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A COMPARATIVE EVALUATION OF FRUITS OF FOLKLORE MEDICINAL PLANTS – EFFECT ON HEP G2 AND 3T3-L1 FIBROBLAST CELLS AND POSSIBLE ROLE IN PROTECTION AGAINST BILE ACID INDUCED CYTOTOXICITY

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ABSTRACT

Garcinia, Citrus and Artocarpusspecies are known for their medicinal properties in the folklore especially in alleviation of biliousness. Chloroform and alcoholic extracts of the fruit parts of Garcinia morella(Mor), G mangostana (MS), Citrus maxima (CMx), C aurantium (CA) andArtocarpusgomezianus (AG) were prepared. The extracts and the bile acids namely, Cholic acid (ChA), Deoxycholic acid (DCA), Chenodeoxycholic acid (CDCA) and Lithocholic acid (LCA) were investigated for their effect on Hep G2 liver carcinoma cells and Murine 3T3-L1 fibroblast cell lines by MTT assay. The ability of the fruit extracts at ameliorating bile acid induced toxicity and effect on adipogenesis in differenting 3T3 cells was studied.

Alcoholic extract of mangostana rind exhibited 30.6 mg of antioxidant capacity equivalent to ascorbic acid in gram of the dried rind which was 5 to 25 times higher in comparison to the other alcoholic extracts tested as per DPPH assay. Dose dependent effect of the extracts was observed on the cells. With the exception of G mangostana, Hep G2 cells were found to be sensitive to the presence of high concentration of the extracts (extract derived from 6mg and 10mg of the dried fruit part per ml, for alcoholic and chloroform extracts respectively). As per the MTT assay results, the metabolic function of the cells was enhanced at lower concentrations of the extracts. Morella extracts were found to exert toxicity to a significant level on both the cell lines at high concentrations. IC_{50} *concentrations of ChA, DCA, CDCA and LCA for 3T3 were 0.53, 0.21, 0.18 and 0.19mM and Hep G2 was more sensitive as the values were 0.38, 0.14, 0.11 and 0.11 mM respectively. Secondary bile acids LCA and CDCA were more toxic than primary bile acids. Alcoholic extract of MS was effective in protecting against the toxicity induced by DCA, LCA and CDCA in both the cells. AG extract appeared to influence adipogenesis in 3T3 cells.*

INTRODUCTION

One of the health issues on the rise is obesity which is associated with sedentary lifestyles and increasing consumption of food rich in fat and sugar. Obesity is currently considered as a major risk factor for type 2 diabetes, hyper- tension, and dyslipidemia. Management of/by bile is one of the major factors affecting digestion process. Cholic acid and Deoxycholic acid are the pimary bile acids synthesized by hepatocytes. In the intestine, the microbial flora partake in conversion of primary bile acids to secondary bile acids.Bile acids (BAs) are known to regulate hepatic de novo lipogenesis, export of TG and plasma turnover, hepatic gluconeogenesis and insulin sensitivity. Dysregulation of cytotoxic Bile acids is associated with obesity and, obesity induced oxidative stress leads to progressive inflammatory responses (Rolo et al, 2000; Perez &Briz, 2010). There is a need to explore and understand the effect of bile acids on living cells. Interaction of bile acids with their receptors is being probed for its potential in treatment of disorders associated with lipid metabolism (Fiorucci 2010)

*Artocarpusgomezianus*and *C aurantium*fruits are recommended in folk lore for reducing biliousness. *Artocarpusgomezianus*is also believed to play a role in glycemic control. Many plants belonging to *Garcinia* and *Citrus* species are recommended and have been studied studied for their contribution towards health (Mabberley, 2004; Jagtap&Bapat, 2010; Varalaxmi*et al*, 2010; Parthasarathy&Nandakishore, 2014; Seethapathy*et al*, 2018). One of the beneficial effects of many of these species is their effect at reducing biliousness. *G morella*,*G mangostana* and *C maxima* however, have not garnered much attention in the scientific community. *G morella* which was once commonly found in and around western ghats is gradually becoming an endangered species. Thus, it would be interesting and worthwhile to rekindle the interest in these plants and explore the influence of the herbal approaches employed by our folklore system which were being used to regulate bile homeostasis and lipid metabolism since ages by our ancestors. We have selected these plants for the study to explore their potential at mitigating bile induced toxicity in animal cell lines namely Hep G2 liver carcinoma and 3T3-L1 fibroblast cell lines.

METHODOLOGY

Materials: Fruit rinds of *Gmorella*(Mor) ,*G mangostana (MS)* and *C maxima (CMx)*, fruit slices of *Citrus aurantium (CA) and Artocarpusgomezianus (AG)* were sundried and stored.

Cell-lines: Hep G2 and Murine 3T3-L1 cell lines were procured from NCCS, Pune. 3T3 and HepG2 were maintained in DMEM and MEM medium respectively containing gentamycin and penicillin-streptomycin mixture.

Preparation of extract: Extracts of the fruit parts were prepared in 50% ethanol and Chloroform. The dried extracts were reconstituted in Dimethyl sulfoxide.

Antioxidant potential: Radical scavenging activity assay and Reducing power assay were carried out by DPPH method and FRAP assay respectively using appropriately diluted samples. Vitamin C was employed as standard. Activity was reported as mg of antioxidant activity or reducing power equivalent to vitamin C per gram dry weight of the sample.

Effect of plant extracts/bile acids on cell viability: Deoxycholic acid (DCA), Chenodeoxycholic acid (CDCA) and Lithocholic acid (LCA) stocks were prepared in ethanol. Cholic acid (ChA) stock was prepared in DMSO.

Cells (3T3/ HepG2) were seeded into 96 well plates at a rate of 10,000 cells/well. After overnight incubation in CO2 incubator at 37ºC, the extracts / bile acids were added. The effect was studied at four different concentrations amounting to 50% ethanolic extract obtained from 6, 1.2, 0.3 and 0.06 mg dry weight of the sample in one ml of the serum free culture medium designated as C1, C2, C3 and C4 respectively. Similarly, effect of chloroform extracts obtained from 10, 2, 0.5 and 0.1 mg dry weight of the sample in one ml of the culture medium designated as C1, C2, C3 and C4 respectively. Effect of the extracts/bile acids were studied after 48h by MTT method.

Effect of plant extracts on bile acid induced cytotoxicity: The effect of the plant extracts on the viability of the cells subjected to bile acid toxicity was studied at two concentrations viz. C2 and C4. The cells were incubated in presence of respective bile acids (DCA, CDCA and LCA – at a concentration where 50-70% of the cell viability was lost) in presence of the plant extracts. MTT assay was carried out after 48h.

Effect of plant extracts on adipogenesis: 3T3 cells were subjected to adipocyte differentiation in 96 well plates. Adipogenesis was induced in differentiation media consisting of glucose, insulin and/ isobutyl-1-methyl xanthine, , dexamethasone and FBS (Zebisch et al, 2012). Effect of AG, MS and CMx extracts on adipogenesis was studied by including these agents in the differentiating medium on 4^{th} day of differentiation. The dose chosen in terms of extract obtained from mg dry weight of the sample in 1ml of differentiation medium is as follows

Extract	CA	AG	MS	CMx	Mor
Alcoholic	4.0	4.0	0.03	1.5	0.06
Chloroform	4.0	4.0	0.1	4.0	0.2

Effect of bile acids on adipogenesis was studied at 0.025mM DCA, CDCA and LCA and 0.1mM ChA. Adipogenesis (oil droplets) was observed microscopically and stained with Oil-red O. The dye extracted in isopropanol was measured at 492 nm for quantification of adipogenesis.

RESULTS AND DISCUSSION:

Fruits belonging to *Garcinia* and *Citrus* species are well known for their antioxidant potential. Both the alcoholic and chloroform extracts of *G mangostana* fruit rind exhibited highest DPPH radical scavenging activity (Table 1) followed by *C maxima* which also showed good antioxidant capacity.

Table 1: Antioxidant potential of the plant extracts

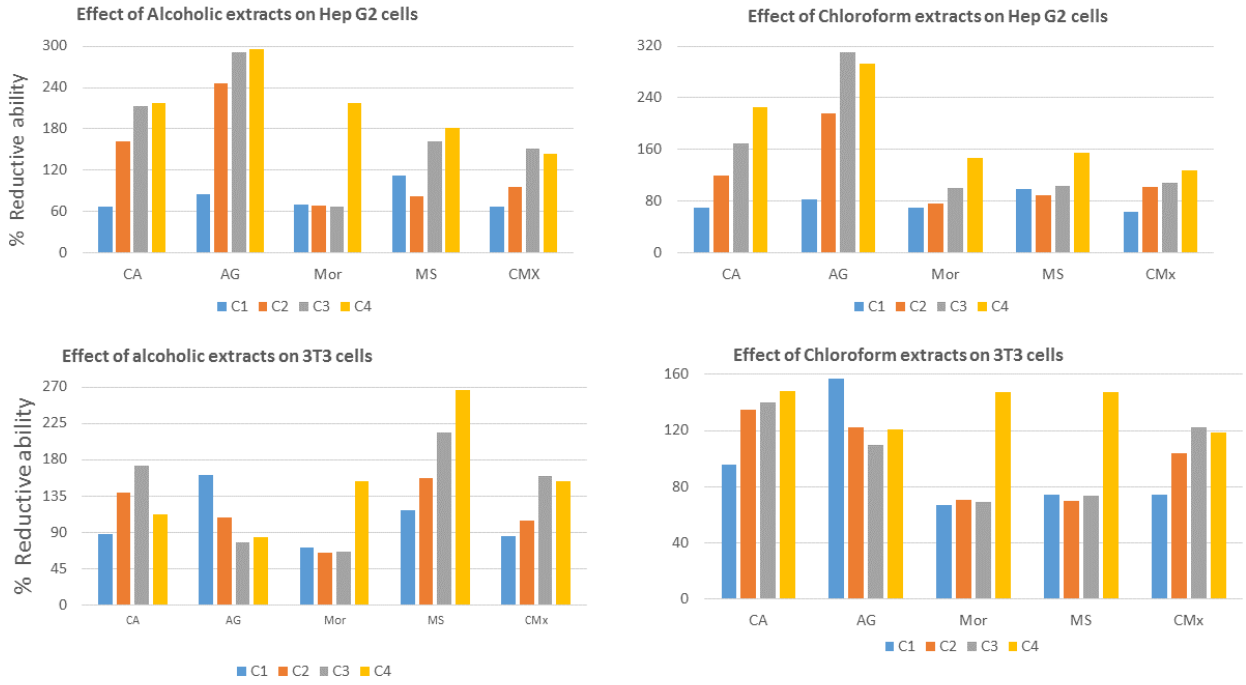
	Reducing power, mg/g⁎	**Antioxidant activity, mg/g⁎**	**Reducing power, mg/g⁎**	**Antioxidant activity, mg/g⁎**
Plant source	Alcohol extract		Chloroform extract	
A gomezianus (AG)	0.84±.2	1.09±0.19	0.04±.01	0.069±0.01
C aurantium (CA)	3.12±0.51	3.8±01.1	0.43±.15	0.18±0.04
G morella (Mor)	1.54±0.04	1.81±0.29	0.12±0.018	0.244±0.004
G mangostana (MS)	3.29±0.38	**30.56±1.33**	0.12±0.002	8.95±0.98
C maxima (CMx)	1.73±0.017	6.24±0.2	0.47±0.001	1.98±0.061
⁎antioxidant potential equivalent to mg of Vitamin C obtained from one g dry weight of the fruit part				

Various bile acids are reported to exert distinct biological effects *in vivo*. Being highly hydrophobic, bile acids, such as DCA and LCA are believed to cause oxidative modification of lipids, proteins, and nucleic acids, eventually leading to cell death. Recent studies have shown that bile acidscan affect intracellular signaling and gene expression, whichmay lead to alterations in cell growth. Bile acids are therefore complex metabolic integrators and signalling factors and hence, bile acid metabolism is being considered in the development of drugs for obesity, type 2 diabetes, hyper triglyceridaemia and atherosclerosis, as well as other associated chronic diseases such as non-alcoholic steatohepatitis (Rolo et al, 2000; Fiorucci*et al.*2010).

Effect of the bile acids on cell viability was studied by MTT assay which measures cell viability based on reductive ability of metabolic enzymes which reduce the tetrazolium dye MTT to its formazan form. The bile acids were found to have toxic effects on both Hep G2 and 3T3 cells. IC50 concentrations of ChA, DCA, CDCA and LCA for 3T3 were 0.53, 0.21, 0.18 and 0.19mM and Hep G2 was more sensitive as the values were 0.38, 0.14, 0.11 and 0.11 respectively. The primary bile acid cholic acid was found to be the least toxic of bile acids. Effect of the plant extracts on the cells was

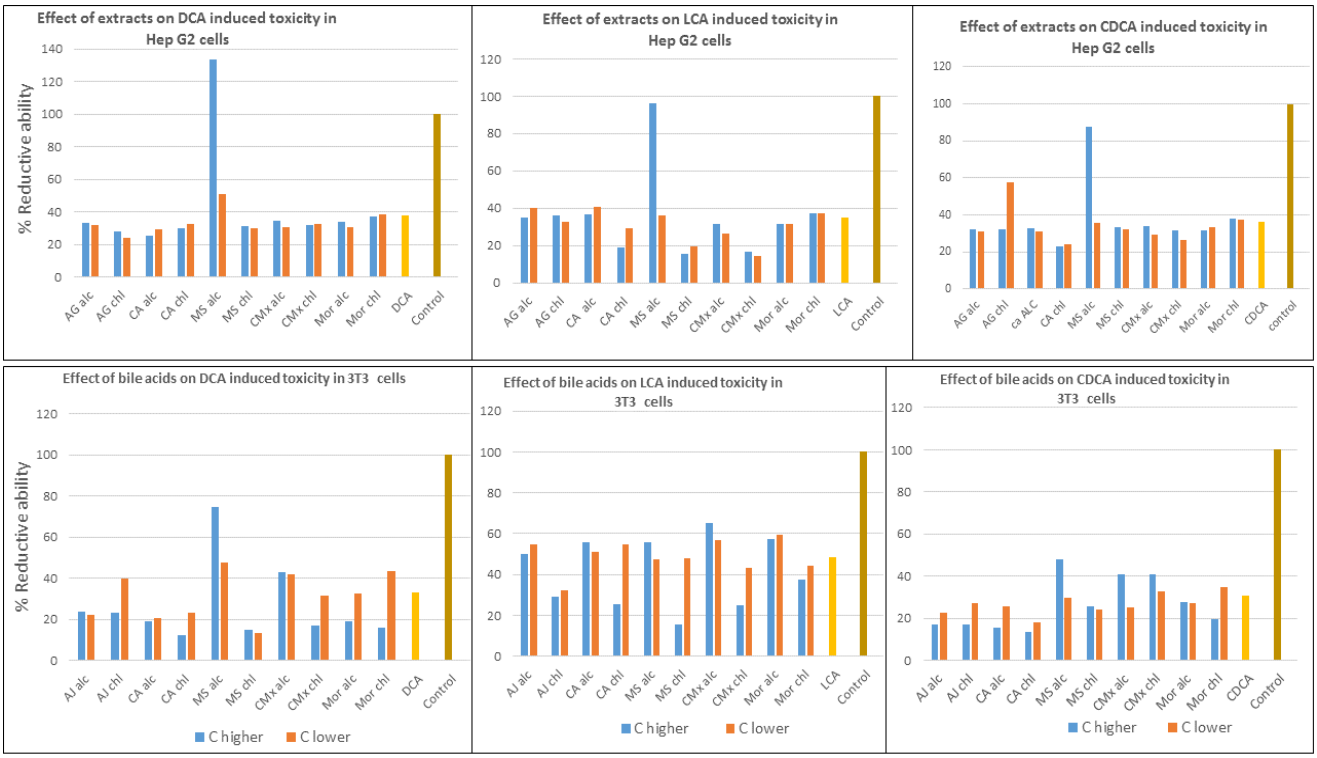
also studied. The results are presented in Figure 1. It was observed that Hep G2 cells were highly sensitive to the presence of higher concentration of the extracts. Reduction of the dye was less at high concentrations implying toxic effect of most of the extracts at higher concentrations. At lower concentrations, the extracts appeared to enhance the reductive ability of the metabolic enzymes in the cells. Similarly, with the exception of AG, higher concentrations of the extracts were found to be toxic to 3T3 cells. It must be noted that AG had shown least antioxidant potential.

Fig 1: Effect of the plant extracts on the reductive ability of metabolic enzymes



A study was undertaken to assess the protective effect of the plant extracts against the cytotoxicity mediated by CDA/DCDA/LCA (0.14mM for Hep G2 and 0.22 mM for 3T3). The concentration selected for the study was the alcoholic extract obtained from 1.2 mg and 0.06 mg dry wt and, chloroform extract recovered from 2 and 0.1mg dry wt of sample. The results are as per Figure 2. Higher concentration of MS alcoholic extract exhibited significant protection against the toxicity induced by the bile acids. Bile acids reportedly give rise to oxidative stress. High antioxidant potential of the mangostana extract could be a contributing factor in the protective action.*C maxima* alcoholic extract which also possesses relatively good antioxidant capacity, appeared to protect 3T3 cells against bile acid induced toxicity. It is possible that the reducing power of these extracts may have been used for scavenging reactive oxygen species produced in response to bile acids, thereby ameliorating the toxic effect of the bile acids.

Figure 2. Effect of the plant extracts on bile acid mediated cytotoxicity.



Bile acid receptors such as farnesoid X receptor is reported to possess dual characteristics: counteracting obesity and diabetes, improved insulin release and sensitivity coupled with accelerated progression of obesity and diabetes. In the present investigation, the effect of the bile acids on adipogenesis was studied in 3T3-L1 cells at sub toxic levels. Increase in adipogenesis to an extent of 1.2 to 1.9 folds was noted. Of the extracts used, AG extracts appear to have anti adipogenic effect affecting around 30-55% inhibition of adipogenesis in differentiating 3T3 cells.

Conclusion:The cells were found to be more sensitive to Morella extracts in comparison to other extracts employed. It was interesting to note that the alcoholic extract of Garcinia mangostana rinds has the potential to protect the cells against the toxic effect of high concentrations of bile acids.

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