INTRODUCTION

Recent studies have focused on the antitumor properties of natural products because of their confirmed pharmacological properties and very low side effects. Rhein (4,5-dihydroxyanthraquinone-2-carboxylic acid) is a natural anthraquinone derivative (Figure-1), that can be extracted from Rheum palmatum, Rheum tanguticum, Cassia angustifolia and Cassia fistula [1-4], and has been reported for diverse pharmacological activities including anti-microbial [5], anti-angiogenic [6], anti-inflammatory [7], anti-cancer activities [8,9]. The extract of rhubarb contained “rhein”, has been utilizing as a mild laxative and astringent since ancient time and rhein 100 mg / Kg / day oral dose of rhein did not cause any harmful effect [10].

Fig. 1: Molecular structure of rhein compound.

Recent past studied have shown that rhein inhibits the growth of Ehrlich ascites tumor [11], rat liver tumor cells [12], human glioma cells [13], tongue cancer cells (SCC-4) [14-16], adenocarcinoma cells (Gaco-2) [17], nasopharyngeal carcinoma cells (NPC) [18], lung cancer cells (A-549) [19], hepatocellular carcinoma cells (BEL-7402) [20], and cervical cancer cells (CaSkii) [21]. Apoptosis is the most important pathway through which many compounds exert their antitumor effects. Previous studied revealed that rhein induced apoptosis in human promyelocytic leukemia cells death (HL-60) via ROS-independent mitochondrial apoptosis pathway [22], whereas SCC-4 cells death via ROS-dependent mitochondrial apoptosis pathway [14]. However, A-549, CaSkii and NPC cells death via Ca2+-dependent mitochondrial apoptosis pathway [19, 21, 23]. Rhein has also been reported to inhibited invasion of cancer cells to the new sites by reducing the expression of matrix metalloproteinase-9 (MMP-9) and vascular endothelial growth factor (VEGF) via inhibition of nuclear transcription factor (NF-kB) in NPC cell line [18]. From the literature survey, the effect of rhein against human cervical cancer (SiHa), breast adenocarcinoma (MCF-7) and hepatocellular carcinoma (HepG2) cells have not been reported. Hence the aim of present study was to investigate the cytotoxicity effects of rhein against human SiHa, MCF-7 and HepG2 cancer cells and underlying molecular mechanisms of apoptosis.

MATERIALS AND METHODS

Reagents and chemicals

Rhein was purchased from Sigma-Aldrich (USA). Tissue culture medium constituents procured from HiMedia (Mumbai, India). All other chemicals and reagents were of analytical grade from Merck (Mumbai, India).

Cell culture

Human cervical cancer (SiHa), breast adenocarcinoma (MCF-7), hepatocellular carcinoma (HepG2) and non-cancer human embryonic kidney (HEK-293) cell lines purchased from the National Center for Cell Science (NCCS, Pune). Cell lines were grown in T-25 flasks and sub cultured twice a week in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and antibiotics (100 U/mL penicillin and 100 µg/mL streptomycin) in a humidified atmosphere of 5% CO2 at 37°C.

Cytotoxicity assay

Cytotoxicity was carried out by MTT (3-(4,5-dimethyl-2-thiazolyl)-2, 5-diphenyl-2H tetrazolium bromide) assays as previously describe [24]. Cells (~2x10⁴ cells/well) were seeded overnight in a flat bottom 96 well plate. After that, cells were treated with various concentrations of rhein (5-115 µM) for 24 and 48 h respectively. At the end of treatment duration, the medium was replaced with fresh medium and added 20 µl of MTT (5 mg/ml PBS) for 4 h. Formazan crystals were formed through mitochondrial reduction of MTT by live cells. Formazan was solubilised in DMSO (150 µl /well) and the absorbance was read at 570 nm on the iMark Microplate Reader (Bio-Rad, USA). The mean percentage (± standard error of mean (SEM)) cell survival was plotted against the rhein concentration and the best fit line was used to calculate IC50.

% Cell survival = (Control OD570 − Experimental OD570) / Control OD570 × 100
Quantification of LDH enzyme

Cells (~2x10^5 cells/well) were seeded overnight in a flat bottom 96 well plate. After that, cells were treated with various concentrations of rhein (5-115 µM) for 24 and 48 h respectively. The LDH enzyme released after cells death in the extracellular medium was quantified through commercially available Cytoscan™-LDH assay Kit (G-Biosciences, USA). The reaction mixture was measured at 490 nm on the iMark Microplate Reader (Bio-Rad, USA). Cytotoxicity was calculated with the following formula:

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\text{% Cytotoxicity} = \left( \frac{\text{Control}_{\text{OD}_{490}} - \text{Soptaneous}_{\text{OD}_{490}}} {\text{Control}_{\text{OD}_{490}}} \right) \times 100
\]

Flow cytometry

Flow cytometry was performed for the analysis of cell cycle arrest according to the standard method [14]. SiHa, MCF-7 and HepG2 cancerous cell lines treated with rhein (IC50) for 24 h. After that, cells were harvested and washed with phosphate buffer saline (PBS). Cells were suspended in 500 µL PBS and fixed with 70% chilled ethanol (700 µL) for 5 min. The fixed cells were washed with PBS and incubated with RNase A (100 µg/mL) for 30 min at 37°C. Finally, cells were washed, added propidium iodide (50 µg/mL) and incubated for 5 min. Cells cycle was sorted on a Becton Dickinson laser-based flow cytometer analysed by inbuilt software (BD Biosciences, USA).

Reverse transcriptase-polymerase chain reaction (RT-PCR)

SiHa, MCF-7 and HepG2 cell lines were treated with various concentration of rhein for 48 h. After that the cells were harvested and washed with PBS. Total RNA was isolated by NP- total RNA Extraction Kit (Taurus & Scientific, USA). 1µg of total RNA was used for cDNA synthesis using RevertAid™ first stranded cDNA synthesis Kit (Fermentas Life Science, USA) with random hexamers. The synthesised cDNA was used for the detection of mRNA expression of GAPDH, p53, Bcl-2, Bax, Casp-3 and -9 genes using specific oligonucleotide primers (PubMed) (Table 1). RT-PCR carried out in a total volume of 25 µL, using 2 µL of cDNA, 1U taq DNA polymerase, 1.5 mM MgCl2, 0.2 mM dNTP and 20 pmole specific oligonucleotide primers (Bio-Rad, USA). A cycle profile consisted of 30 sec at 94°C annealing, and 30 sec at 72°C for extension in amplification, and finally 30 sec at 72°C for extension in amplification.

Table 1: Oligonucleotide primers

| DNA fragment assay |

DNA fragmentation assay was performed according the protocol of DNA ladder assay kit (G Biosciences, USA). Rhein (IC50) treated cells were harvested, washed with PBS and used for isolation of genomic fragmented DNA. The isolated fragmented DNA was separated on 1.8% (w/v) agarose gel electrophoresis and visualized on the Gel Doc system (Bio-Rad, USA).

Statistical analysis

All the experiments were carried out on triplicate and data were presented as mean ± standard error of mean (SEM). Statistical significance was examined with ANOVA test. The p < 0.05 was considered as statistically significant.

RESULTS

Cytotoxicity effect of rhein

Rhein showed cytotoxicity effect on cancer cell lines dose and time dependent manner (Figure 2). IC50 value of rhein against SiHa, MCF-7 and HepG2 cell lines were 54.28 ± 0.17, 49.37 ± 0.23, and 36.34 ± 0.14 µM respectively. However, in LDH assay, rhein showed 45.54, 45.65 and 49.42% cells death at IC50 concentration. Similarly, after 24 h, cytotoxicity of rhein against SiHa, MCF-7 and HepG2 cell lines were 41.60, 40.36 and 41.02% in MTT assay and 40.12, 37.45 and 39.25% in LDH assay. Both cytotoxicity assays were significant correlation (r > 0.974; p < 0.05).

To determine whether normal cell growth was also inhibited, we investigated cell viability in the non-cancer human embryonic kidney cells (HEK-293). IC50 value of rhein against HEK-293 was 74.95 ± 0.31 µg/mL. The order of cell lines affected by the activity of rhein compound was HepG2 > MCF-7 > SiHa > HEK-293.

Expressional analysis of p53, Bcl-2 and Bax genes

Treatment of rhein induced up-regulation of p53 gene in a dose-dependent manner (Figure 3-5). At LC50, p53 gene up-regulated by 9.75 fold in SiHa, 10.16 fold in MCF-7, and 11.11 fold in HepG2 cell lines as compared to the untreated control (p < 0.022). In addition, rhein treatment showed down-regulation of Bcl-2 gene and up-regulation of Bax gene in a dose-dependent manner (p < 0.03) (Figure 3-5).

The ratio of Bax to Bcl-2 expression level plays a role in determination of the process whether the cells die via apoptosis or undergo proliferation. At low concentration, rhein did not show significant changed in Bax and Bcl-2 genes expression. However, with the increase of rhein concentration, expression level of Bax gene was increased and Bcl-2 gene was decreased in all three cell lines. At IC50, Bax to Bcl-2 ratio was increased in SiHa (3.1), MCF-7 (7.0) and HepG2 (3.2) celllines.

Fig. 2: Cytotoxicity (%) trends of rhein against HepG2, MCF-7 and SiHa cell line determined by MTT assay after 48 h.

Expressional analysis of Casp-3 and -9 genes

For efficient apoptosis, caspases family genes need to activate whether the signal is initiated by the intrinsic or extrinsic apoptotic pathway. In present study, Casp-3 and -9 genes
expression was increased in a dose dependant manner in all three cell lines (Figure 6-8). At IC50, SiHa, MCF-7 and HepG2 cell lines showed up-regulation of Casp-3 gene by 2.5, 2.4 and 3.3 fold whereas Casp-9 gene by 4.3, 3.5 and 4.5 fold respectively (p < 0.028).
Cell cycle analysis
Rhein treated all three cell lines increased S-phase population and decreased G1 and G2 phase population (Figures 9). Untreated cells of SiHa, MCF-7 and HepG2 showed S-phase population of 15.58, 49.27, and 53.97% respectively. However, rhein treated increased S-phase population of 51.51, 64.01, 65.64% respectively.

Genomic DNA fragmentation
As shown in Figure 10, the DNA fragments were clearly observed as a ladder pattern in the rhein treated all three cell lines as compared to the untreated control. DNA ladder is characteristic associated with apoptotic process in which the genomic DNA is cleaved into fragments by the endogenous endonucleases.

![Fig. 10: Fragmentation of genomic DNA in untreated and treated SiHa (C1, T1), MCF7 (C2, T2), and HepG2 (C3, T3) cell lines.](image)

DISCUSSION
Rhein reported to induced cell cycle arrest [14]. DNA damage [16], inhibits MAP kinase and p-AKT [17], vascular endothelial and epidermal growth factors [25], and DNA synthesis in cancer cell lines [26]. Anti-proliferative concentration of rhein against SiHa, MCF-7 and HepG2 cell lines were acceptable range and consistent with the previous findings [27, 28]. The cytotoxicity effect of rhein against HepG2 cell lines was more than the MCF-7 and SiHa cell lines. Rhein treated SiHa, MCF-7 and HepG2 cell showed round morphology with small wrinkles and broken debris are indicative of cell death process.

The morphological change in response to rhein was correlated with previous finding in which rhein-induced alteration of the cytoskeleton and thereby affects the plasma membrane and intracellular membranes [29]. A previous report indicated that rhein induced S-phase arrest of SCC-4 cell line accompanied by the alteration of various cell cycle regulated proteins [14]. We also confirmed that the rhein decreased G1 and G2 phases and relatively increased S-phase population in order of SiHa > MCF-7 > HepG2 cells (Figure 9). The result is consistent with that rhein induced cell cycle S-phase arrest in hepatocellular carcinoma (BEL-7402) [20]. Hence, cell cycle S-phase arrest population differ in all cancer type in response to rhein could be due to cells specificity [9, 30].

The response of genes associated with apoptosis signaling pathway was evaluated at various dose of rhein (Figure 3-8). Rhein up-regulates p53, Bax genes and down-regulates Bcl-2 gene in all three cell lines. The expression level of p53 gene was high in HepG2 cell line whereas Bax gene was high in SiHa cell line. Our result is consistent with the previous findings in which rhein activated p53/p21 signaling pathway of apoptosis in CaSkI and A-549 cells [19,21] and p53 and CD95/CD95L apoptotic pathway system in Hep G2 cells [31]. Similarly, rhein induced apoptosis in breast cancer cells via activation of NF-kB and p53 signaling pathway [9]. Hence, activation of p53 gene might be played important roles in apoptosis by activation of p53 signaling pathways in SiHa, MCF-7 and HepG2 cell lines. Down regulation of Bcl-2 gene in the rhein treated cells, MCF-7 and HepG2 cell lines is also consistent with the down regulation of Bcl-2 protein in CaSkI and A-549 cell lines [19,21]. p53 gene is negative regulator of Bcl-2 gene and transcriptional activator of the Bax gene [32]. The up-regulation of p53 gene and Bax to Bcl-2 ratio resulted in the activation of cytochrome-c and caspase enzymes that induced apoptosis. The expression level Cassp-3, and -9 genes was in order of HepG2 > SiHa > MCF-7 cell lines (Figure 6-8). The SCC-4 and NCP cells also activated caspase-9 and -3 enzymes level in response to rhein [14,23]. The caspase-9 initiates the cascade of apoptosis after the release of mitochondrial cytochrome-c and activation of apaf-1 [33,34]. Since caspase-3 is the main downstream effector that plays a pivotal role in the terminal phase of apoptosis [35]. Therefore, these findings indicate that rhein induced activated Casp-9 consequently leads to the activation of the caspase cascade, such as Casp-3 and induced genomic DNA fragmentation (Figure 10) [36]. Hence, our results suggested that the rhein-induced apoptosis in SiHa, MCF-7 and HepG2 cell lines by p53 signaling pathway which activating caspase cascade. Present result and previously findings proved that rhein is a potential inhibitor of cancer cell proliferation and induced apoptosis regulatory genes.

CONCLUSION
In conclusion, the present study demonstrated that rhein inhibited cancer cell proliferation, up-regulated the p53, Bax, Casp-3 and -9 genes and down regulated the Bcl-2 gene and ultimately leads to genomic DNA fragmentation. We therefore, suggest that rhein is a natural compound might be useful in cancer drug development and chemotherapy.

DISCLOSURE
The authors have no any conflicts of interest in this work.

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REFERENCES


