

Original Research Article

Cynodon dactylon (L) Pers (Poaceae) root extract induces apoptotic cell death via the cyclin D1 pathway in human nasopharyngeal carcinoma cells in vitro and in vivo

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Abstract

Purpose: To investigate the activity of *Cynodon dactylon* root extract as a potential anticancer agent.

Methods: Human nasopharyngeal carcinoma HNK-1 cells were treated with ethanol extract of *C. dactylon* roots. After treatment with *C. dactylon* root extract, cells were assessed for their cytotoxicity, metabolic status, apoptosis and cell cycle progression. The anticancer activity of the extract was also determined in vivo using a mouse model.

Results: *C. dactylon* extract exhibited significant cytotoxicity and arrested cell cycle in HNK-1 cells in a dose-dependent manner. Fluorescence-activated cell sorting (FACS) data revealed that it also induced apoptotic cell death. Rb phosphorylation and mRNA levels of cyclin E were significantly reduced. Moreover, *C. dactylon* extract caused a significant reduction in tumor size and volume in vivo.

Conclusion: *C. dactylon* root extract induces apoptosis in nasopharyngeal cancer cells via cyclin D1 pathway and inhibits tumor growth in vivo.

Keywords: *Cynodon dactylon*, Anticancer, Cyclin D1 pathway, Apoptosis, Nasopharyngeal carcinoma

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INTRODUCTION

Nasopharyngeal carcinoma (NPC) is a discrete type of cancer that is common among 50- to 69-year-old individuals. The disease, which is endemic to Southeast Asia and Southern China, is considered a major public health issue. Although radiotherapy is the primary method of treatment, surgery is preferred when there is nodal involvement [1]. Local recurrence is usually managed by a combination of radiotherapy and chemotherapy. The morbidity rate of malignant tumors has increased; moreover, the mortality rate has also increased due to invasion and metastasis, particularly during the late development of a malignant tumor [2].

Therefore, it is of utmost importance to understand the mechanisms of tumor invasion and metastasis to develop new therapeutic targets. Approximately 80 % of the global population use traditional medicine, which is based primarily on plants [3], and plant products are the preferred treatment choice for cancer in traditional medicine. Diverse cancer studies have been conducted using traditional medicinal plants, which lack undesirable side effects, in conjunction with current chemotherapeutic agents [4].

Cynodon dactylon (family: Poaceae), a common weed grass in India, is highly regarded in the

field of traditional medicine. The whole plant is believed to possess various medicinally active ingredients, and the plant extract shows antimicrobial and antihypertensive activities. It has also been used for the treatment of weak vision, urinary tract infection, calculi, and prostatitis.

The aqueous plant extract is used as an anti-inflammatory, antidiabetic, diuretic, antiemetic, and purifying agent [5]. More than 20 compounds have been characterized from *C. dactylon*, of which, hydroquinone (69.49 %), levoglucosenone (2.72 %), and furfural (6.0 %) are the most abundant [6]. Nadkarni reported the use of *C. dactylon* roots by practitioners of traditional medicine in India for curing certain types of cancer [7]. The anticancer activity of *C. dactylon* against NPC was investigated in the present study.

EXPERIMENTAL

The human NPC cell line HNK-1 was purchased from the American Type Culture Collection (ATCC; Rockville, MD, USA). Cells were grown in RPMI-1640 medium. All culture media were supplemented with 10 % fetal bovine serum (FBS), 100 U/mL penicillin, and 100 mg/mL streptomycin. Cells were grown in a humidified atmosphere containing 5 % CO₂ at 37 °C. All reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise indicated.

Extraction

The ethanolic extract of *C. dactylon* root was used in the present study. Plant samples were collected from a local botanical garden. The material was identified using available databases [8,9]. The roots were washed and dried in the shade, powdered using a grinder, and subjected to hot extraction with ethanol in a Soxhlet apparatus. The sample was subjected to preliminary phytochemical screening, and the remaining extract was stored in a refrigerator.

Cell culture/tumor cells

The obtained cell lines were maintained through subculturing (10⁵ cells/mL) at 37 °C in RPMI 1640 medium containing 10 % FBS in a humidified chamber. A control sample was maintained under the same conditions. Cells were first subjected to treatment with MG132 (1.0 μM), insulin (200 nM), or LiCl (30 mM) for 1 h before extraction. The cells were pretreated with 0.5 μg/mL cycloheximide (CHX) for 30 min to block *de novo* protein synthesis.

In vitro cytotoxicity assay

Cells were washed with phosphate-buffered saline (PBS), harvested by trypsinization, plated onto 96-well plates, and incubated in 5 % CO₂ and 95 % air at 37 °C for 24 h. Cells were then treated with different concentrations of plant extract. Dilutions of stock solutions were adjusted to a final concentration of 0.1 % in the culture medium with dimethyl sulfoxide (DMSO); this concentration of DMSO did not affect cell viability. Control cells were incubated in culture medium only. The experiment was conducted in triplicate.

MTT assay

Tumor cell growth was quantified based on the ability of living cells to reduce the yellow dye 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) to a blue formazan product. At the end of incubation, the medium in each well was replaced with MTT solution (20 cells/well, 5 mg/mL in PBS).

The plates were then incubated for 4 h in 5 % CO₂ and 95 % air at 37 °C. MTT reagent was removed, and the formazan crystals produced by viable cells were dissolved in DMSO (100 μL) and gently shaken. The absorbance was then determined on an enzyme-linked immunosorbent assay (ELISA) reader at 492 nm.

Apoptosis assessment

The Active Caspase-3-FITC Antibody Apoptosis Kit was used to examine apoptotic cells. The permeability of tumor cells was increased using a buffer that was subsequently incubated with antibodies supplied with the kit. Flow cytometry was subsequently used to quantify the percentage of apoptotic cells.

Cell cycle assessment

The cell cycle was assessed using flow cytometry with the assistance of a BrdU-7-AAD (bromodeoxyuridine-7-amino-actinomycin D) kit, which uses an immunofluorescence stain to measure the cell cycle by labeling the DNA with BrdU and antibodies conjugated to fluorescein isothiocyanate (FITC) or allophycocyanin (APC).

Furthermore, with the use of 7-AAD, a two-color flow cytometer can determine the phase of the cell cycle (i.e., the transition from the G₀ to the G₁ phase, which further transitions to the S phase, or from the G₂ to the M phase in cells that are actively synthesizing DNA).

In vivo assessment

Twenty-four female albino, line-bred mice (weighing 19 to 22 g, 8 weeks old) were used as test animals. Mice were raised in an environment of 23 ± 5 °C and a relative humidity of 55 ± 5 % with an alternating 12 h dark/light cycle in polypropylene cages under aseptic and hygienic conditions. Commercial feed pellets and water were supplied *ad libitum* on a daily basis. The study was conducted in accordance with ethical principles that complied with international guidelines [10] and was approved by the institutional ethical committee of The Second Affiliated Hospital of Fujian Medical University, Quanzhou City, Fujian, China (Ethical Permit no. 04/Q0301/28).

The mice were divided into three groups: pretreatment, treatment, and control, each of which consisted of eight mice. Tumor cells were cultured in a suitable medium and then trypsinized, centrifuged, isolated, and re-suspended in plasma to maintain a concentration of 1×10^7 cells/mL. The cells were then infused subcutaneously into mice at a predefined location at the posterior side (Table 1).

Specimens were collected from the tumor section after sacrificing the mice upon completion of 8 weeks of treatment.

Tumor growth was calculated as tumor volume (V) as follows: $V = 1/2AB^2$,

where A and B are the minimum and maximum tumor diameters, respectively.

Western blot analysis

The tumors were carefully isolated in equal sections and homogenized. The cells were then lysed with RIPA buffer for protein extraction. Chemically-induced cell lysis was performed using 20 mM Tris buffer, pH 7.5, 150 mM NaCl, 1

mM PMSF, 2.5 mM sodium pyrophosphate, 1 mM β -glycerophosphate, 1 mM EDTA, 1 mM EGTA, 1 % Triton X-100, 1 mM sodium vanadate, and a protease inhibitor cocktail. The extracted proteins were quantified and separated, and the expression levels of cyclin D and Rb were detected by Western blot analysis as described previously [11–13]. Briefly, an equal fraction of proteins was separated by means of sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS–PAGE) using a Mini-PROTEAN II system before transfer to a nitrocellulose membrane. Blocking of the nitrocellulose membrane was performed with 5 % fat-free milk in Tris-buffered saline containing Tween 20 (TBST; 10 mM Tris–HCl, pH 7.5, 100 mM sodium chloride, and 0.1 % Tween 20) and subsequently probed with appropriate primary antibodies. The membrane was developed with the aid of enhanced chemiluminescence reagents. The Kodak Image Station 4000MM Pro was used to detect the specific bands.

Statistical analysis

All pre- and post-treatment data were analyzed using Student's paired t-test and are presented as the mean \pm standard error of the mean (SEM). A *P*-value < 0.05 was considered statistically significant. All variables were analyzed with Chi-square test, and Graph Pad Prism 6 was used for all statistical analyses.

RESULTS

Cytotoxicity

The cytotoxicity assay performed on HNK-1 cells revealed that *C. dactylon* extract was effective at a concentration > 50 μ M. The extract had a cytostatic effect at concentrations > 50 μ M and exhibited > 50 % growth relative to the control at the indicated concentrations (Figure 1).

Table 1: Treatment protocol and observations from *in vivo* studies

Variable	Pretreatment group	Treatment group	Control group
Diet before exposure	Conventional feed + extract (0.5% w/w)	Conventional feed + extract (0.5 %w/w)	Conventional feed
Diet after exposure	Conventional feed	Conventional feed + extract (0.5% w/w)	Conventional feed
Duration of exposure	8 weeks	8 weeks	8 weeks

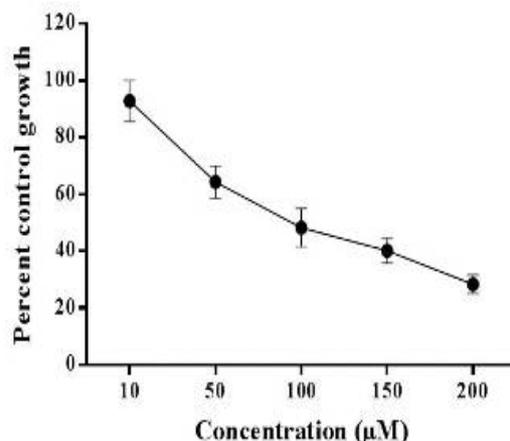


Figure 1: Cytotoxicity of ethanol extract of *C. dactylon* roots on HNK-1 cells

Cell cycle arrest

Flow cytometric analysis of the DNA from *C. dactylon*-treated cells revealed promising activity with regard to suppressing the cell cycle during the transition from the G1 to the S phase. *C. dactylon* caused cell cycle arrest in dose-dependent manner; it increased the number of

cells in the G1 phase but decreased the number in the S phase (Fig. 2). These results indicate that *C. dactylon* root induces apoptosis in a dose-dependent manner.

HNK-1 cell apoptosis induced by *C. dactylon* was analyzed by FACS and annexin V/propidium iodide (PI) staining. *C. dactylon* induced apoptosis in a dose-dependent manner. The percentage of apoptotic cells increased with increasing concentrations of extract. The percentage of apoptotic cells in the control was 3.3 %, but it increased to 77.4 % at the highest concentration of extract tested (150 µM, Figure 3).

***C. dactylon* extract induces phosphorylation, cyclin D1 deactivation, and reduction in transcription factors**

In our study, Rb phosphorylation was reduced, indicating activation of the CDK4/6 pathway. Moreover, the two main transcriptional targets, cyclin A and E, were also reduced (Figure 4).

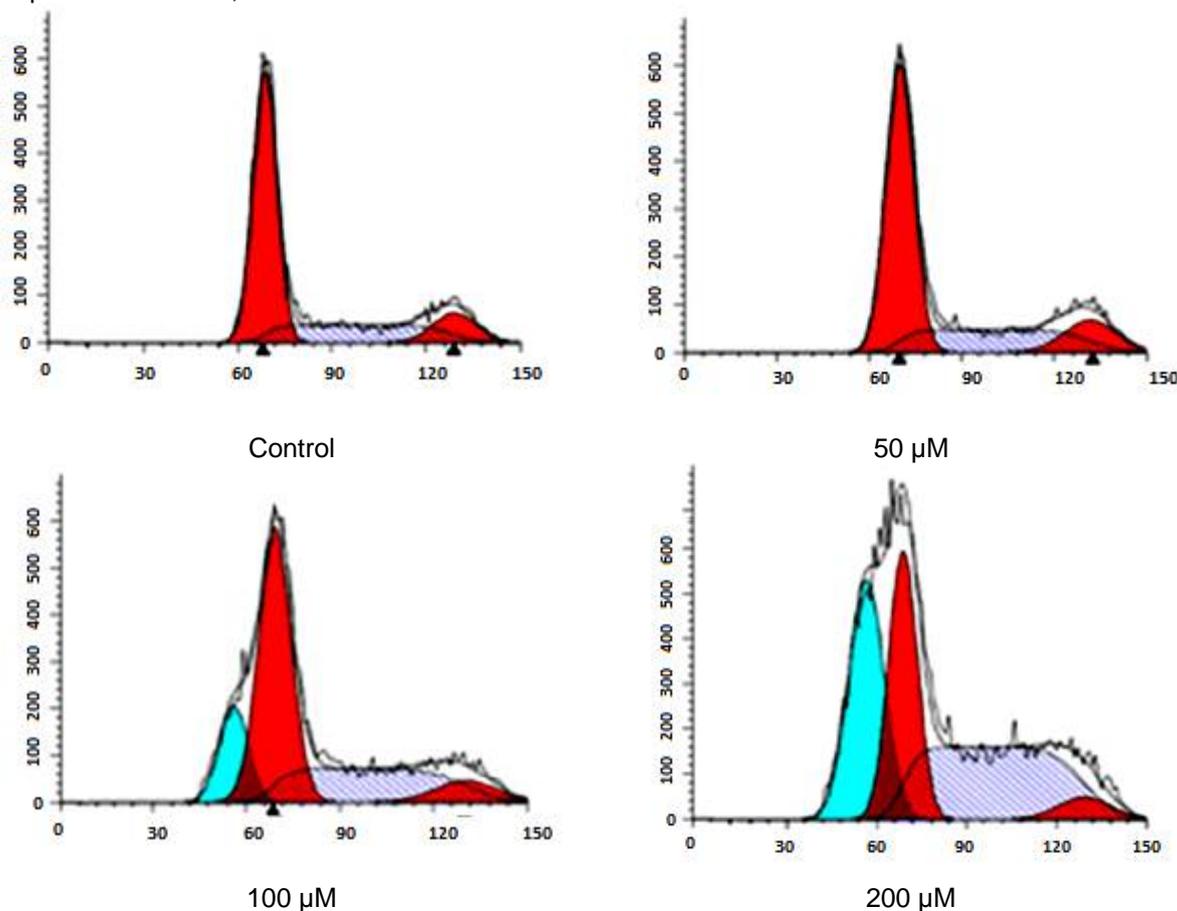


Figure 2: Effect of *C. dactylon* extract on cell cycle

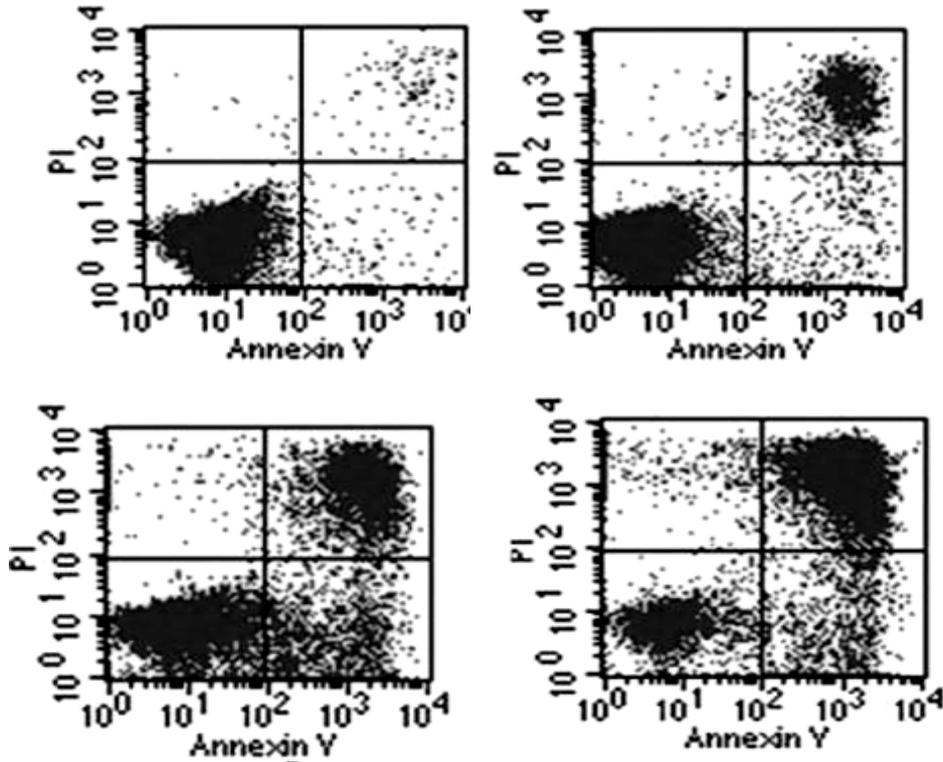


Figure 3: Determination of apoptotic and necrotic cells using annexin-V and propidium iodide staining, respectively

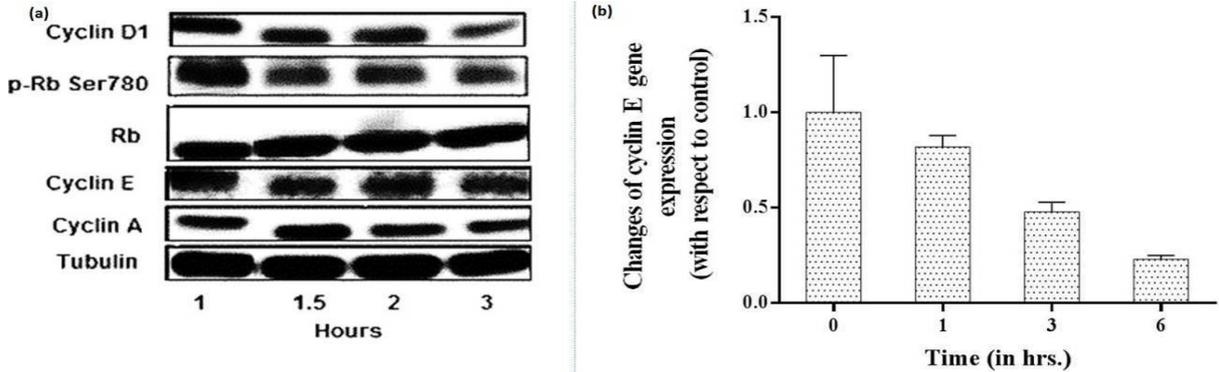


Figure 4: (a) Immunoblot analysis of proteins associated with the cell cycle and (b) down-regulation of cyclin E mRNA expression in HNK-1 cells treated with *C. dactylon* root extract

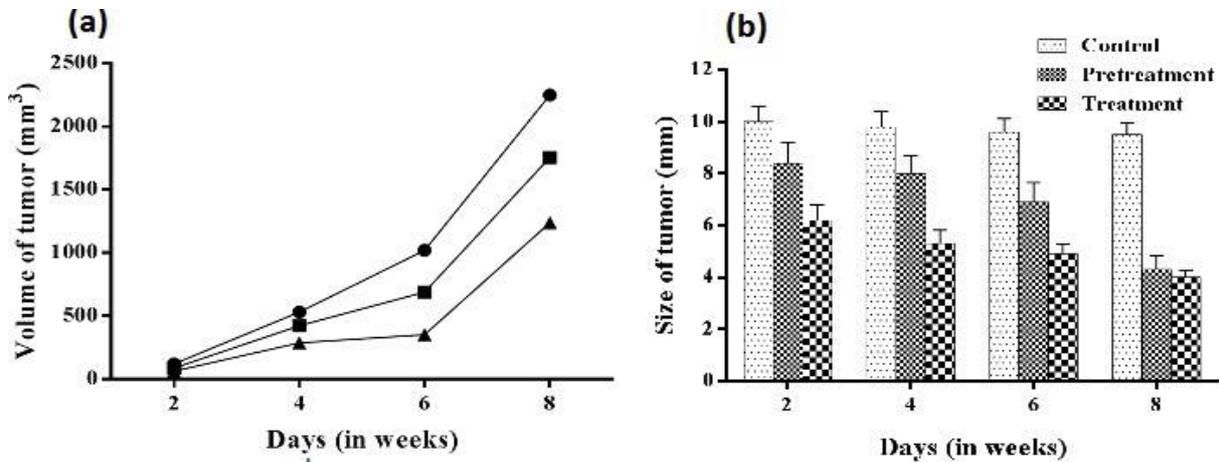


Figure 5: Tumor growth in mice fed the same conventional diet and maintained under the same environmental conditions for 8 weeks. (a) Tumor volume (● control, ◆ pretreatment, and ▲ treatment group) and (b) size decreased following treatment

***In vivo* activity**

The *in vivo* assessment also showed a promising effect of the extract when subjected to sequential treatment with *C. dactylon*. There was a significant reduction in tumor size and volume compared to the pretreatment group, revealing the efficacy of *C. dactylon* as an antitumor agent for the treatment of NPC (Figure 5).

DISCUSSION

Herbal medicine and the use of plants as therapeutic agents for cancer treatment have received much attention in recent years due to their safety and relatively few undesirable side effects [14]. *C. dactylon* exhibits various pharmacological activities, acting, for example, as an anticancer, antibacterial, and antihypertensive agent [15]. A previous study revealed that *C. dactylon* induces cell death in colorectal carcinoma by DNA fragmentation [16]. However, the potential mechanism of *C. dactylon* against NPC cells remains unknown.

For the first time, we demonstrated that *C. dactylon* inhibits the proliferation of NPC cells *in vitro*. *C. dactylon* induced a G1 arrest of cells and aided in the downregulation of cyclin D and the phosphorylation of Rb protein. It also targeted the cyclin signaling pathways, induced apoptotic cell death, and caused death in more than 70 % of HNK-1 cells. *C. dactylon* also significantly reduced tumor size and volume at the highest concentration tested (150 μ M) *in vivo*.

C. dactylon significantly inhibited the growth of NPC cells in a dose-dependent manner. Previous reports have indicated that it inhibited the growth of colorectal carcinoma, hepatocarcinoma, human colon adenocarcinoma, and breast cancer cells [16–18]. However, there has been no information on the effects of *C. dactylon* extract on NPC cells to date.

Extracellular signals involved in the gene expression and protein regulation required for cell division play a major role in mammalian cell proliferation [19]. Cyclin D1 plays an important role in cell cycle regulation and other related pathways. Upon phosphorylation, cyclin D1 loses its repressive activity for the E2F transcription factor, which then activates the transcription of several genes required for transition from the G1 to the S phase and for DNA replication. Cyclin D1 plays a major role in cancer by inducing several physiological processes during malignant cell transformation, including abnormal growth, angiogenesis, and resistance to apoptosis [20].

In our study, *C. dactylon* extract showed promise for suppressing the cell cycle during the transition from the G1 phase to the S phase, which was accompanied by cyclin D1 inactivation; this was further accompanied by a decrease in cyclin E expression, which explains the mechanism involved.

Cyclin D1 controls cell cycle progression. It was inferred that the influence of *C. dactylon* extract led to a reduction of cyclin D1 at the protein level. It has been reported that promoters of cyclin E and cyclin D1 are activated by E2F gene products, whereas E2F activity is directly regulated by Rb and associated proteins [21]. E2F1 and cyclin E provide an autoregulatory loop that controls critical steps that affect the cell cycle. Thus, according to our results, the accumulation of cyclin D1 appears to be an important component of Rb phosphorylation and the release of transcription factor E2F1 to initiate cell cycle progression to S phase.

CONCLUSION

The findings of the present study indicate that *C. dactylon* induces apoptosis in NPC cells. The apoptotic role of phytochemicals is associated with a higher activated level of cyclin and p-Rb proteins in cyclin D1 pathway. Further research on the specific roles of the active ingredients of *C. dactylon* extract on cell cycle regulation will contribute to the development of safer therapeutic targets.

DECLARATIONS

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Conflict of Interest

No conflict of interest associated with this work.

Contribution of Authors

The authors declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by them.

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