Journal of Advanced Zoology
ISSN : 0253 –7214
Volume 44 Issue S-5 Year 2023 Page 3260 : 3268

Studies On 3/7 Caspase Activity And Apoptosis Induction By Diarylheptaniods Isolated From Garuga Pinnata Roxb

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Article History

Received: 25/10/2023
Revised: 23/12/2023
Accepted: 29/12/2023

Abstract

This study aims to evaluate the stimulation of caspase 3/7, 8 and 9 activity and induction of apoptosis by the diarylheptanoids isolated from Garuga *pinnata* (G. *pinnata*) RoxB. Garuganin 1, 3, 4 and 5 which were previously reported for their isolation have tested for their anticancer potencies by different caspase activation and apoptosis induction in MCF-7 and HCT-15 cell lines. However, based on the MTT assay results (Previously reported) Garuganin 3 and 5 were selected for this study. from the stem bark of G. *pinnata*. The activation of caspases 3/7, 8, and 9 is a conformational process of cancer cell death. Such activation of caspases by different concentrations (05, 10, 15, 20μg/ml) of the isolated compounds Garuganin 3 and 5 have been evaluated. According to the results, both the compound significantly initiated the activation of 3/7, 8, and 9 caspases at 05, 10 and 15μg/ml and slightly dropped at 20 μg/ml of the compound concentration. Garuganin 5 resulted high 3/7 and 8 caspase activity percentage in HCT-15 cells with 81.3% and 77.8% respectively noted at 15 μg/ml of compound concentration. The 3/7 and 8 caspase activity percentage of Garuganin 5 in MCF-7 showed 79.3% and 74.0% respectively recorded at 15μg/ml of compound concentration. On the other hand, Garuganin 3 noted 72.9% and 66.5% of 3/7 and 8 caspase activity percentage in HCT-15 cells respectively noted at 15μg/ml of compound concentration. On the other hand, Garuganin 3 and 5 tested for apoptosis induction in the HCT-15 and MCF-7 cells noticed significant cell death in the early and late apoptotic stages. The results were concentration dependent (05, 10, 15, 20μg/ml). Garuganin 5 at 15μg/ml is highly active against HCT-15 in the early and late apoptosis with 18.5% and 33.5% apoptosis induction percentage respectively. Following, the apoptotic induction percentage of Garuganin 5 was found significant against MCF-7 in the early and late apoptosis with 14.5% and 28.0% apoptotic induction percentage respectively.

Keywords: Diarylheptanoids, Garuganin 3, Garuganin 5, caspase, Apoptosis, MCF-7, HCT-15

1.0 INTRODUCTION

Caspases are the *c*ysteine-*asp*artic prote*ases*, immensely play significant role in different mechanisms of cell death and as well inflammation. The regulatory pathways of caspases during cell death were identified with the discovery of *ced-3* (an executioner of cell death) (Ellis and Horvitz, 1986) [1]. Following, interleukin-1-beta-converting enzyme (ICE) and Nedd2 are the mammalian homologs of *ced-3* to be identified and renamed as caspase-1, (Yuan *et al*., 1993)[2] and caspase-2 (Kumar *et al*., 1992)[3] respectively. There are 18 mammalian caspases that are recognised till today (Eckhart *et al*., 2008) [4]. However, with the exception of caspase-16, placental animals lack the recently discovered caspases-15, -17, and -18. It's also vital to remember that mice lack caspase-5. Moreover, the murine and bovine orthologues of caspase-4 recognized are caspase-11 and -13 (Eckhart *et al*., 2008) [4]. In humans, caspase-12 is present in both truncated and full-length alleles, whereas in rodents, it is a full-length caspase (Fischer *et al*., 2002) [5]. Caspase-14 is primarily involved in cornification and protecting the skin's deeper layers, and it is expressed in the epidermis (Hoste *et al*., 2011) [6].

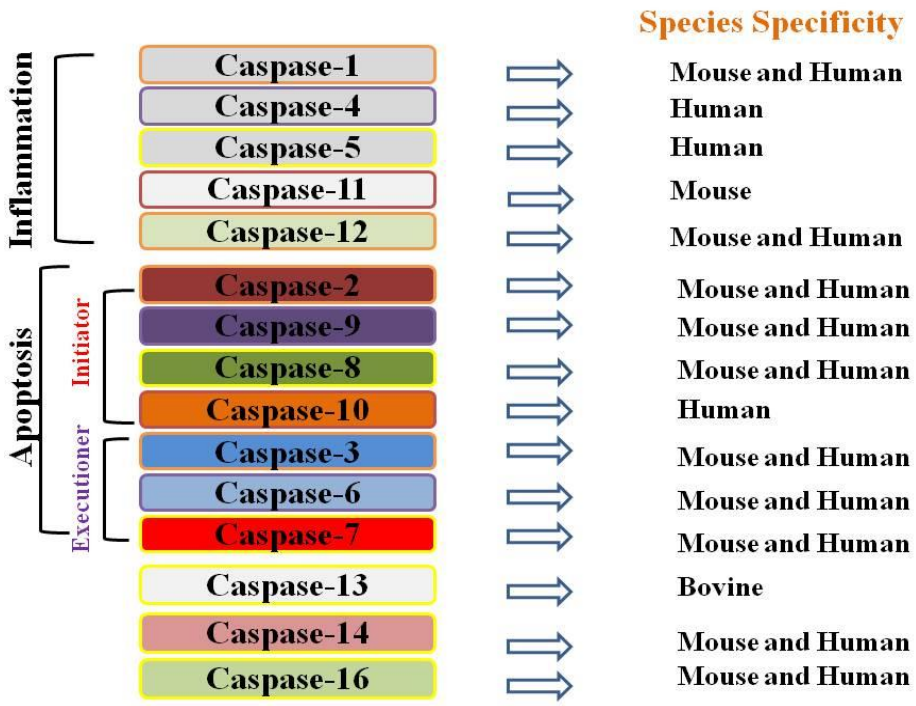

Fig 1 Classification of placental mammalian caspases.

The activation pathways of caspases is most important, Caspases may activate in two different pathways such as Induced Proximity Model and Mitochondrial Pathway. In accordance to Induced Proximity Model, the caspase 8 is activated by Death Inducing Singling Complex (DISC) and caspase 9 is activated at Apoptosome complex (Schulze-Osthoff *et al*., 1998) [7]. Caspase activation can begin at a variety of entrance points, including the plasma membrane via the death receptor route or the mitochondria (via the mitochondrial pathway) (Hengartner, 2000) [8].

The present investigation is an extension of our previously reported work (Srilekha *et al*., 2023) [9]. The objective of this investigation include the evaluation of 3/7, 8 and 9 caspase activation and induction of apoptosis mechanism by the diarylheptanoids isolated from stem bark of G. *pinnata*.

2.0MATERIAL AND METHODS

2.1 CASPASES 8, 9, 3/7 ASSAY

Based on the significant anti-proliferative activity of Garuganin 3 and Garuganin 5 (Srilekha *et al*., 2003) [9] these isolated compounds were further evaluated for their 3/7, 8, and 9 caspase activation. The caspase 3/7, 8, and 9 assay was performed based on the method instructions found in the manufacturer's kits. Briefly, add 100 μL approx (5 × 104 HCT15 and MCF-7 cells/mL) were transferred to 96-well microtiter plates and maintained in incubator for 24 h. Compounds at different concentrations (05, 10, 15 and 20 μg/mL) are transferred to the cells and incubated for 24 h. Add Caspase-Glo 3/7, 8, and 9 reagents and plates were

agitated gently for 30 sec. After one hour of incubation luminescence signal was recorded using GloMax-Multi Detection System.

2.2 EFFECT OF DIARYL HEPTANOIDS COMPOUNDS ON EARLY AND LATE STAGE APOPTOTIC CELL POPULATIONS

Apoptotic cells were detected using a FITC Annexin V Apoptosis Detection Kit ((BioLegend, CA, USA), according to the manufacturer's instructions. Briefly, 24 h after treatment with JPCF, floating and adherent cells were collected from three wells and washed with cold PBS. The cell pellet was resuspended in 1× binding buffer (1 × 106 cells/mL). The cells were then stained with 5 µL of FITC Annexin V and 5 µL PI and incubated for 15 min in the dark. Later, 400 µL of binding buffer was added, and cells were examined immediately after staining (within an hour) using a FACScan flow cytometer (Cytomics FC 500; Beckman Coulter, Brea, CA, USA).

3.0 RESULT

3.1 CASPASES 8, 9, 3/7 ASSAY

The activation of caspases 3/7, 8, and 9 is a conformational process of cancer cell death. Such activation of caspases by different concentrations (05, 10, 15, 20μg/ml) of the isolated compounds Garuganin 3 and 5 have been evaluated. According to the results, both the compound significantly initiated the activation of 3/7, 8, and 9 caspases at 05, 10 and 15μg/ml and slightly dropped at 20 μg/ml of the compound concentration. Garuganin 5 resulted high 3/7 and 8 caspase activity percentage in HCT-15 cells with 81.3% and 77.8% respectively noted at 15 μg/ml of compound concentration. The 3/7 and 8 caspase activity percentage of Garuganin 5 in MCF-7 showed 79.3% and 74.0% respectively recorded at 15μg/ml of compound concentration. On the other hand, Garuganin 3 noted 72.9% and 66.5% of 3/7 and 8 caspase activity percentage in HCT-15 cells respectively noted at 15μg/ml of compound concentration. On the other side, the 3/7 and 8 caspase activity percentage of Garuganin 3 in MCF-7 showed 67.5% and 59.7% respectively recorded at 15μg/ml of compound concentration. The tested compounds (Garuganin3 and 5) showed minimum activation of caspase 9 activity. Garuganin 5 noted 44.8% and Garuganin 3 resulted 38.6% of caspase 9 activation in HCT-15 cells noted at 15μg/ml of compound concentration. Following, the caspase 9 action by Garuganin 5 and 3 in MCF-7 cell found average with 32.9% and 27.3% respectively (Table1.1 and Fig 1.2-1.8)

Table 1.1 Activation of caspases3/7,8 and 9 by Garuganin 3 and 5 in HCT-15 and MCF-7 cell lines

Compounds	Con (μg/ml)	HCT-15						MCF-7					
		3/7 Caspase activity		8 Caspase activity		9 Caspase activity		3/7 Caspase activity		8 Caspase activity		9 Caspase activity	
		T	UT	T	UT	T	UT	T	UT	T	UT	T	UT
Garuganin-3	05	27.2	--	21.6	--	15.5	--	24.5	--	18.2	--	23.1	--
	10	41.8	--	49.9	--	32.9	--	40.7	--	31.3	--	24.5	--
	15	72.9	--	66.5	--	38.6	--	67.5	--	59.7	--	27.3	--
	20	63.4	--	55.1	--	27.6	--	60.8	--	53.1	--	25.6	--
Garuganin-5	05	31.2	--	25.5	--	20.2	--	28.1	--	23.6	--	26.7	--
	10	46.9	--	42.8	--	35.8	--	44.5	--	35.1	--	29.1	--
	15	81.3	--	77.8	--	44.8	--	79.3	--	74.0	--	32.9	--
	20	77.6	--	70.2	--	40.9	--	72.5	--	70.6	--	30.8	--
T-Treated with isolated compounds; UT-Untreated (Negative control)													

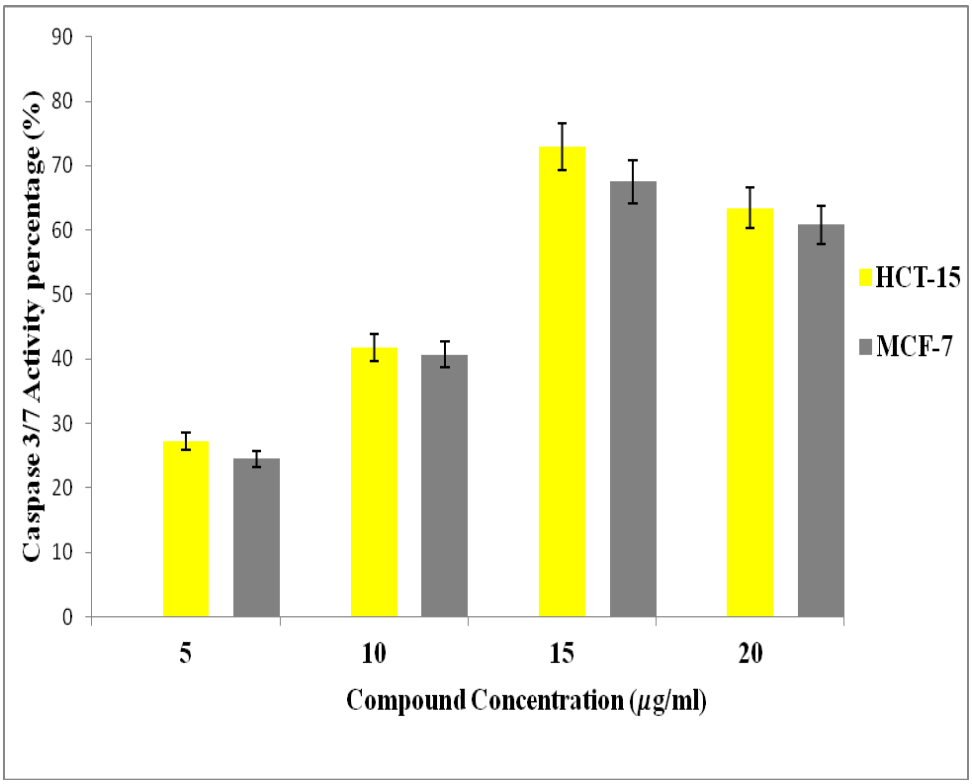

Fig 1.2 Caspase 3/7 activation by the compound Garuganin-3 in HCT-15 and MCF-7 cancer cell lines

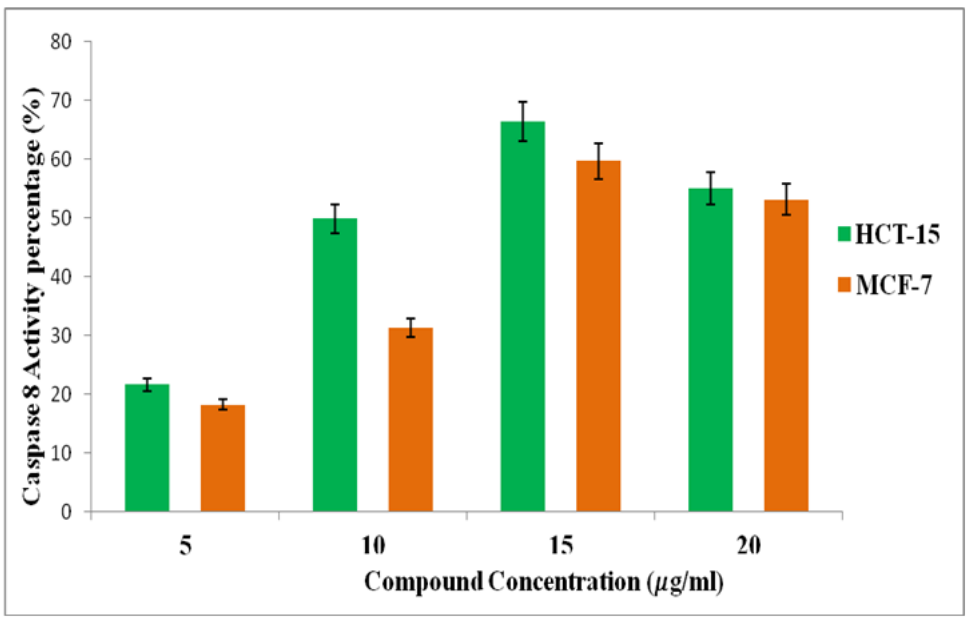

Fig 1.3 Caspase 8 activation by the compound Garuganin-3 in HCT-15 and MCF-7 cancer cell lines

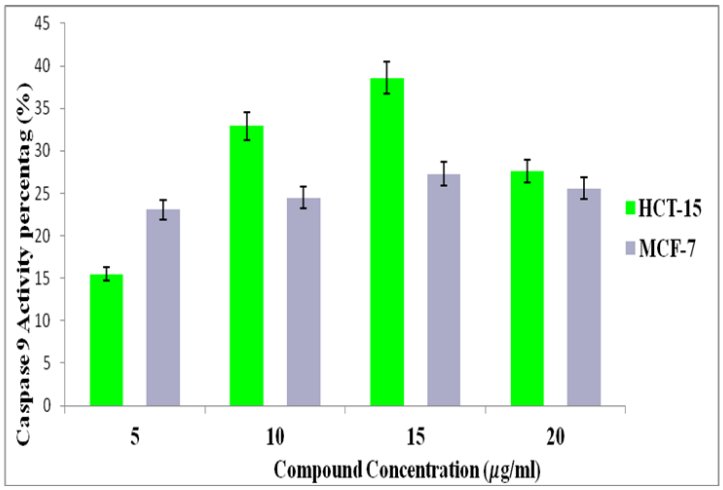

Fig 1.4 Caspase 9 activation by the compound Garuganin-3 in HCT-15 and MCF-7 cancer cell lines

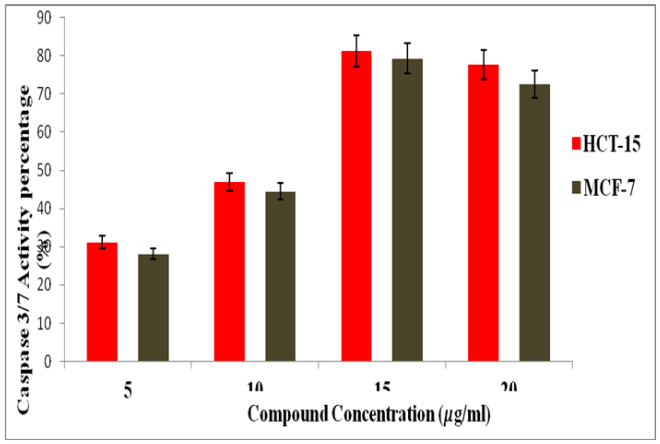

Fig 1.5 Caspase 3/7 activation by the compound Garuganin-5 in HCT-15 and MCF-7 cancer cell lines

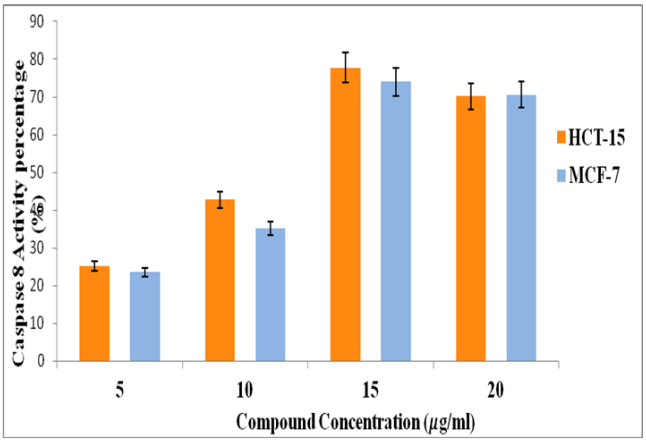

Fig 1.6 Caspase 8 activation by the compound Garuganin-5 in HCT-15 and MCF-7 cancer cell lines

3.2 EFFECT OF DIARYL HEPTANOIDS COMPOUNDS ON EARLY AND LATE STAGE APOPTOTIC CELL POPULATIONS

Garuganin 3 and 5 tested for apoptosis induction in the HCT-15 and MCF-7 cells noticed significant cell death in the early and late apoptotic stages. The results were concentration dependent (05, 10, 15, 20μg/ml). Garuganin 5 at 15μg/ml is highly active against HCT-15 in the early and late apoptosis with 18.5% and 33.5% apoptosis induction percentage respectively (Table 1.3 and Fig. 1.8). Following, the apoptotic induction percentage of Garuganin 5 was found significant against MCF-7 in the early and late apoptosis with 14.5% and 28.0% apoptotic induction percentage respectively (Table 1.3 and Fig 1.8). On the other hand, Garuganin 3 showed significant against HCT-15 in the early and late apoptosis with 15.5% and 29.5% apoptosis induction percentage respectively (Table 1.2). The apoptotic induction percentage of Garuganin 3 was found significant against MCF-7 in the early and late apoptosis with 12.5% and 25.5% apoptotic induction percentage respectively (Table 1.2).

However, the apoptotic activity of the Garuganin3 and 5 was dropped at 20μg/ml against tested cell lines. Garuganin 5 at 20μg/ml is slightly active against HCT-15 in the early and late apoptosis with 14.0% and 26.5% apoptosis induction percentage respectively (Table 1.3). Following, the apoptotic induction percentage of Garuganin 5 was also found average against MCF-7 in the early and late apoptosis with 13.5% and 10.0% apoptotic induction percentage respectively (Table 1.3). On the other side, Garuganin 3 was weakly active against HCT-15 in the early and late apoptosis with 10.5% and 24.0% apoptosis induction percentage respectively (Table 1.2). The apoptotic induction percentage of Garuganin 3 was also averagely active against MCF-7 in the early and late apoptosis with 10.5% and 8.5% apoptotic induction percentage respectively (Table 1.2). The activity of Garuganin 3 and 5 at 05μg/ml was found least against HCT-15 and MCF-7 cells (Table 1.2 and 1.3).

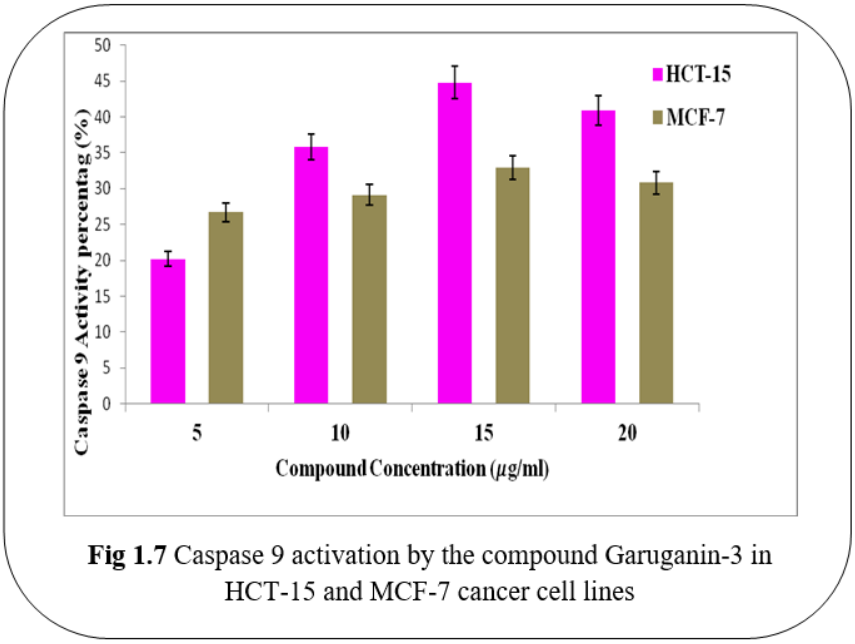

Fig 1.7 Caspase 9 activation by the compound Garuganin-3 in HCT-15 and MCF-7 cancer cell lines

Table 1.2 Induction of Apoptosis by Garuganin 3 in HCT-15 and MCF-7 cell lines

	Conc (μg/ml)	HCT-15				MCF-7			
		A1	A2	A3	A4	A1	A2	A3	A4
Garuganin-3	05	4.0	3.5	81	11.5	2.0	3.5	86	8.5
	10	5.5	8.5	71	15.0	4.0	6.0	79.5	10.5
	15	25	29.5	30	15.5	21	25.5	41	12.5
	20	20.5	24.0	45	10.5	13	8.5	68	10.5
A1-Necrosis, A2-Late Apoptosis, A3-Viable, A4-Early Apoptosis									

Table 1.3 Induction of Apoptosis by Garuganin 5 in HCT-15 and MCF-7 cell lines

	Conc (μg/ml)	HCT-15				MCF-7			
		A1	A2	A3	A4	A1	A2	A3	A4
Garuganin-5	05	5.5	7.5	72	15.0	3.0	5.5	81	10.5
	10	8.0	10.0	61.5	20.5	5.5	7.5	75	12.0
	15	30	33.5	18	18.5	25	28.0	32.5	14.5
	20	22.5	26.5	37	14.0	15.5	10.0	61	13.5
A1-Necrosis, A2-Late Apoptosis, A3-Viable, A4-Early Apoptosis									

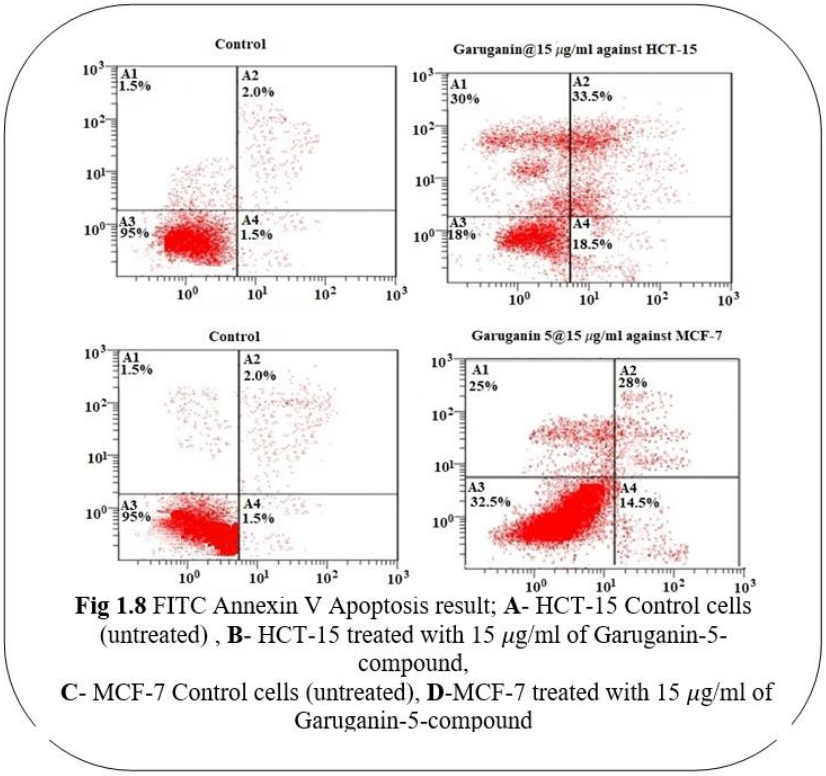

Fig 1.8 FITC Annexin V Apoptosis result; **A**- HCT-15 Control cells (untreated) , **B**- HCT-15 treated with 15 μg/ml of Garuganin-5-compound,
C- MCF-7 Control cells (untreated), **D**-MCF-7 treated with 15 μg/ml of Garuganin-5-compound

4.0 DISCUSSION

This study was framed out to evaluate two main objectives of the Garuganin 3 and 5 Diarylheptanoids isolated from the stem bark of G. *pinnata*. The first objective includes the activation of caspase 3/7, 8 and 9. Second objective includes the determination of apoptosis. The study was carried out using HCT-15and MCF-7 cancer cells. In accordance to the results, both the compounds exhibited concentration dependent activation of tested caspases and as well induction of significant percentage of apoptosis in the selected cancer cells. However, the exponential raise of the caspase activation or apoptotic induction was observed from 05 to 15μg/ml and finally, dropped at 20μg/ml concentration of the Garuganin 3 and 5 compounds. Caspase activation and apoptotic induction is an integrated process involved in the targeting in the tumour cell death. For instance, caspase-3 is an important component in certain apoptosis pathways and as well essential for the B-cell homeostasis regulation. Caspase-8 a mammalian family caspase has been found to involve in death-receptor-induced apoptosis. Moreover, this caspase possess dual role in Apoptosis and as well Necrosis. In addition, caspase-8 also plays a vital role in extrinsic apoptosis. Stress induced apoptotic pathway is mainly concerned to caspase-12 which regulates ER-specific apoptosis pathway.

It has been demonstrated that the activation of apoptosis in the target cells is the mechanism by which a variety of cytotoxic treatments, such as anticancer medicines, beta-irradiation, suicide genes, or immunotherapy, kill tumor cells (Debatin, 1997; Herr and Debatin, 2001) [10-11]. Under specific physiological and pathological conditions, separate, intrinsic cell death pathways are activated, leading to apoptosis, also known as programmed cell death (Hengartner, 2000) [8]. Only imperfectly known is the underlying mechanism that causes cytotoxic therapy to trigger an apoptotic response in response to varied stimuli. However, it appears that some inducers frequently cause damage to DNA or to other important molecules and/or subcellular structures as a first strike, which is then spread via the cellular stress response (Hengartner *et al*., 2000) [8]. A number of stress-inducible molecules, such as JNK, MAPK/ERK, NF-B, or

ceramide, may have a significant impact on the pathways leading to apoptosis (Leppa and Bohmann, 1999; Davis, 2000) [12-13]. Contrarily, substances like granzyme B, which is released by cytotoxic T cells or natural killer (NK) cells, can directly activate a cell's own apoptosis effector mechanisms (Hengartner, 2000) [8]. The classic morphological and molecular signs of apoptosis include membrane blebbing, cell shrinkage, and nuclear DNA fragmentation (Hengartner, 2000) [8]. Caspases and other proteolytic enzymes are crucial effector molecules in apoptosis, including cytotoxic therapy-induced cell death (Kaufmann, 1989; Earnshaw *et al*., 1999) [14-15].

Now-a-days, rapid emergence of multi drug resistant cancer cells causes severe threat in the field of cancer therapy. There is an urgent alarm for the development or the discovery of the drugs as been gain the movement. In addition to the multi drug resistance, the side effects and cost effective of the chemically derived drugs are also in the view of drug discovery. Plants are generally considered as factories for therapeutic compounds with safe and affordable. In this context, the present investigation is focussed to evaluate the caspase activation and apoptotic induction by the isolated compounds (Garuganin 3 and 5) from G. *pinnata* stem bark. Basing on the results we confirmed that Garuganin 5 and 3 are most significant in activation of caspase 3/7 and caspase 8. These are most important caspase which play important role as executioner in the cancer therapy. Thus results suggest that, the isolated compounds can be further evaluated for different tests that are essential for drug discovery against human breast and collateral cancer.

5.0 CONCLUSION

The results suggest that the diaryheptanoids (Garuganin3 and Garuganin 5) isolated from G. *pinnata* are potent against HCT-15 and MCF-7 cancer cells. However, Garuganin 5 exhibited higher caspase and apoptotic induction comparing to Garuganin

ACKNOWLEDGEMENTS

The authors are grateful to the Chancellor, Chaitnya Deemed to be University, for his cooperation and encouragement and also thankful to the Department of Biotechnology, NITW for providing the facilities for this research work.

CONFLICT OF INTEREST

The authors disclose no conflict of financial or nonfinancial interest.

ETHICAL STATEMENTS

This study does not involve any human beings or animals.

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