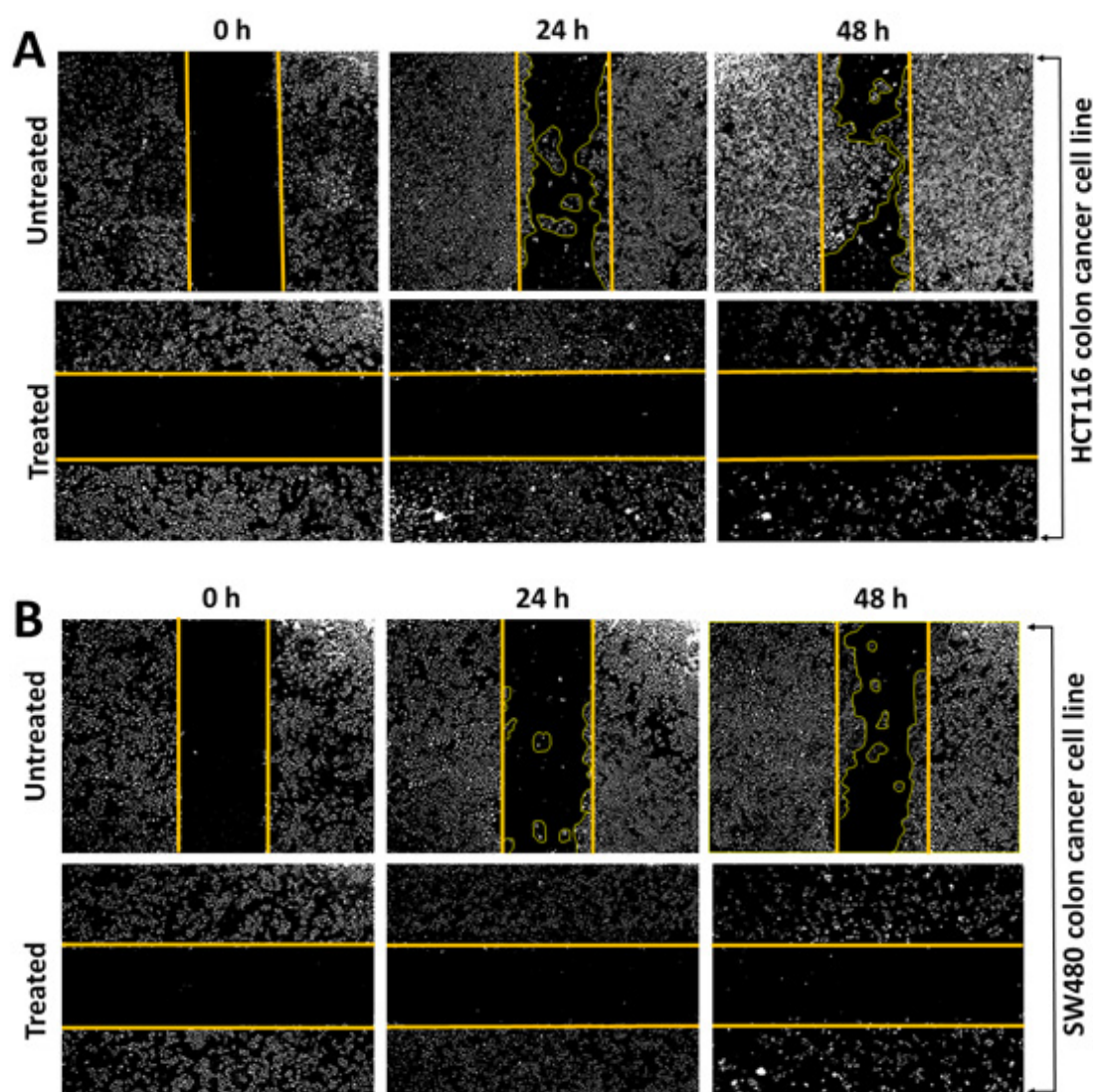
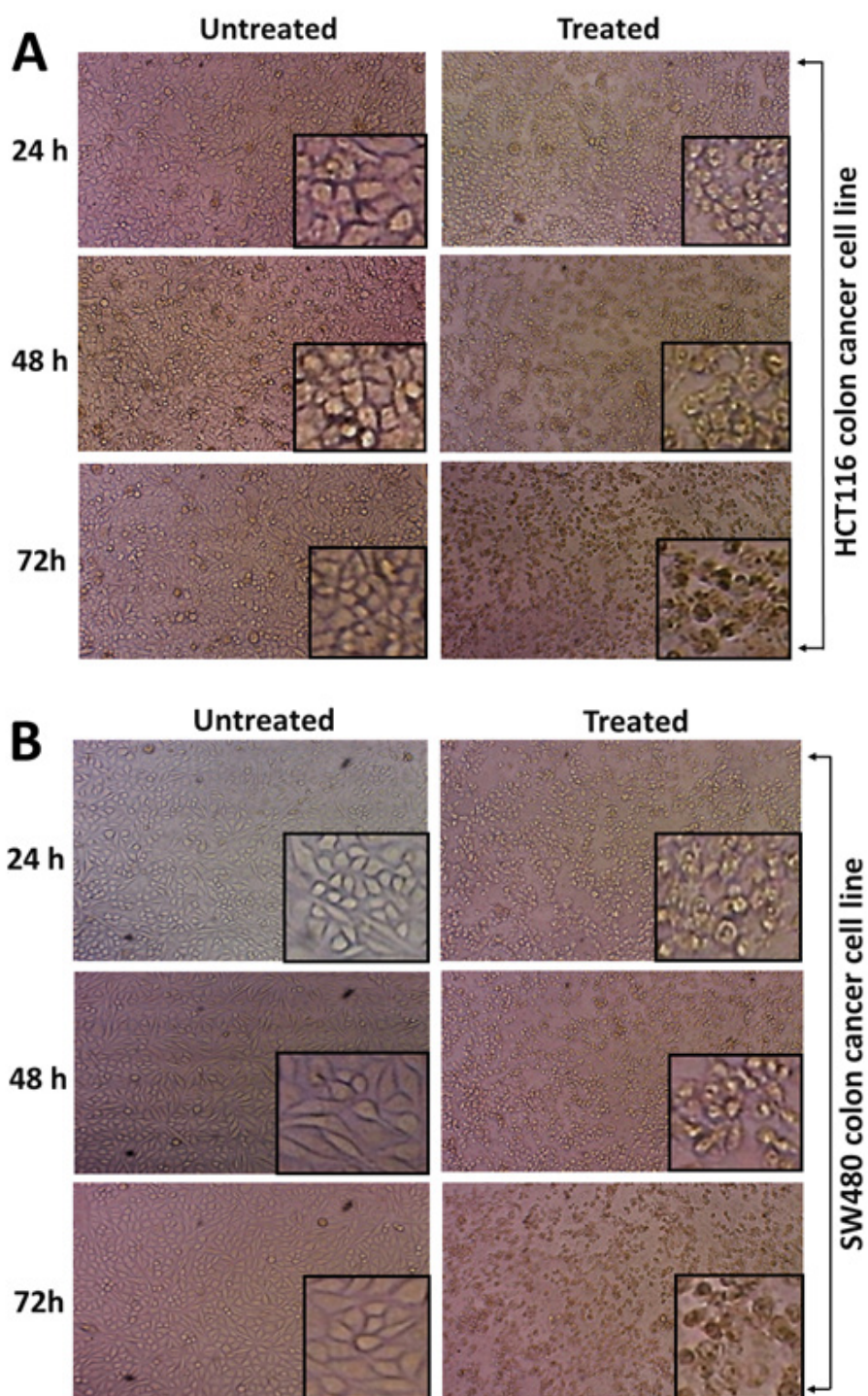
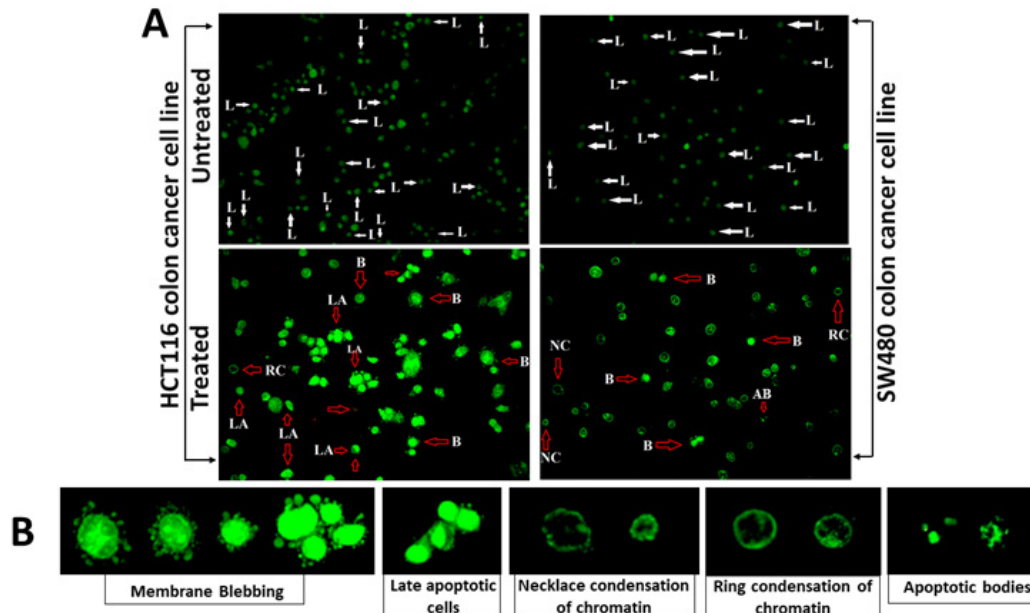


Fig. 5. The cell migration assay of treated and untreated (vehicle-controlled) HCT116 (A), and SW480 (B) colon cancer cell lines

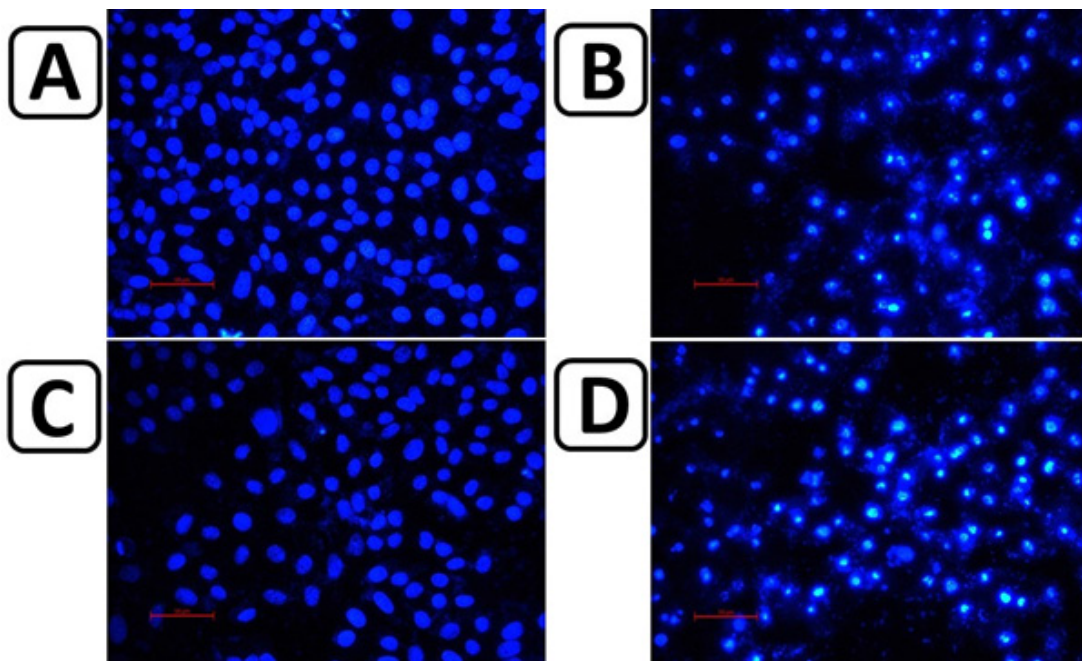




**Fig. 6.** Treated (with fraction-1) and untreated (vehicle-controlled) colon cancer cells underwent morphological alterations (A. HCT116, B. SW480)



**Fig. 7.** (A) The AO/EB fluorescence staining of treated and untreated colon cancer cells (HCT116 and SW480). (B) Enlarged images of special apoptotic features found in experimental group of cells. L: viable or live cells, B: membrane blebbing, LA: late apoptosis; NC: necklace condensation of chromatin; RC: ring condensation of chromatin; AB: apoptotic bodies



**Fig. 8.** DAPI staining of HCT116 and SW480 cells in response to fraction-1. Stained untreated (vehicle-controlled) colon cancer cells (A: HCT116, C: SW480) with no DNA fragmentation and chromatin condensation; stained treated (with fraction-1) colon cancer cells (B: HCT116, D: SW480) showed DNA fragmentation and chromatin condensation after 48 hrs of incubation with respective  $IC_{50}$  concentrations

**Table 1.** Identification of major phytochemical compounds present in the flash chromatography-derived fractions of methanolic extract of *Parkia javanica* bark (MEPJB) from the mass spectral data

Compound No	m/z ratio	Molecular weight	Molecular formula	Area % in respective fractions	Identified Chemical Compounds in MEPJB
C: 1	191.1774	206	C <sub>14</sub> H <sub>22</sub> O	F1(50.740%) F2(21.277%) F3 (7.859%)	Phenol,2,4-bis(1,1-dimethylethyl)- OR 2,4-Di-tert-butylphenol (2,4-DTBP)
C: 2	73.9704	340	C <sub>22</sub> H <sub>44</sub> O <sub>2</sub>	F1 (2.552%)	Methyl 14-methyl-eicosanoate
C: 3	149.0541; 72.8706	652	C <sub>38</sub> H <sub>68</sub> O <sub>8</sub>	F1 (4.023%) F3 (3.533%)	L-(+)-ascorbic acid 2, 6-dihexadecanoate
C: 4	56.9816	276	C <sub>17</sub> H <sub>24</sub> O <sub>3</sub>	F1 (3.387%)	7,9-di-tert-butyl-1-oxaspiro(4,5) deca-6,9-diene-2,8-dione
C: 5	149.1226	278	C <sub>16</sub> H <sub>22</sub> O <sub>4</sub>	F1 (6.636%)	Dibutyl phthalate (DBP)
C: 6	73.9704	256	C <sub>16</sub> H <sub>32</sub> O <sub>2</sub>	F2 (3.300%)	Methyl 9-methyltetradecanoate
C: 7	72.9394	256	C <sub>16</sub> H <sub>32</sub> O <sub>2</sub>	F2 (5.716%)	n-Hexadecanoic acid
C: 8	149.0541	352	C <sub>23</sub> H <sub>44</sub> O <sub>2</sub>	F2 (5.281%)	Methyl 11-Docosanoate
C: 9	56.9128	436	C <sub>31</sub> H <sub>64</sub>	F2 (4.171%)	Hentriacontane
C: 10	56.9128	914	C <sub>54</sub> H <sub>108</sub> Br <sub>2</sub>	F2 (10.106%)	Tetrapentacontane, 1,54-dibromo-
C: 11	56.9128	696	C <sub>41</sub> H <sub>77</sub> O <sub>2</sub> F <sub>5</sub>	F2 (4.900%)	Octatriacontyl pentafluoropropionate
C: 12	59.8720	180	C <sub>6</sub> H <sub>12</sub> O <sub>6</sub>	F3 (20.870%)	D-Allose
C: 13	73.9017	228	C <sub>14</sub> H <sub>28</sub> O <sub>2</sub>	F3 (3.590%)	Methyl 11-Methyl-Dodecanoate
C: 14	54.9166	296	C <sub>19</sub> H <sub>36</sub> O <sub>2</sub>	F3 (4.066%)	Methyl 13-Octadecenoate
C: 15	56.9816	322	C <sub>23</sub> H <sub>46</sub>	F3 (2.352%)	5-Methyl-Z-5-Docosene

C: compound; F: fraction

**Table 2.** IC<sub>50</sub> concentrations (µg/ml) of the flash chromatography-derived fractions of methanolic extract of *Parkia javanica* bark (MEPJB) on colon cancer and normal cell lines

Cell lines	Incubation period	IC <sub>50</sub> concentrations (µg/ml)		
		Fraction-1	Fraction-2	Fraction-3
HCT116 (Human colon cancer cell line)	24 h	16.25	43.75	> 200
	48 h	9.94	35.71	> 200
	72 h	9.38	34.38	166.67
SW480 (Human colon cancer cell line)	24 h	35	67.86	> 200
	48 h	20.14	36.02	> 200
	72 h	19.71	21.63	155.26
FIBROBLAST (Normal human connective tissue cell line)	24 h	68.18	82.5	> 200
	48 h	50	71.87	177.78
	72 h	45	65.91	67.5

cancer cell lines (HCT116 and SW480) and a normal human fibroblast cell line (GM00637) revealed that all three fractions have cytotoxic effects on both the colon cancer cell lines (Fig. 4 A-C; D-F). Out of three, fraction-1 was found to be the most active, followed by fraction-2 and fraction 3. 5-FU showed a less cytotoxic effect

on colon cancer cell lines compared to that of fraction-1 and 2 (Fig. 4 A-C; D-F). Furthermore, the IC<sub>50</sub> concentrations of 5-FU on cancer cells showed toxicity in normal human fibroblast cell line (Fig. 4 G-I). The IC<sub>50</sub> concentrations of fraction-1 and 2 (Table 2) on cancer cell lines were almost nontoxic to human fibroblast cell line

(Fig. 4 G-I). In a time and dose-dependent study, fraction-1, the most active fraction, demonstrated significant results in inhibiting cell motility, altering cell morphology, inducing apoptosis and altering nuclear morphology of the treated colon cancer cells HCT116 and SW480 (Fig. 5, 6, 7 and 8).

When the chromatographic fractions of MEPJB were analysed by GC-MS, it revealed the presence of a total of 15 chemical compounds having an area percentage >2% (Table 1). The major compound found in fraction-1 (50.740%) and fraction-2 (21.277%) was 2,4-DTBP, which was also present in fraction 3 in a limited amount (7.859%) along with 20.870% of D-Allose. The gradation of the presence of 2,4-DTBP in different fractions was well corroborated by the gradation of anticancer activities of the fractions. In fraction-3, the effect of D-Allose was not very effective. The anticancer activity of 2,4-DTBP has been reported by many authors<sup>36, 37</sup>. Therefore, 2,4-DTBP may be the major contributor to the anti-colon cancer activity of the fractions. Further work in this direction is warranted.

### CONCLUSION

Taking together all the results of the study indicated to anti-colon cancer potential of the active chromatographic fraction of *Parkia javanica* crude bark extract and 2,4-DTBP may be the major contributing compound for this activity.

### ACKNOWLEDGEMENT

The authors acknowledge the technical support of Guwahati Biotech Park, Guwahati, Assam, India. The authors also acknowledge the technical help of Central Instrumentation Centre and Biotech Hub of Tripura University, Tripura, India. We are thankful to Indian Council of Medical Research (ICMR) for providing the Senior Research Fellowship to Partha Saha, Ph.D. student at Tripura University, Tripura, India.

### Conflict of interest

The authors declare that they have no competing interests.

### Funding Source

ICMR Senior Research Fellowship of Partha Saha, Ph.D. student at Tripura University.

### Data Availability

All the data are available upon request to the corresponding author.

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