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Evaluation of the mitochondrial membrane potential of Draksha amruthadi kashaya in Paracetamol induced HepG2 cells

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ABSTRACT
Liver is a vital organ in human body which is significantly involved in maintaining and regulating the homeostasis of the body.So a healthy liver is a crucial factor of overall health and wellbeing. But Liver is continuously exposed to environmental toxins, affected by poor drug habits, intake of prescribed and over the counter drugs, consumption of alcohol etc. Considering the global, national and regional data from previous studies liver diseases and death due to liver diseases, are some of the major medical concerns in the present world. Indiscriminate and uncontrolled usage of medicines have greatly contributed to it especially in the situation of Pandemic. Even though there is tremendous advancement in health system, more effective and safe hepatoprotective drugs are still a need of present era which clearly signifies the importance of studies to evaluate, develop plant based hepatoprotective drugs. Here the study was conducted to find out the mitochondrial membrane potential ofHepG2 cells treated with an Ayurvedic medicine,DrakshaamruthadikashayamentionedinKamalarogachikitsainthe textbookSahasrayoga through in vitro cell line study.Draksha, Guduchi, Ikshu are the ingredients contained in this formulation which waspreparedaccordingtothekashayakalpanamethodexplainedinSarangadharaSamhitha. Paracetamol was used to induce hepatotoxicity in HepG2 cells.Here the flow cytometry assessment of mitochondrial membrane potential was donein paracetamol induced HepG2 cells treated with Draksha amruthadi Kashaya through in vitro cell line study. From the study the mitochondrial membrane potential of the cells were well maintained by the protective action of Draksha amruthadi kashaya.
Key words: Mitochondrial membrane potential, kashaya, HepG2 cell line

I. INTRODUCTION

Liver is a vital organ in human body which plays fundamental role in diverse processes, among which storage, secretion, metabolism and detoxification are the major ones. Though liver has the ability to regenerate, sustained infections and fat deposits can limit the regenerating ability of the liver. But with the improvement in living standards, the prevalence of metabolic liver diseases including non-alcoholic fatty liver disease and alcohol-related liver disease is set to rise, eventually leading to more cases of end-stage liver diseases (liver failure, cirrhosis, and liver cancer). Burden of obesity also plays a significant role[1]. According to the latest WHO data published in 2017, Liver disease deaths inIndia has reached 2.95% of total deaths[2]. Liver disease is considered as one among 10leading causes of death in India. According to a recent study 5000-10000 people inKeraladie from liverdisorders, compared to the national averageof 70000[3].Also,it isestimated that out of all gastroenterology hospital admissions, almost 2.5% cases aredue to DILI in India. Due to the pandemic situation, the problem has increased due to the in discriminate and uncontrolleduse of various drug sinrecent times[4].Paracetamoloverdose is the leading cause of drug-induced acute liver failure in many developedcountries[5]. Research studies are being conducted for the intervention of more effective and less toxic hepatoprotective drugs. In Ayurveda single as well as combined herbal preparations are mentioned in this context and many of them are being used in clinical practice. But it is necessary to research and revalidate the hepatoprotective effect of such drugs.In Ayurveda,Draksha amruthadi kashaya is one such formulation explained in kamala roga chikitsa in the textbook Sahasrayoga[6] which contains only three ingredients draksha, amrutha and ikshu. In vitro studies can explore the biological effects of drugs and pharmaceuticals

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outside the living organismHere Paracetamol is used to induce hepatotoxicity in this study. It is a widely used analgesic and antipyretic drug that has been established to develop acute liver toxicity above therapeutic doses.Use of combination of medicines in the form of pain killers and antihistamines etc , makes it difficult to identify the hepatotoxicity caused by paracetamol in specific[7].Liquid form of paracetamol with a prefixed dose and exposure time was used to induce hepatotoxicity in this study. HepG2 which is a human hepatoma derived cell line was used to conduct this study. Here the flow cytometry assessment of mitochondrial membrane potential was done in paracetamol induced HepG2 cells treated with Draksha amruthadi Kashaya through in vitro cell line study.

II. MATERIALS AND METHODS

Collection of the raw drugs

The raw materials required for the preparation of the Draksha amruthadi kashaya were procured fromauthentic source. The drugs were further verified from the department of Dravyaguna Vijnana, Government Ayurveda College, Tripunithura. The identity, purity and strength were assessed.

Figure1: StemofGuduchi

Figure2:FruitofDraksha

Figure3:StemofIkshu

Preparation of Kashaya

After washing of raw drugs and size reduction, kashaya preparation was done using Sarangadhara Samhita reference[8]. Outer skin of Ikshu was removed, made into small pieces and crushed .Crushing of draksha was done .Outer skin of Guduchi was removed, made into small Pieces and crushed. All ingredients were taken in equal quantity and kept in a clean steel vessel 16 times of water was added into it. Heating was done under mild flame with stirring Heating was continued until the quantity reduced to 1/8th part Prepared kashaya was filtered through a clean piece of cloth into a glass bottle

Serial No	ingredients	Botanical name	Part used	Quantity
1	Draksha	Vitis vinifera L	Fruit	1 part
2	Guduchi (Amrutha)	Tinospora cordifolia (Thunb.) Miers	Stem	1 part
3	Ikshu	Saccharum officinarum L	Stem	1 part

Table 1 : Ingredients of Draksha amruthadi kashaya

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Figure 4:IngredientsforKashayapreparation

Figure5: PreparationofDrakshaamruthadiKashaya

Figure6: Drakshaamruthadikashaya

Procedure

Cellculturing

HepG2 cell line was maintained in DMEM (Dulbecco's modified Eagles media),purchased from National centre for Cell Sciences (NCCS), Pune. The cell line wasculturedin25cm^{2}tissuecultureflaskwithDMEMsupplementedwith10%FBS(foetalbovine serum), L-glutamine, sodium bicarbonate and antibiotic solution containing:Penicillin(100U/ml),Streptomycin(100µg/ml),andAmphoteracinB(2.5µg/ml).Culturedcellli

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neswerekeptat37ºCinahumidified5%CO_2(carbondioxide)incubator.

Cellsseedingin96wellplate:
Twodaysoldconfluentmonolayerofcellsweretrypsinizedandthecellsweresuspendedin10%growthmedium,100µlcellsuspension(5x10^3cells/well)wasseededin 96 well tissue culture plate and incubated at 37ºC in a humidified 5% CO_2 (carbondioxide)incubator.

Determinationofmitochondrialmembranepotentialbyflowcytometry

Afterattainingsufficientconfluency,theHepG2cellsweresubjectedtopreconditioningwith12.5µMkashayaforaperiodof24hours,followedbyactivation with 20 µM paracetamol and incubated for 24 hours at 37ºC in a humidified 5% CO2incubator.The dose 12.5µM of Kashaya has already fixed through MTT assay. NontreatedcontrolcellsandParacetamolinducedcellswerealsomaintained.After,incubation,thecellsweretrypsinizedandsubjectedtoflowcytometryas per thefollowingprocedures:
Working solution was prepared by diluting the Muse™ MitoPotential Dye 1:1000

-

in1Xassaybuffer.Thecellsaftercentrifugationwasresuspendedin1Xassaybufferand added with 95µl of mitopotential working solution. Mixed thoroughly by pipetting upandincubatedthecellsfor20minutesina37°CCO2incubator.Afterincubation5µLof Muse MitoPotential 7- AAD was added to each well. Then mix thoroughly bypipetting up and down or vortexing for 3 to 5 seconds and kept for incubation for 5minutes.Thesampleswereloadedontoaflowcytometer(Millipore,USA)andeventswereacquired aftergatingandcorrelated withcontrols.
Fourpopulationsofcellswhichcanbedistinguished:

- (LL) Live cells with depolarized mitochondrial membrane:MitoPotential(-) and 7-AAD (-)
- (LR) Livecellswithintactmitochondrialmembrane:MitoPotential(+) and 7-AAD (-)
- (UR) Dead cells with depolarized mitochondrial membrane:MitoPotential(+) and 7-AAD(+)
- (UL) Dead cells with intact mitochondrial membrane:MitoPotential(-) and 7-AAD (+)

III. RESULT

a. Controlcells

The control cells showed 86.68 % Live cells, 3.25 % Depolarized live cells, 10.07 % dead cells.Depolarized dead cells were found to be absent.

Cells	%Gated	Cellconcentration(cells/mL)
Live(LR)	86.68 %	2.99E+06
Depolarized/Live(LL)	3.25 %	1.12E+05
Depolarized/Dead(UL)	0.00 %	0.00E+00
Dead(UR)	10.07 %	3.47E+05
TotalDepolarized	3.25 %	1.12E+05

Table2:MMPof controlcells

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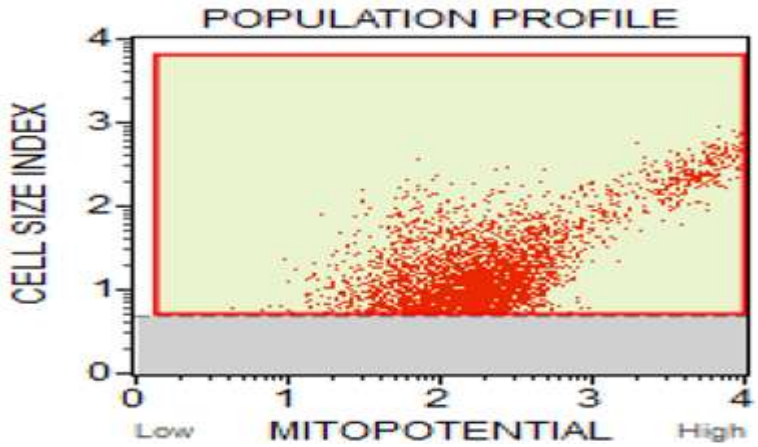

Figure7:Populationprofileofcontrolcells

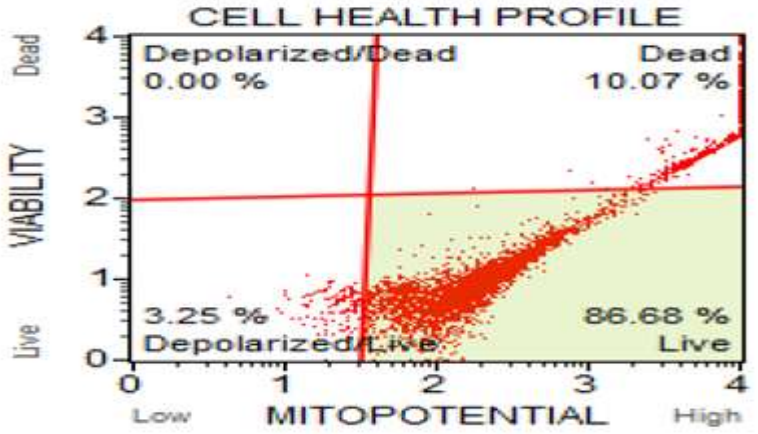

Figure8:Cellhealthprofileofcontrolcells

b. Paracetamoltreatedcells

Paracetamol only treated cells showed 66.72 % live cells, 17.00 % depolarized live cells, 16.27 % dead cells.Depolarized dead cells were found to be absent.

Table53:MMPof paracetoltreatedcells

Cells	%Gated	Cellconcentration(cells/mL)
Live(LR)	66.72 %	1.63E+06
Depolarized/Live(LL)	17.00 %	4.16E+05
Depolarized/Dead(UL)	0.00 %	0.00E+00
Dead(UR)	16.27 %	3.98E+05
TotalDepolarized	17.00 %	4.16E+05

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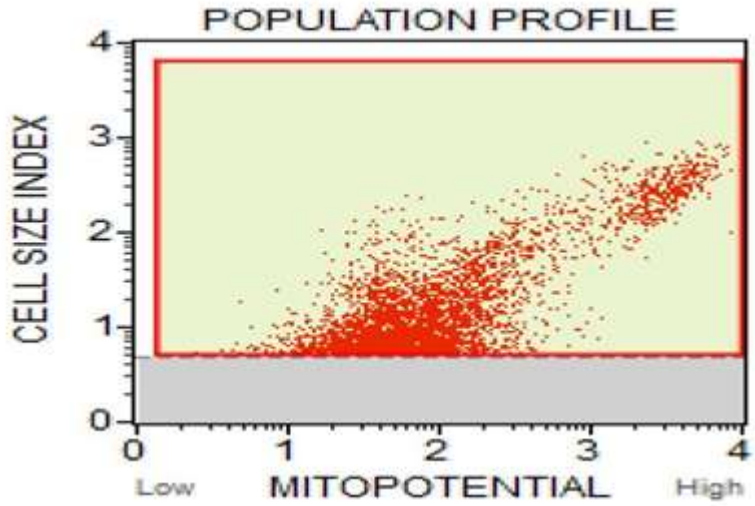

Figure9:PopulationprofileofParacetamol treatedcells

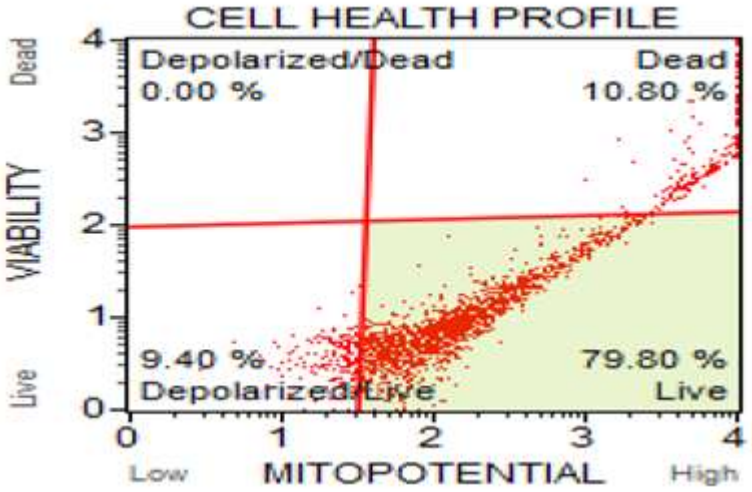

Figure10: Cellhealthprofileof Paracetamoltreatedcells

c. Paracetamol+Kashayatreatedcells

Paracetamol and Kashaya treated cells showed 79.80 % live cells, 9.40 % depolarized live cells, 10.80 % dead cells.Depolarized dead cells were found to be absent.

Cells	%Gated	Cellconcentration(cells/mL)
Live(LR)	79.80 %	2.18E+06
Depolarized/Live(LL)	9.40 %	2.56E+05
Depolarized/Dead(UL)	0.00 %	0.00E+00
Dead(UR)	10.80 %	2.95E+05
TotalDepolarized	9.40 %	2.56E+05

Table54:MMPofParacetamol+Kashayatreatedcells

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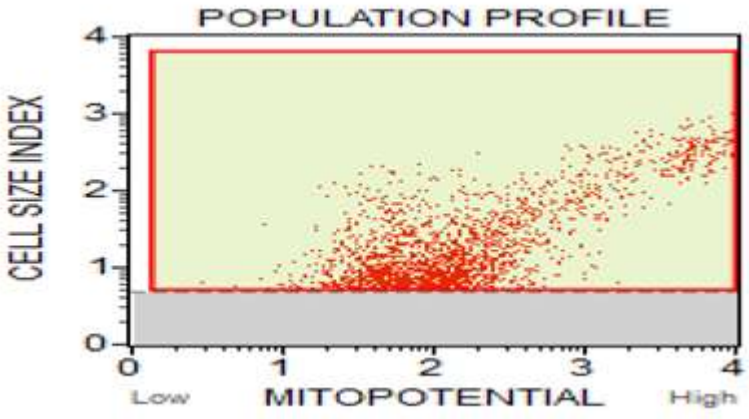

Figure11: PopulationprofileofParacetamol+Kashayatreatedcells

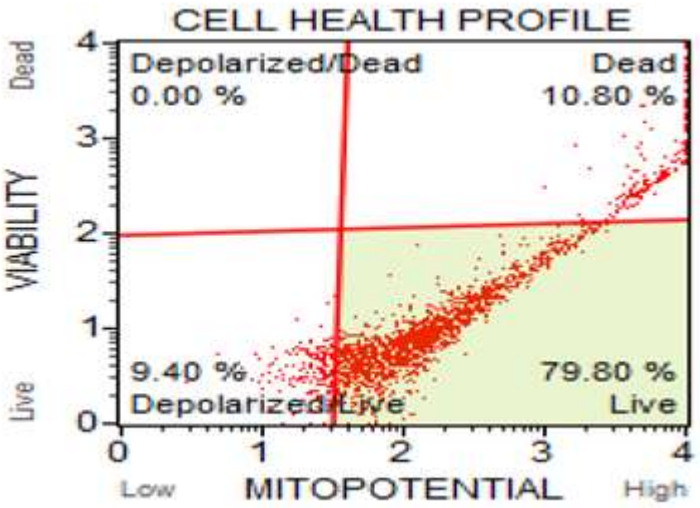

Figure12:CellhealthprofileofParacetamol+Kashayatreatedcells

In the determination of MMP, percentage of live cells in paracetamol onlytreated cells was reduced to 66.72% compared to untreated control cells where it was86.68%. When the cells were treated with draksha amruthadi kashaya, the percentageof live cells was found to be 79.80%. Depolarized live cells was found to be 17% inparacetamol only treated cells, while it was only 3.25 % in control group. In the grouptreated with Draksha amruthadi Kashaya, the percentage of depolarized live cells wasfound to be 9.40%. Depolarized dead cells was absent in all the three groups. Thepercentage of dead cells in paracetamol only treated group was elevated to 16.27%compared to control group where it was 10.07%. In case of the group treated withDrakshaamruthadikashaya, percentageof deadcells was found to be10.80%.

IV. DISCUSSION

The proper function and quality of mitochondria is one among the primarydeterminants of cellular health during paracetamol induced hepatotoxicity. The changeshappening to the mitochondrial morphology and mitochondrial membrane potential is an indicator of ongoing hepatocellular necrosis. Mitochondria play a central role inparacetamolinducedcelldeathandinjury.Intheassessmentofmitochondrialmembrane potential using flow cytometry, the control HepG2 cells showed 86.68%livecells,3.25%depolarizedlivecellsand10.0

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7%deadcells.Whenitwastreatedwith20 mM paracetamol, it showed 66.72% live cells, 17.00% depolarized live cells and16.27% deadcells.HepG2 cellswhichareпreconditionedwith12.5µL Drakshaamruthadi kashaya, it showed 79.80% live cells, 9.40% depolarized live cells and10.80% dead cells. Depolarized dead cells was found to be absent in all the threegroups. The percentage of live cells decreased on inducing toxicity with paracetamol,whichgotincreasedbypreconditioningwithkashaya.Alsothepercentageofdeadcellsand depolarized live cells were found to decrease in the kashaya treated group. Thefindingsgivetheevidencethatthekashayaishaving aprotectiveactiononmitochondrial functioningandmaintenanceofmitochondrialmembranepotentialwhichismandatoryforhealthylivecells.

V. CONCLUSION

The flow cytometry assessment of MMP in non treated control cells and paracetamol only treated cells were done and it was compared with that of draksha amruthadi kashaya pretreated cells. Theresultsshowedtheabilityofthekashayatorestoreth emitochondrialmembrane potential on inducing the hepatotoxicity.It clearly indicates the hepato protective activity of Kashaya on inducing hepatotoxicity by paracetamol.

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Conflict of Interest: Nil

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