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ORIGINAL ARTICLE

Rehmanniae Radix Leaves Stimulates ROS-Induced Apoptosis on Human Mammary Cancer Cells through Suppressing PI3K/AKT/mTOR and GSK3β Signaling Pathway

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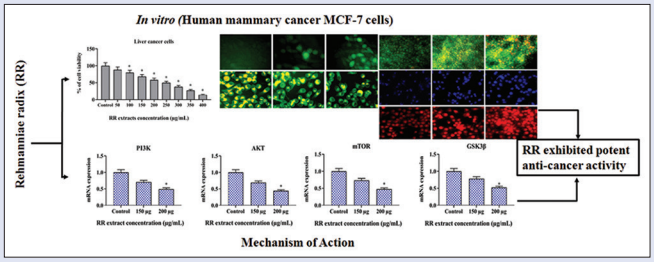
ABSTRACT

Background: Mammary carcinoma is the most communal carcinoma of higher women mortality. *Rehmanniae Radix* (RR) a traditional Chinese medicine. **Objectives:** To examine the anti-proliferative and anti-apoptotic effect of RR plant extract on mammary cancer cells MCF-7 and its action on PI3K/AKT/mTOR and GSK3 β mechanisms. **Materials and Methods:** The anti-proliferative of plant extract of RR was calculated, and morphological changes were measured by 3-(4,5-dimethylthiazol-2-yl) -2,5-diphenyltetrazolium bromide assay. ROS (dichloro-dihydro fluorescein diacetate), MMP (Rh-123), apoptosis by Propidium Iodide, 4′ 6-diamidino-2-phenylindole, and Acridine Orange/Ethidium Bromide staining assays assessed cellular cell death in MCF-7 cells. Western blotting analyses exhibited both phosphorylated forms compared with total PI3K/AKT/mTOR and GSK3 β were recognized. **Results:** The inhibitory action of breast cancer cells that RR extracts designated in a concentration manner. The IC_{50} value of RR extract on MCF-7 cells at 150 µg/mL concentration. Furthermore, staining assays seemed that RR extract-treated at 150 and 200 µg/mL, ROS production and apoptosis were augmented, and MMP and cell development were meaningfully reduced compared to control cells. Western blot analysis severely reduced the expression of p-PI3K, p-AKT, p-mTOR, and p-GSK3 β, but all total was extremely expressed than control cells. Altogether, the initiation of programmed cell death and suppression of cell propagation by the action of RR extract through the PI3K/AKT/mTOR mechanism recommended the anti-proliferative action of RR. **Conclusion:** Our results gave an innovative place of anti-tumor effect of RR extract is a gifted therapeutic and preventive agent in mammary cancer cell treatment.

Key words: Apoptosis, mammary cancer, *Rehmanniae radix*

SUMMARY

- Mammary cancer is the most communal occurrence and most life-threatening health problem of mortality in women globally
- The induction of apoptosis and suppression of cell proliferation by the action of *Rehmanniae radix* (RR) extract through the PI3K/AKT/mTOR mechanism in human mammary cancer MCF-7 cells proposed the anti-proliferative action of RR.



Abbreviations used: RR: *Rehmanniae radix;* Rh-123: Rhodamine-123; EB: Ethidium Bromide; DCFH-DA: Dichloro-dihydro fluorescein diacetate; AO: Acridine Orange; MTT: 3-(4,5-dimethylthiazol-2-yl) -2,5-diphenyltetrazolium bromide; DAPI: 4′ 6-diamidino-2-phenylindole; PI: Propidium Iodide; MCF-7: Michigan Cancer Foundation-7.

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INTRODUCTION

Mammary cancer is the most mutual occurrence and most life-threatening health problem of death rate in women worldwide[1] and is more reports connected with female humans with their receptors.[2] Estrogen hormones have an inordinate growth undergoes during cancer.[3] Hence, estrogen-positive mammary carcinoma is devised for nearly 80% of all breast carcinoma. Widely, about 20% of human epidermal growth factor receptor (HER) positive cancers so high synthesis of HER2 expressions in breast cancer. Additional type of breast cancer is triple-negative; their estrogen receptors do not have over-expression HER protein.[3] MCF-7 cells are the well-known estrogen-positive illustrative mammary carcinoma cell lines.[4]

The over-expression of PI3K orderly regulates the phosphorylated form of AKT/mTOR, and AKT, which are more significant to stimulate cell development and multiplication of cancer cells.[5,6] Cancer marks of cell proliferation, cell death, survival, and metastasis are permitted in the signaling cascade of PI3K/AKT/mTOR regulations on breast cancer.[7,8] Some reports have been clarified the natural sources suppress PI3K/AKT/mTOR regulating cancer cells.[9-12] The key approach for the inhibition of carcinoma is mainly targeting the PI3K/AKT/mTOR signaling.

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Rehmanniae radix (RR) is a traditional and classical Chinese medicine, its actions essentials of *Mateira medica* and the grand compendium of *Materia media*.[13] RR edible herb and numerous medicinal properties cover as Dihuang in Chinese (DC), which is typically found through the *Rehmannia glutinosa* (Gaerfn) DC, perennial plant of root lesions, it was stated that clinically valid more than 3000 years.[14,15] Recent pharma compound research hypothesized the RR has more than 140 individual compounds, some of them phenethyl alcohol glycosides, monoterpenoids, and triterpenes.[16,17] RR has vaccines pharmacological protective roles in numerous diseases such as diabetes, gynecological,[18] osteoporosis,[19] and hematological diseases.[20] This study presents the RR extracts effect on MCF-7 mammary cancer cells on cytotoxicity, apoptosis ROS formation, and mitochondrial membrane potential via morphological analysis. Further, we verified RR leaf extracts and their anti-cancer mechanisms to excite the mammary carcinoma cells (MCF-7) apoptosis and cell growth suppression via PI3K/AKT/mTOR GSK3 β signaling pathway.

MATERIALS AND METHODS

Chemicals

Rhodamine 123 (Rh-123), ethidium bromide (EB), dichloro-dihydro fluorescein diacetate (DCFH-DA), acridine orange (AO), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), phosphate buffer saline (PBS), 4′,6-diamidino-2-phenylindole (DAPI), and propidium iodide (PI) were obtained from Sigma-Aldrich (USA).

Preparation of plant materials and extracts

The RR leaves were attained, and the leaf was washed with the running tap water, and plant leaves were dried under at 27°C ± 2°C. The plant leaves dried blended to powdered form; 1 L of organic solvents of ethanol 300 g of the plant powder were detached using with a Soxhlet extractor boiling point ranges 60°C–80°C for 8 h. The RR leaves extracts were sieved by filter paper (Whatman No. 1). The crude plant leaf extracts were vanished to dryness in a rotary vacuum evaporator. After abandon of ethanol, we in a straight line prepared a working standard absorption for the research, i.e. 50, 100, 200,250, 300, and 350 μg/mL, respectively.

Cell culture and proliferation

MCF-7-human breast cancer cells were acquired through ATCC, USA, and culture in nutrient medium (RPMI 1640) through FBS, streptomycin, penicillin (1%) (Invitrogen, NY, USA). Breast cancer cell lines were molded as monolayers and then incubated in a moistenedCO_2 incubator at 37°C in CO_2 (5%). The nutrient RPMI medium was altered every 2 or 3 days. Separate cells using trypsin-EDTA 0.25% in PBS.

Cytotoxicity assay

Human mammary tumor cells 1×10^4 density loaded in 96 well plates for 24 h. For 24 h, MCF-7 cells were treated with vehicle control DMSO (0.1%) and RR leaves extracts at concentrations of 50, 100, 200, 250, 300, and 350 g/ml. MTT dye (1.2 mg/mL) was then pragmatic to the 96 well plates, which were then incubated at 37°C for 24 h. The MTT formazan crystals dissolve using DMSO and then calculate the absorbance at 490 nm by assist fluorescence.

Measurement of intracellular ROS production

ROS accretion restrained using the dye of DCFH-DA, which enters into the intracellular cytosol region. When ROS augmented, it becomes oxidized into fluorescence dichlorofluorescein (DCF). Then the range of ROS production was consequently relative to fluorescence expression power. MCF-7 cells were located in six-well plates and then treated with RR leaf extract at different concentrations 150 and 200 μg/mL and then incubator at CO_2 around 24 h. Later, DCFH-DA dye was added into MCF-7 cells nearly 10 min incubation. Finally, the fluorescence intensity was assessed through a filter like exudation 485 ± 10 nm and emission filter 530 ± 92.50 nm. The data were composed according to the percentage of intensity of fluorescence from the fluorescence microscope.

Evaluation of mitochondrial membrane potential (Δψm)

The ROS-induced Δψm alterations were estimated through lipophilic dye in the change of cation (Rh-123). The MCF-7 cells were complemented with RR extracts (150 and 200 μg/mL) around 30 min. An inverted fluorescent microscope was used to observe the MCF-7 cells. However, the data were uttered as mean standard deviation in a thrice replicated data set in the control cells at the end of the results.

Apoptotic morphological examination

The apoptotic stages were measured by the staining process of AO/EB depicted the apoptotic findings via structural changes by the procedure.[21] Mammary cancer cells were treated with two different concentrations of RR leaf extracts (150 and 200 μg/mL) for 24 h, control and treated MCF-7 cells were PBS cleaned and stained 1:1 ration AO/EB dye mixture at 27°C for 5 min. The reaction mixture was analyzed under a fluorescence microscope exudation filter 510-590 nm at 40x magnification.

4′,6-diamidino-2-phenylindole staining assay

DNA content analyses in both live and necrotic cells were asses by using dye DAPI. MCF-7 cells 1×10^5 were established in 6 well plates and incubated at 5% CO_2 for 24 h, and RR extracts treatment were done at 150 and 200 μg/mL doses of MCF-7 cells at 37°C. After PBS wash using the 4% formaldehyde fixed and 10 μg/mL con of DAPI. After stained 5–10 min, MCF-7 cells were inspected under a fluorescence microscope.

Propidium iodide staining assay

MCF-7 cells (5×10^5) were permitted to settle on a six-well plate and incubated 24 h treatment with 150 and 200 μg/mL of RR extracts. These cells were then secluded and added PI staining 5 μl with 10 mg/mL concentration kept in cool bath dark conditions. The supernatant from the cell disruption (10 μl) was pragmatic to the PI and a fluorescence glass slide was used. Slide imagined under a fluorescence microscope for 30 min.

Real-time quantitative polymerase chain reaction

Using 6 well plates the cells grown at 3×10^5 cells concentration consist 80% of confluences, then the cells were then incubated with the RR plant extract at 150 and 200 μg/mL for 24 h. total RNA from the cells were isolated by using Qiagen RNeasy Mini Kit manufacturer orders. The reverse transcription system (Bio-Rad S 1000 Thermocycler, USA) was practical to give cDNAs, and it stowed cooling conditions until-20. The primers were procured for the reactions in the Integrated Technologies, USA. Every experiment was completed in the thrice trial.

Statistical analysis

The statistical data was designed using the analysis of variance, and the differences were analyzed using Dunnett's test. The statistical analyses were carried out using SPPS 11 with a significance level of $P < 0.05$.

RESULTS

Effect of *Rehmanniae Radix* extract cytotoxicity effect on MCF-7 cells

The MTT method was employed to measure the cytotoxicity of the RR extract. Figure 1 designated that RR extracts suppressed the growth

of MCF-7 cells at different concentrations when augmented the dose of extract reduced the cell count and cell viability in 24 h treatment. Fluorescence microscopical morphological identifications established these findings.

Effect of *Rehmanniae Radix* extract on intracellular ROS production on MCF-7 cells

Using the DCFH-DA dye, we examine how well RR extracts produce ROS in MCF-7 cells [Figure 2]. ROS synthesis was connected in RR extract-treated MCF-7 cells via the protuberant emission of fluorescence intensity compared with control cells.

Effect of *Rehmanniae Radix* extract on ΔψmDepolarisation in MCF-7 cells

Δψm is a symbol of cellular physiological status. Further, it responded as a key role of Δψm and dropped which initiation states lead to apoptosis. RR extract lessens Δψm when treated on MCF-7 cells, which was stained using Rh-123 dye [Figure 3]. Emission of green color fluorescence indicated that the mitochondrial membrane was polarized when augmented the control cells. In contrast, RR extract at 150 and 200 µg/mL treated on MCF-7 cells presented reformed Δψm and diminished the green fluorescence emission. Finally, the DCFH-DA dye method clearly perceived that improved ROS synthesis in MCF-7 cells resulted from RR extract treatment.

Effect of *Rehmanniae Radix* extract induced apoptosis in MCF-7 cells

AO/EB, PI, and DAPI staining were aided in assessing RR leave extracts' action. As portrayed in Figures 4-6, RR extracts were treated with 150, and 200 µg/mL concentration for 24 h displayed a marked stimulation of apoptosis. DAPI, dual AO/EB, and PI staining show stimulation of cell death in MCF-7 cells treated with 200 g/mL RR leaves extracts comparison with control cells.

Effect of *Rehmanniae Radix* leaves extracts effect on the PI3K/AKT/mTOR signaling pathway

We recognized the mRNA expression status of PI3K, AKT, mTOR, and GSK3 to regulate the action of RR leaf extracts on the stimulation of the PI3K/AKT/mTOR pathway in mammary cancer MCF-7 cells [Figure 7]. Our present results were presented that RR leaf extracts were repressed the gene expressions of PI3K, GSK3 β, AKT, and mTOR at 150 and 200 µg/mL different concentrations of RR extracts.

DISCUSSION

Typically, the research has gauged that phytochemicals were influenced to suitable therapeutics properties, mainly encouraging cancer cell death, including anti-inflammation, anti-proliferative and antioxidant functions.[22,23] In the current verdicts, we hypothesis that RR extract has inhibition of cell proliferation on mammary cancer cells. This RR extract augmented PI3K, GSK3 β, AKT, and mTOR expression, finally causes to mammary cancer cell death. The anti-proliferative effect of RR leaf extracts on various concentration treated MCF-7 cells is illumined in Figure 1. The current findings seemed the past of mammary cancer cells to RR extracts at 48 h settled significantly induced cell death of cancer

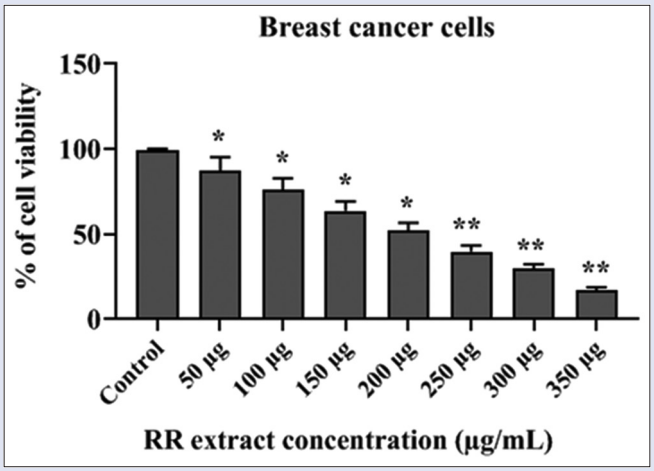

Figure 1: Effect of *Rehmanniae Radix* plant extracts on cell cytotoxicity of MCF-7 cells was examined through the 3-(4,5-dimethylthiazol-2-yl) -2,5-diphenyltetrazolium bromide method. Results are assessed as MCF-7 cells treated with either control and R leaves extract (50–350 µg/mL) for 24 h. Values were presented as mean ± standard deviation of 3separate experiments analysis of variance analysis of variance followed by DMRT. Asterisks indicate statically different from control: *, **$P < 0.05$

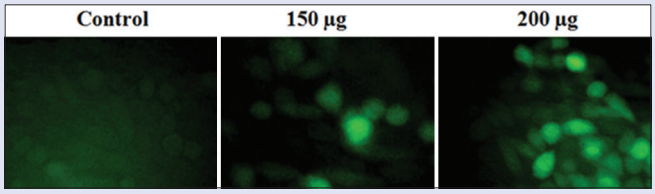

Figure 2: Effect of *Rehmanniae Radix* plant extracts induced ROS in MCF-7 cells via dichloro-dihydro fluorescein diacetate staining method for 24 h. Control (green fluorescence) and leaves extracts150 µg depicted weak background fluorescence, and 200 µg showed bright dichlorofluorescein florescence in MCF-7 cells treated with *Rehmanniae Radix* leaf extracts in a concentration manner

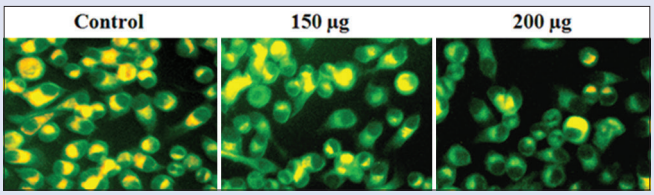

Figure 3: Effect of *Rehmanniae Radix* leaves extract on the Δψm of MCF-7 cells. Breast cancer cells were treated with dose-dependent manner of *Rehmanniae Radix* extracts 150 and 200 µg/mL for 24 h, stained with suitable Rh-123 and the mitochondrial depolarisation changes of MCF-7 cells were measured

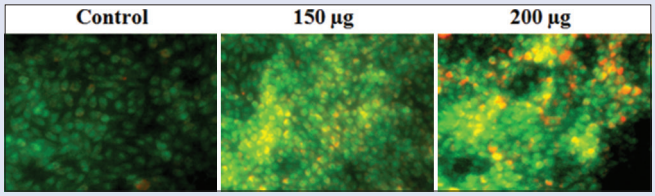

Figure 4: Effect of *Rehmanniae Radix* extracts induced apoptosis on breast cancer cells. Dual acridine orange/ethidium bromide dye for resulting green, yellow, orange, and red color depicts to live, early, late apoptotic, and DNA damaged necrotic cells were treated with *Rehmanniae Radix* leaves extracts 150 and 200 µg/mL for 24 h

cells MCF-7. Thus, these findings established that the RR leaves extracts to suppress cell proliferation and depend on the concentration.

ROS production was increased or depleted of endogenous antioxidants by induction of the cell death process were previously well recognized.[24]

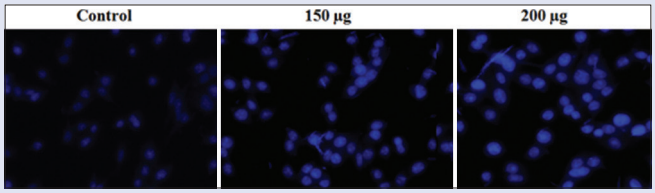

Figure 5: Effect of *Rehmanniae Radix* extracts induced apoptosis through 4′ 6-diamidino-2-phenylindole staining. MCF-7 cells were exposed to control and *Rehmanniae Radix* leaf extracts (150 and 250 µg/mL) for 24 h. The breast cancer cells stained with 4′ 6-diamidino-2-phenylindole dye were examined under a fluorescence microscope

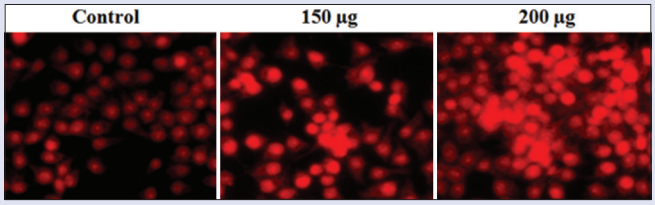

Figure 6: Effect of *Rehmanniae Radix* leaves extracts induced in nuclear morphology changes in MCF-7 cells by PI staining. The MCF-7 cells were treatment with *Rehmanniae Radix* leaf extracts at different doses (150 and 200 µg/mL) for 24 h and stained with PI depicts apoptotic cells and fragmented nuclei

Cells stayed survive conditions in a lower level of ROS, but a relative then supports programmed cell death. ROS modifying drugs are being conceived as therapeutics tactics to goal cancer cell death.[25] Our present examination analyzed the RR extract knowingly induced ROS production in MCF-7 cells in concentration-based mode.

Numerous studies have established that anti-tumor drugs induced cytotoxic effects on carcinoma cells via apoptotic induction through mitochondrial function loss and assessed Δψm.[26,27] Our contemporary studies informed that RR extract condensed the Δψm status in MCF-7 cells at diverse concentrations manner.[28] Elucidated his studies already that similar effects were pragmatic as anti-cancer drug action due to Δψm reduction, which named that intrinsic signaling pathway. These data established that the RR extracts lowered proliferation of mammary carcinoma cells by stimulation cell apoptotic mechanism detected in PI, AO/EB, and DAPI staining morphological appearance cell death stimulation is measured as a concept of control as reported results seemed that were repressed the nonencapsulated from of RR extract in MCF-7 cells.[29,30]

To assess the mechanisms through RR extract, suppress cell development, and stimulation of programmed cell death in MCF-7 cells, we reviewed the PI3K, GSK3 β, AKT, and mTOR signaling pathways treated with RR leaf extract. Cell proliferative intracellular signaling mechanisms play a crucial role in developing and multiplication cells, movement metabolism, and cell death. The PI3K, AKT, and mTOR pathways were found to be abnormally regulated in a diversity of carcinomas, counting mammary cancer.[31,32] The proper therapeutic testing developments of PI3K/AKT/mTOR suppressors have caused the targeting to be closed down. The regulation of AKT, PI3K, and mTOR was a viable remedy for averting mammary cancer. In the present results, change treatment with the RR leaves extracts, PI3K, GSK3 β, AKT, and mTOR expressions status were professionally stimulated of apoptosis in breast

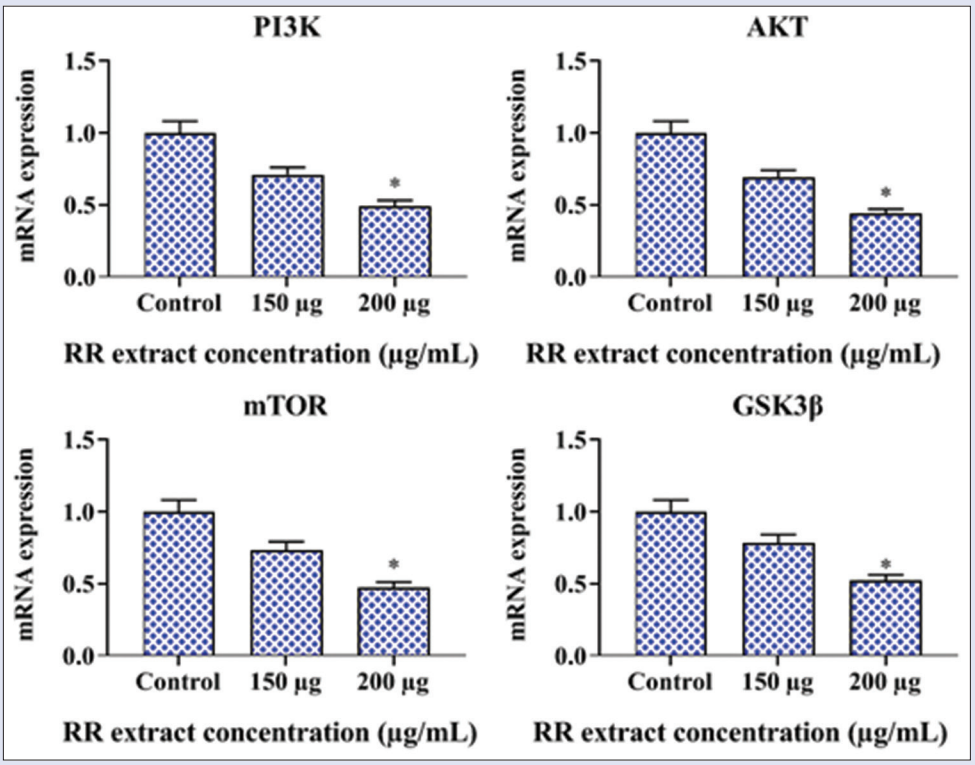

Figure 7: Effect of *Rehmanniae Radix* leaves extracts induced in nuclear morphology changes in MCF-7 cells by PI staining. The MCF-7 cells were treatment with *Rehmanniae Radix* leaf extracts at different doses (150 and 200 µg/mL) for 24 h and stained with PI depicts apoptotic cells and fragmented nuclei

cancer cells. This hypothesis arbitrates that mammary carcinoma cell lines suppressions are more pertinent when persuaded RR leaf extract suppresses the PI3K signaling mechanism. RR leaves extracts stimulated the mammary cancer cell induction of apoptosis through PI3K/AKT/mTOR and GSK3 β mechanism by other pathways.

CONCLUSION

The current hypothesis confirms the RR extract suppresses cell development and stimulates programmed cell death in the mammary cancer MCF-7 cells culture method. The RR leaf extracts actioned on the development of cell growth suppressions are most interrelated with its capability to suppress regulation of AKT, PI3K, mTOR, and GSK3 β signaling actions. These results recommended that the RR leaf extracts can give an alternative facet for mammary cancer cells. Further, our findings are desired to analyzed all ingredients in an RR leaves extracts and classify *in vivo* effects of these plant extracts permitted in an animal model to inspect the RR plant extracts therapeutic target in different action sites.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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