In Vitro Anticancer Activity Of Quercetin Isolated From Carmona retusa (Vahl.) Masam on Human Hepatoma Cell Line (Hepg2)

Chandrappa C.P.1, Govindappa M.1, Anil Kumar N.V.2, Channabasava R.1, Sadananda T. S.1 and Sharanappa P.3

1Natural Product Laboratory, Department of Biotechnology, Shridive Institute of Engineering and Technology, Tumkur, Karnataka, India. 
2Department of Chemistry, Manipal Institute of Technology, Manipal, Karnataka, India. 
3Department of Studies in Biosciences, Hemagangothri, University of Mysore, Hassan, Karnataka, India.

Abstract: Quercetin was isolated from an ethanol extract of Carmona retusa (Vahl.) Masam and was analyzed for its anticancer activity on HepG2 cell lines by MTT assay, Hoechst 33342 staining and Caspase-3 colorimetric assay. Plant extract yield of flavonoid quercetin from Carmona retusa (Vahl.) Masam showed significant and concentration-dependent anticancer activity at 100 μg/ml and 80 μg/ml doses after 24 and 48 h of treatment on HepG2 cell line in MTT assay, significant cell apoptosis have shown at 53μg/ml concentration of extract yield of flavonoid quercetin from Carmona retusa (Vahl.) Masam in Hoechst 33342 staining and a significant activation of caspase-3 observed at 100 μg/ml of extract yield of flavonoid quercetin from Carmona retusa (Vahl.) Masam after 24 h and even 48 h of incubation. Our findings suggesting that the plant extract yield of flavonoid quercetin from Carmona retusa (Vahl.) Masam could be used as a promising anticancer agent.

Key Words: Carmona retusa, anticancer, HepG2 cell lines, MTT assay, Hoechst 33342, Caspase-3 colorimetric assay.

I. Introduction

The plants that possess therapeutic properties and exerting beneficial pharmacological effects on the animal body are generally designated as medicinal plants [1]. Plants have a long history of use in the treatment of cancer and they have played a vital role as a source of effective anticancer agent. It is significant that over 60% of currently used anticancer agents are derived, in one way or another, from natural sources, including plants, marine organism, and microorganisms [2]. The use of bioassay offers various scientific strategies like screening of extracts, fractions and compounds obtained from plants, which are often used in phytochemical research [3].

It has been shown that quercetin treatment caused cell cycle arrests such as G2/M arrest or G1 arrest in different cell types. Moreover, quercetin-mediated apoptosis may result from the induction of stress proteins, disruption of microtubules and mitochondrial, release of cytochrome c and activation of caspases [4]. Quercetin is the principal flavonoid compound (3, 30, 40, 5, 7-penta hydroxyl flavanone) commonly extracted from cranberries, blueberries, apple and onions. It possesses a wide spectrum of biopharmacological properties and may offer promising new options for the development of more effective chemopreventive and chemotherapeutic strategies because of its powerful antioxidant and free-radical scavenging properties. Quercetin treatment has been associated with selective antiproliferative effects and induction of cell death, probably through an apoptotic mechanism, in breast or other cancer cell lines but not in normal cells [5].

Quercetin is able to regulate cell cycle by directly binding several molecular targets and depending on the cell type and tumor origin, it blocks the cell cycle at G2/M or at the G1/S transition. At the G1/S transition, quercetin blocks cell-cycle progression through the up-regulation of p21 and p27 and p53. p53 exerts an inhibitory activity on several CDKs. In particular, p21 inhibits CDK2-cyclin E, with the consequent inhibition of CDK2-dependent phosphorylation of pRb and the sequestration of E2F1, thus inhibiting gene transcription induced by E2F1 and progression into and through S phase. p21 also inhibits CDK2-cyclin A and CDK1-cyclin B, which are essential for progression through S Phase and G2, respectively. p27 exerts several effects on cell cycle, but only under certain conditions it can inhibit the complexes CDK4-cyclin D and CDK6-cyclin D. The tumor suppressor p53, once activated, can induce several different cellular responses, including growth arrest and apoptosis. Growth arrest is essentially elicited through the up-regulation of the genes that encode for inhibitors of cell-cycle progression, including p21 and p27. In different cellular models, quercetin stabilizes p53 both at mRNA and protein levels. Apart from blocking cell growth through the direct action on key modulators of cell cycle, quercetin is able to induce apoptosis via mitochondrial pathway: indeed, quercetin can disrupt
MMP (Mitochondrial Membrane Potential), which in turn provokes the release of cytochrome c in the cytoplasm, a phenomenon that activates multiple caspases, such as caspase-3 and -7 [6]. The overall effects of quercetin on molecular cell cycle are shown in Fig 1.

Apoptosis is a physiologic intracellular process involving a well-ordered signaling pathway that leads to cell death and clearance of the dead cells by neighboring phagocytes, without inflammation. Cytotoxic drug-induced damage to the cells, especially in the DNA, triggers apoptosis through two signaling mechanisms, the activation and release of mitochondrial pro-apoptotic proteins known as caspases under the control of a Bcl-2 family of proteins or up-regulated expression of pro-apoptotic receptors on cancer cells, whose subsequent interaction with their legends activates apoptotic signaling pathways. These receptors include the Fas (also called APO-1 or CD95) and TNF (Tumor Necrosis Factor - related apoptosis-inducing ligands (TRAIL) receptors [7].

Literature survey is suggesting that there is no any report on the effects of quercetin isolated from Carmona retusa and number of liver cancer cases is reported in India [8]. Hence, it is aimed to be evaluating the anticancer activity of extract yield of flavonoid quercetin from Carmona retusa on HepG2 cell line in our study.

II. Materials And Methods

2.1 Plant Material

Fresh stem of Carmona retusa was collected from Hemagangothri Campus, Hassan district of Karnataka, India. The collected plant was authenticated with herbarium, Government Ayurvedic College, Mysore, India and Department of Studies in Botany, University of Mysore, Mysore. The collected stem was cleaned with deionized water and dried under shade for two weeks at room temperature (26±2°C). Dried stem was grounded and filtered using 0.3mm mesh

2.2 Preparation Of Sample

S solvent system used for the extraction was ethanol. Flask extraction procedure was adapted for extraction. 25g of the powdered stem sample was soaked in the conical flask containing solvent, wrapped with aluminum foil and placed in shaker for 48h at 120-130 rpm. After 48h, the extract was filtered using Whatman filter paper No 1. Concentrated the solvent extract in an air circulating oven at 54°C until total dryness

2.3. Chemicals And Instruments

MTT [3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide], Dulbecco's Modified Eagle Medium), DMSO, doxorubicin, Hoechst 33342 (2'-[4-ethoxyphenyl]-5-[4-methyl-1-piperazinyl]-2,5'-bi-1H-benzimidazoletrihydrochloride trihydrate), human hepatoma cell lines (HepG2), cell lysis buffer, 1X assay buffer, Caspase-3 Substrate (Ac-DEVD-pNA), pNA standard and florescent microscope.

2.4 Mtt Assay

MTT [3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] based cytotoxicity assay was first described by Mosmann in 1983[10]. This colorimetric assay is depends on the ability of a mitochondrial dehydrogenase enzyme of viable cells to cleave the tetrazolium rings of the pale yellow MTT and form dark blue formazan crystals which is largely impermeable to cell membranes, thus resulting in its accumulation within healthy cells. The number of surviving cells is directly proportional to the level of the formazan product created [11].

Plant extract yield of flavonoid quercetin from Carmona retusa have prepared in the following concentrations (triplicates for 24 h and 48 h were set for each concentration), (1) 100µg, (2) 80 µg, (3) 60 µg, (4) 40 µg, (5) 20 µg, (6) 10 µg, (7) 5 µg, (8) positive control (doxorubicin- 50 µg) (9), vehicle control -5% DMSO and (10) media control.

2.5 Hoechst 33342 Staining

Hoechst stains are part of a family of blue fluorescent dyes used to stain DNA [12, 13]. Hoechst 33342(2’-[4-ethoxyphenyl]-5-[4-methyl-1-piperazinyl]-2,5’-bi-1H-benzimidazoletrihydro chloride trihydrate) is a cell-permeable DNA stain that is excited by ultraviolet light and emits blue fluorescence at 460 to 490nm. Hoechst 33342 binds preferentially to adenine-thymine (A-T) regions of DNA. This stain binds into the minor groove of DNA and exhibits distinct fluorescence emission spectra that are dependent on dye: base pair ratios. Hoechst 33342 is used for specifically staining the nuclei of living or fixed cells and tissues. This stain is commonly used in combination with 5-bromo-2-deoxyuridine (BrDU) labeling to distinguish the compact chromatin of apoptotic nuclei, to identify replicating cells and to sort cells based on their DNA content [14].
2.6 Caspase-3 Colorimetric Assay
Caspase-3 colorimetric assay was carried out at Raghavendra Biotechnologies, Bangalore, Karnataka, India. Caspase-3, also known as CPP-32, Yama or Apopain is an intracellular cysteine protease that exists as a proenzyme, becoming activated during the cascade of events associated with apoptosis. Caspase-3 cleaves a variety of cellular molecules that contain the amino acid motif DEVD (The amino acid sequence Asp-Glu-Val-Asp) such as poly ADP-ribose polymerase (PARP- involved in apoptosis), the 70 kD protein of the U1-ribonucleoprotein and a subunit of the DNA dependent protein kinase. The presence of caspase-3 in cells of different lineages suggests that caspase-3 is a key enzyme required for the execution of apoptosis [15, 16, 17].

Caspase-3 colorimetric activity assay kits provide a simple and convenient means for assaying the activity of caspases that recognize the sequence DEVD. The assay is based on spectrophotometric detection of the chromophore p-nitroaniline (pNA) after cleavage from the labeled substrate DEVD-pNA. The free pNA can be quantified using a spectrophotometer or a microtiter plate reader at 405 nm. Comparison of the absorbance of pNA from an apoptotic sample with an uninduced control allows determination of the fold increase in caspase-3 activity [18].

III. Results And Discussion

3.1 MTT Assay
From MTT assay, after treatment with various concentrations of extract yield of flavonoid quercetin from Carmona retusa, parameters such as cell viability and percentage cytotoxicity were compared with untreated cells (vehicle). The photographs of anticancer activity of extract yield of flavonoid quercetin from Carmona retusa at various concentrations are presented in Fig 2 and 3. Decrease in cell viability and increase in cytotoxicity by extract yield of flavonoid quercetin from Carmona retusa was observed on HepG2 cell lines in a dose dependent manner, but a significant increase in cytotoxicity was observed for 100 µg/ml and 80 µg/ml doses of extract yield of flavonoid quercetin from Carmona retusa after 24 and 48 h of treatment on HepG2 cell line. Extract yield of flavonoid quercetin from Carmona retusa has shown maximum percentage of the cell death up to 96 % and 94 % after 24 and 48 h of treatment respectively. The least percentage of the cell death was observed at 5 µg/ml concentration of extract yield of flavonoid quercetin from Carmona retusa and it was only 7%. The IC₅₀ values of extract yield of flavonoid quercetin from Carmona retusa were found to be 53.07µg/ml and 54.31µg/ml for 24 and 48 h of incubation respectively and are presented graphically represented in Fig 4 and 5. There is no significant variation in % of the cell death at 24 and 48 h of incubation and hence it can be considered the 24 h of incubation for further work.

In HepG2 human hepatoma cells, quercetin blocks cell-cycle progression at the G1 phase, and exerts this effect through the increase of p21 and p27 and p53 [6]. Collectively, the proapoptotic effects of quercetin may result from multiple pathways. First, in MDA-MB-231 cells (breast adenocarcinoma), quercetin treatment increases cytosolic Ca²⁺ levels and reduces the MMP, thus promoting activation of caspase -3, -8 and -9 [19].

Many of the workers have followed the MTT assay to evaluate the anticancer activity of natural products such as hydroalcoholic extract of Tabernaemontana diversicarie on Hela cell lines [20], the crude extract and fractions of Viburnum foetens are evaluated against human breast and colorectal cancer cell lines [21], extracts of Rubia cordifolia, Plumbago zeylanica and Calophyllum inophyllum against MCF-7 and HT-29 cell lines [22], resveratrol isolated from grapes against HL60 cells and normal PBL.[23].

3.2 Hoechst 33342 Staining
HepG2 cells treated with 53µg/ml of extract yield of flavonoid quercetin from Carmona retusa were examined by fluorescence microscopy after Hoechst 33342 staining to evaluate the effect of quercetin on the induction of apoptosis. As depicted in Figure 6, the cells showed marked morphological changes such as condensed and fragmented chromatin and the formation of apoptotic bodies after treatment for 24 h and 48 h. Quercetin may be a potential chemo preventive or therapeutic agent in hepatocarcinoma cells and further efforts to investigate these possibilities are needed [24]. Quercetin-mediated apoptosis may result from the induction of stress proteins, disruption of microtubules and mitochondrial, release of cytochrome c and activation of caspases [25, 26, 27].

Most of the workers have applied the Hoechst 33342 staining earlier to screen the novel anticancer agents. Antiproliferative and proapoptotic activities of triptolide (PG490) have been reported against human prostatic epithelial cells by Hoechst 33342 staining [28], anticancer property of cisplatin have evaluated against NCI-H460 lung cancer cells in vitro [29].

3.3 Caspase-3 Colorimetric Assay
The determination of anticancer activity of natural products by caspases assay is an important biochemical feature in apoptotic signaling and further determination to investigate whether the apoptosis was induced by the extracts [30]. We checked the effect of quercetin on the cascade of caspases that are crucial

www.iosrjournals.org
In vitro anticancer activity of quercetin isolated from Carmona retusa (Vahl.) Masam on... initiators in the cell death pathways. Since caspase-3 is the main downstream effector caspase that is present in most cell types and plays an important role in the execution of apoptosis cell death by cleaving the cellular substrates [31], we then compared treated and untreated control cells in order to ensure the effect of extract yield of flavonoid quercetin from Carmona retusa. Enzymatic activity of caspase-3 was increased gradually after 24 h and even 48 h of incubation as the concentration increased (5-100 µg/ml) and it is graphically represented Fig 7 and 8. A significant activation of caspase-3 observed at 100 µg/ml of extract yield of flavonoid quercetin from *Carmona retusa* after 24 h and even 48 h of incubation.

Quercetins that significantly decrease HepG2 cell viability have similar effects on other cancer cell cultures such as leukemia, human colon, prostate, breast, lung cancer cells, and murine hepatoma [32]. The result showed that caspase-3 was significantly activated in the treated cells. Moreover, the activity was blocked by the broad range caspase-3 inhibitor (Ac-DEVD-CHO), suggesting that the apoptosis signaling induced by the extracts was a caspase-dependent pathway. Due to the defect in the apoptosis process in normal cells causes normal cells to turn into immortalized cancer cells. Hence, the induction of apoptosis in cancer cells is an important step that is expected for the development of novel anticancer drug [30]. Anticancer activity of zerumbone, a natural compound isolated from Zingiber zerumbet evaluated by caspase - 3 assay [33] and berberine, a natural product against human prostate carcinoma cells [34].

### IV. Figures

![Fig 1: Effects of quercetin on molecular events of cell cycle (Courtesy: www.hindawi.com)](image)

Fig 1: Effects of quercetin on molecular events of cell cycle (Courtesy: www.hindawi.com)

![Fig 2 A): Effect of Media on HepG2 Cell line.  B) Effect of Vehicle (5 % DMSO) on HepG2 Cell line](image)

Fig 2 A): Effect of Media on HepG2 Cell line.  B) Effect of Vehicle (5 % DMSO) on HepG2 Cell line
**In vitro anticancer activity of quercetin isolated from Carmona retusa (Vahl.) Masam on...**

**Fig 3:** Antitumor activity of extract yield of flavonoid quercetin from *Carmona retusa* on HepG2 cells at various concentrations. A) 100 µg, B) 80 µg, C) 60 µg, D) 40 µg, E) 20 µg, F) 10 µg and G) 5 µg.

**Fig 4:** Antitumor activity of extract yield of flavonoid quercetin (µg/ml) from *Carmona retusa* on HepG2 cells at various concentrations after 24 h incubation.

**Fig 5:** Antitumor activity of extract yield of flavonoid quercetin (µg/ml) from *Carmona retusa* on HepG2 cells at various concentrations after 48 h incubation.

**Fig 6:** Treated cells were stained with nuclear Hoechst 33342 and visualized under a fluorescence microscope after a 24 h and 48 h treatment. Red arrows indicate apoptotic bodies. Representative areas were photographed with 200X magnification. (a) – 24 h incubation, (b) – 48 h incubation and (c) – control.
In vitro anticancer activity of quercetin isolated from Carmona retusa (Vahl.) Masam on HepG2 cell lines

V. Conclusion
In the present work, the quercetin was evaluated for its anticancer activity against HepG2 cell lines. Quercetin have shown a significant effect in all the assays applied in the study. Hence, the plant extract yield of flavanoid quercetin could be used as anticancer agent which required further in vivo studies.

Acknowledgment
Authors are thankful to Dr. M.R.Hulinaykar, Managing Trustee, Sri Shridevi Charitable Trust (R.), Tumkur and Dr. K.Sukumaran, Principal, Shridevi Institute of Engineering and Technology, Tumkur for providing laboratory facilities. Authors are acknowledged to Dr. P. Sharanappa, Assistant Professor, Department of Studies in Biosciences, University of Mysore, Hassan for identification and collection of our plant.

References
In vitro anticancer activity of quercetin isolated from Carmona retusa (Vahl.) Masam on...