

 ISSN NO. 2320-5407	Journal Homepage: -www.journalijar.com INTERNATIONAL JOURNAL OF ADVANCED RESEARCH (IJAR) Article DOI:10.21474/IJAR01/7432 DOI URL: http://dx.doi.org/10.21474/IJAR01/7432	 INTERNATIONAL JOURNAL OF ADVANCED RESEARCH (IJAR) ISSN 2320-5407 Journal Homepage: http://www.journalijar.com Journal DOI:10.21474/IJAR01
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RESEARCH ARTICLE

HEPATOPROTECTIVE EFFECT OF COMBINATION OF TENDER LEAVES OF *MANGIFERA INDICA* LINN AND TENDER COCONUT WATER IN HEP G2 CELLS.

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Manuscript Info

Manuscript History

Received: 17 May 2018
Final Accepted: 19 June 2018
Published: July 2018

Keywords:-

M.indica, Tender coconut water,
Hepato-protective, HepG2 cells.

Abstract

Liver is an important organ of metabolism and mortality due to liver diseases in India is 22.93%. Ayurveda claims the combination of paste of tender leaves of *Mangifera indica* Linn (M.indica) and tender coconut water is useful in liver disorders. But scarce scientific data is available on the protective effect of this combination in liver disorders. Hence the present study evaluated hepato-protective effect of this combination in HepG2 cells against carbon tetra chloride induced hepato-toxicity by using MTT assay. Aqueous and alcoholic extracts of the combination of drug was prepared as per the standard procedure. HepG2 cells were purchased from NCC, Pune. The experiments done with different concentrations of aqueous and alcoholic extract of the combination. Aqueous & alcoholic extract of tender leaves of *M.indica* and tender coconut water also tested separately. Silymarin was taken as positive control and untreated cells as negative control. All the experiments were done in triplicates and the percentage of cell viability was evaluated based on average of optical density readings. Data obtained were analysed statistically by ANOVA test. All the tested samples had hepatoprotective effect when compared to negative control and positive control at particular concentrations. The percentage of viability was more in combination especially at lower concentration of aqueous extract than that of silymarin. Thus the present study gathered scientific data on hepato protective activity of the combination of Tender leaves of *M. indica* and tender coconut water and thus traditional knowledge is validated.

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Introduction:-

Liver is considered to be one of the most important organ of metabolism. Death rate due to liver diseases in India is 22.93%^[1]. The risk of the liver intoxication is increased by the higher exposure to the environmental toxins, pesticides, frequent use of chemotherapeutics and alcoholic abuse, drugs etc. The organ liver can be correlated with *Yakrth* in terms of position, function, pathology etc. In Ayurvedic literature, *yakrth*(liver) is considered as an important *anga*(organ) of the human body right from the vedic period. The liver diseases are generally managed in lines with *Yakrthvikara* (hepato-biliary disorder), *Kamala* (Jaundice), etc. The combination of Tender leaves of

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Mangifera indica Linn (Amrapallava) and Tender coconut water (Nalikerodaka) is mentioned in the context of *kamalachikitsa* (Juandice) in *Chikitsamanjari* (Ayurvedic traditional book)^[2].

In Ayurveda, the disease kamala represents a group of liver disorders. *Kamala* is a *pithananathmaja* (Forty specific diseases of *pitha*)^[3] and *rakthapradoshaja vyadhi* (Disease due to vitiation of blood tissue)^[4]. Since *yakrth* is the seat of *pitha*^[5] and *moolasthan* of *rakthavahasrotas*^[6], it is clear that the disease *kamala* is caused due to the derangements of *yakrth*. So the drugs having *pithashamana*, *seethaveerya* (cold in potency), *kashaya* (Astringent) & *madhura* (Sweet) *rasa* (taste) property can be used for treatment of *Yakrthvikara* (liver disorder).

Amrapallava, which is identified as tender leaves of mango tree (*Mangifera indica* Linn) are used for various ailments in Ayurvedic system of medicine since ancient time. *Mangifera indica* Linn belongs to the family Anacardiaceae. The younger leaves are reported to have anti-diabetic activity^[7], antioxidant activity^[8], Gastro protective^[9], antibacterial activity^[8]. According to Ayurveda, the *Amrapallava* (tender leaves of *Mangifera indica* Linn) is having *kashaya* (Astringent) in taste and *Seetha* (Cold) in potency, and is having *kaphapithashamana*^[10] property.

Tender coconut water obtained from coconut tree is a natural isotonic beverage with the same level of electrolytic balance as we have in our blood. It's considered as the fluid of life. In Sanskrit, the coconut palm is called as *Naliker* and the tender coconut water is called as *Nalikerodaka*. According to Ayurveda, *nalikerodaka* (Tender coconut water) is said to be *Sheetala* (Cold), *hrudya* (cardioprotective), *deepana* (digestive stimulant), *shukrala* (Aphrodisiac), *laghu* (light). It relieves *pitha*, *pipasa* (thirst) and *bastishuddhikara* (diuretic)^[11].

In vitro bioassays are important in the evaluation of plants with possible hepatoprotective effects. In the present scenario so many herbal drugs are screened for hepatoprotective activity using in vitro cell line method. HepG2 cell lines are more sensitive and easy to operate with reliable results and it can be a lead for further preclinical evaluation. In vitro evaluation of hepato protective activity of drug is fundamental for further testing in animal models.

Tender leaves of *Mangifera indica* Linn and *Nalikerodaka* Tender coconut water are having pharmacological properties which are essential to protect liver damages. Both the drugs are easily available and cost effective. So the combination of these drugs may be a good choice of hepatoprotective activity. Therefore a scientific validation is necessary to prove the efficacy of the combination of *Amrapallava* (Tender leaves of *Mangifera indica* Linn) and *Nalikerodaka* (Tender coconut water) and also that of the individual drugs in liver disorders.

Materials And Methods:-

Collection of Plant material:-

The tender leaves of *Mangifera indica* Linn. and tender coconut water for the study were collected from same area of Kollam district. For the present study the fresh drug was taken and it was later dried in shade. After drying it was pounded to coarse powder. Powdered drug was stored in an air tight container.

Experimental material:-

The human liver-derived HepG2 cell lines were obtained from National Centre for Cell Sciences, Pune, India. The cells were maintained in DMEM containing 10% FBS, at 37°C and 5% CO₂.
Standard- Silymarin, MTT assay kit

Preparation of sample for Assay:-

Test drug 1- Water soluble extractive of tender leaves of *Mangifera indica* Linn (TLMI-AQ)

The tender leaves of *Mangifera indica* Linn was dried in shade and powdered. The extract of the drug was prepared using 5gm drug in 100ml Chloroform water in a closed flask for 24 hours, shaking frequently. Filtered rapidly, taking precautions against loss of solvent. Evaporated the filtrate to dryness in a tired flat bottomed shallow dish and dried at 105°C, to constant weight and weighed.

Test drug 2:-TCW

Tender coconut water

Test drug 3:-Alcohol soluble extractive of tender leaves of *Mangifera indica* Linn (TLMI-AL)

The tender leaves of *Mangifera indica* Linn was dried in shade and powdered. The extract of the drug was prepared using 5gm drug in 100ml ethanol in a closed flask for 24 hours, shaking frequently. Filtered rapidly, taking precautions against loss of solvent. Evaporated the filtrate to dryness in a tared flat bottomed shallow dish and dried at 105⁰c, to constant weight and weighed.

Test drug 4:-Alcohol soluble extractive of Tender leaves of *Mangifera indica* Linn & tender coconut water (TLMI+TCW-AL)

Kalka of *Amrapallava* (Tender leaves of *Mangifera indica* Linn) was prepared into paste by rubbing it on stone plate by adding sufficient quantity of tender coconut water to it. Then it was dried in shade and powdered. The extract of the drug was prepared using 5gm drug in 100ml ethanol in a closed flask for 24 hours, shaking frequently. Filtered rapidly, taking precautions against loss of solvent. Evaporated the filtrate to dryness in a tared flat bottomed shallow dish and dried at 105⁰c, to constant weight and weighed.

Test drug 5:-Water soluble extractive of Tender leaves of *Mangifera indica* Linn& tender coconut water (TLMI+TCW-AQ)

Kalka of *Amrapallava* (Tender leaves of *Mangifera indica* Linn) was prepared into paste by rubbing it on stone plate by adding sufficient quantity of tender coconut water to it. Then it was dried in shade and powdered. The extract of the drug was prepared using 5gm drug in 100ml Chloroform water in a closed flask for 24 hours, shaking frequently. Filtered rapidly, taking precautions against loss of solvent. Evaporated the filtrate to dryness in a tired flat bottomed shallow dish and dried at 105⁰c, to constant weight and weighed.

Hepatoprotective effect study on HepG2 cell line:-

The screening of hepatoprotective activity is based on the protection of human liver-derived HepG2 cells against CCl₄-induced damage -determined by estimating mitochondrial synthesis using the tetrazolium assay

Two days old confluent monolayer of cells were trypsinized and the cells were suspended in 10% growth medium, 100µl cell suspension (5x10⁴ cells/well) wasseeded in 96 well tissue culture plate and incubated at 37°C in a humidified 5% CO₂ incubator. After 24 hours of incubation at 37°C in 5% CO₂ to allow cell attachment. Then the media were removed. The cells were then exposed to toxicant medium containing 1% CCl₄ along with or without various concentrations of the total alkaloid fraction or the media alone

250 µg/ml of standard Silymarin& Prepared extracts in 5% DMEM were five times serially diluted by two fold dilution (100µg, 50µg, 25µg, 12.5µg, 6.25µg in 100µl of 5% DMEM) and each concentration of 100µl were added in triplicates to the respective wells and incubated at 37°C in a humidified 5% CO₂ incubator.

Entire plate was observed at an interval of each 24 hours; up to 72 hours in an inverted phase contrast tissue culture microscope and microscopic observation were recorded as images.

At the end of the period, cytotoxicity was assessed by estimating the viability of the HepG2 cells by the MTT reduction assay. After 24 hours of incubation period, the sample content in wells were removed and 3 0µl of reconstituted MTT solution was added to all test and cell control wells, the plate was gently shaken well, then incubated at 37°C in a humidified 5% CO₂ incubator for 4 hours. 100µl of MTT Solubilization Solution (DMSO) was added and the wells were mixed gently by pipetting up and down or shaken well in order to solubilize the formed formazan crystals

This colorimetric assay involves the conversion of MTT to a purple formazan derivative by mitochondrial succinate dehydrogenase, which is present only in viable cells. The absorbance values were measured by using microplate reader at a wavelength of 570 nm.

The percentage of growth inhibition is calculated using the formula:

$$\% \text{ of viability} = \frac{\text{Mean OD Samples} \times 100}{\text{Mean OD of control group}}$$

Results And Discussion:-

Effect of Amrapallava (Tender leaves of *Mangifera indica* Linn.) along with Nalikerodaka (Tender coconut water) in carbon tetrachloride induced hepatotoxicity in HepG2 cells:-

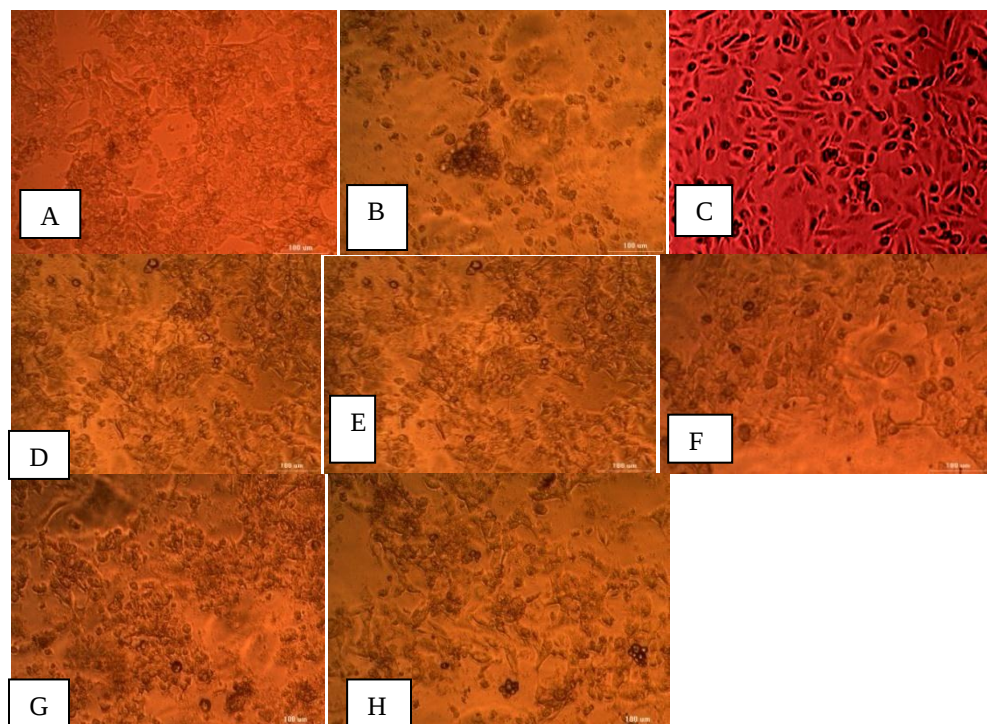
The exposure of HepG2 cells to 1% (v/v) CCl₄-induced significant cell death. The cell viability was almost half of control after 24 h exposure (44.95 ± 0.96). After exposure with CCl₄, the cells were exposed with various concentrations (6.25–100 µg/ml) of test drug TLMI(AQ), TCW, TLMI(AL), TLMI+TCW(AQ) and TLMI+TCW(AL). Silymarin was taken as the standard drug. The average absorbance of different concentrations of the test drugs were recorded at 540nm and their percentage viability was calculated. The result of cell viability depicted in table no.1

Protective effect of various fractions of test drugs and standard drug silymarin on CCl₄ induced toxicity in HepG2 cell line Table no.

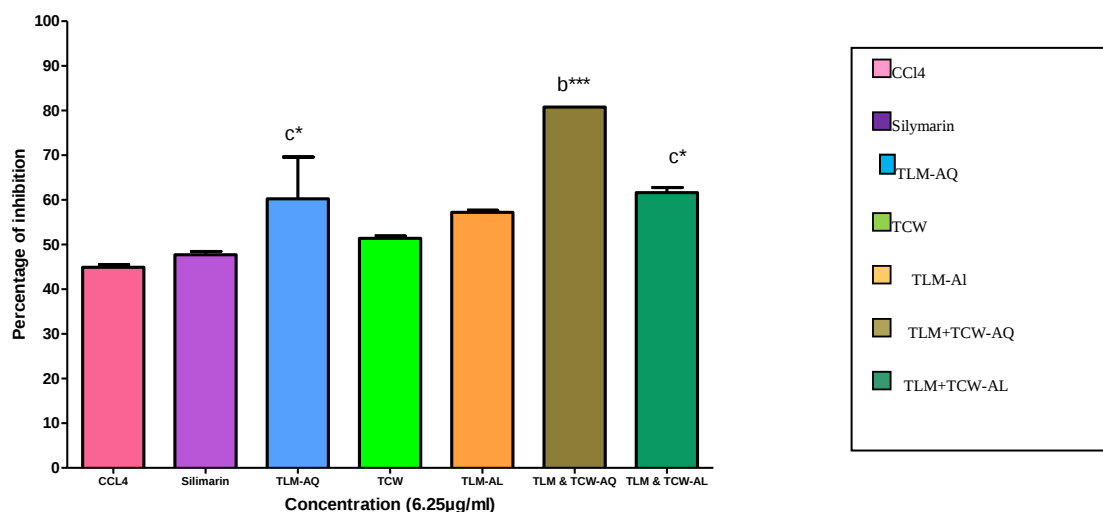
CCl₄ induced toxicity in HepG2 cell line Table no.1

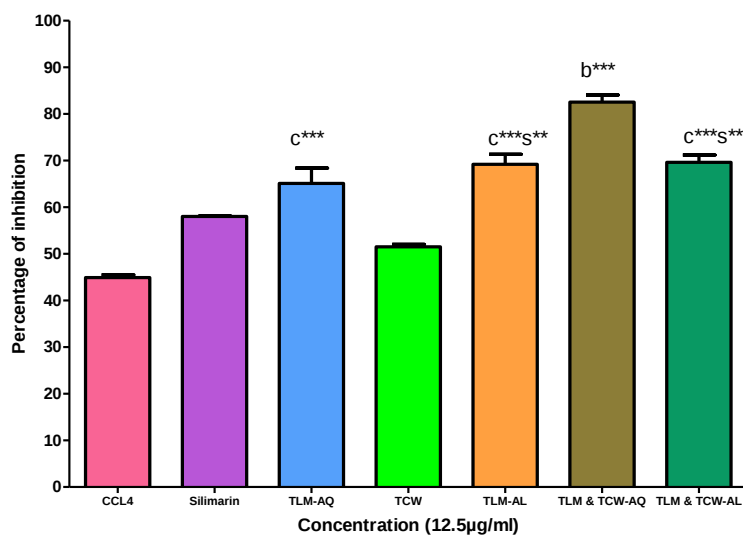
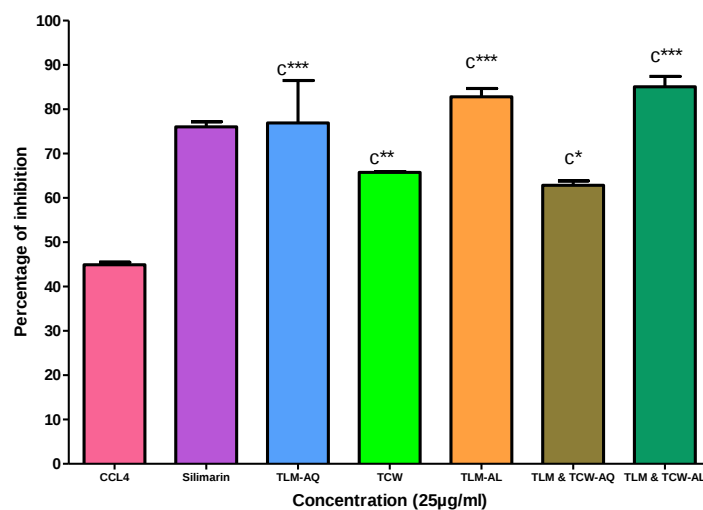
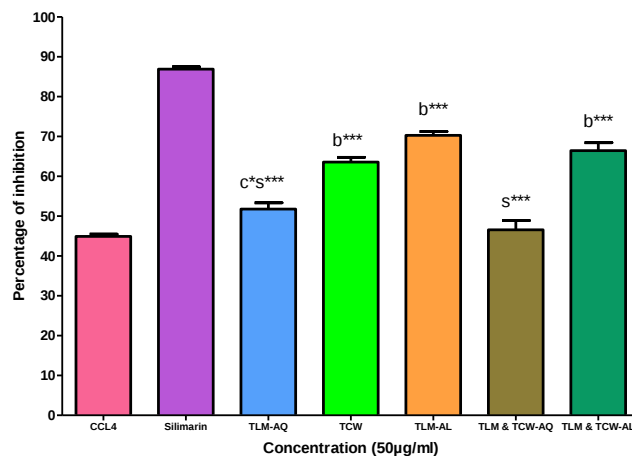
Group	Experimental groups	Cell viability(%)
Control	Normal control	100
Toxicant control	CCl ₄ control	44.95
Silymarin treated	Silymarin (6.25 µg/ml) + CCl ₄ (1% v/v)	47.73
	Silymarin (12.5 µg/ml) + CCl ₄ (1% v/v)	58.04
	Silymarin (25 µg/ml) + CCl ₄ (1% v/v)	76.04
	Silymarin (50 µg/ml) + CCl ₄ (1% v/v)	86.89
	Silymarin (100 µg/ml) + CCl ₄ (1% v/v)	93.88
Tender leaves of <i>Mangifera indica</i> Linn. (Aqueous extract)	TLMI AQ (6.25 µg/ml) + CCl ₄ (1% v/v)	60.21
	TLMI AQ (12.5 µg/ml) + CCl ₄ (1% v/v)	65.14
	TLMI AQ (25 µg/ml) + CCl ₄ (1% v/v)	76.98
	TLMI AQ (50 µg/ml) + CCl ₄ (1% v/v)	51.81
	TLMI AQ (100 µg/ml) + CCl ₄ (1% v/v)	46.90
Tender coconut water(TCW)	TCW(6.25 µg/ml) + CCl ₄ (1% v/v)	51.37
	TCW(12.5 µg/ml) + CCl ₄ (1% v/v)	51.53
	TCW(25 µg/ml) + CCl ₄ (1% v/v)	65.77
	TCW(50 µg/ml) + CCl ₄ (1% v/v)	63.57
	TCW(100 µg/ml) + CCl ₄ (1% v/v)	65.61
Tender leaves of <i>Mangifera indica</i> Linn. (Alcoholic extract)	TLMI AL (6.25 µg/ml) + CCl ₄ (1% v/v)	56.82
	TLMI AL(12.5 µg/ml) + CCl ₄ (1% v/v)	67.01
	TLMI AL (25 µg/ml) + CCl ₄ (1% v/v)	83.04
	TLMI AL (50 µg/ml) + CCl ₄ (1% v/v)	70.71
	TLMI AL (100 µg/ml) + CCl ₄ (1% v/v)	50.73
Tender leaves of <i>Mangifera indica</i> Linn + Tender coconut water(Aqueous extract)	TLMI+TCW-AQ (6.25 µg/ml) + CCl ₄ (1% v/v)	80.76
	TLMI+TCW-AQ (12.5 µg/ml) + CCl ₄ (1% v/v)	84.02
	TLMI+TCW-AQ (25 µg/ml) + CCl ₄ (1% v/v)	62.04
	TLMI+TCW-AQ (50 µg/ml) + CCl ₄ (1% v/v)	48.29
	TLMI+TCW-AQ (100 µg/ml) + CCl ₄ (1% v/v)	44.47
Tender leaves of <i>Mangifera indica</i> Linn + Tender coconut water (Alcoholic extract)	TLMI+TCW-AL(6.25 µg/ml) + CCl ₄ (1% v/v)	61.65
	TLMI+TCW-AL(12.5 µg/ml) + CCl ₄ (1% v/v)	69.63
	TLMI+TCW-AL(25 µg/ml) + CCl ₄ (1% v/v)	85.13
	TLMI+TCW-AL(50 µg/ml) + CCl ₄ (1% v/v)	66.47
	TLMI+TCW-AL(100 µg/ml) + CCl ₄ (1% v/v)	46.52

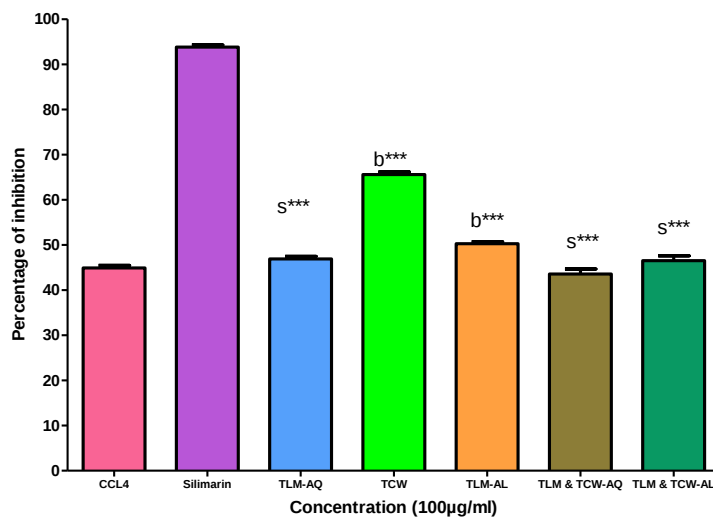
From the table among all the 5 Samples, aqueous extract of tender leaves of *Mangifera indica* Linn, Tender coconut water, alcoholic extract of tender leaves of *Mangifera indica* Linn, alcoholic extract of combination of tender leaves of *Mangifera indica* Linn and tender coconut water showed maximum percentage viability at 25 μ g/ml concentration. Aqueous extract of combination of tender leaves of *Mangifera indica* Linn showed maximum percentage viability at 12.5 μ g/ml concentration. Standard drug silymarin showed maximum viability at 100 μ g/ml concentration.



Figno.1 A) Normal control cells B)Carbon tetrachloride(CCL41%) treated C) Silymarin treated at 25 μ g/ml concentration D) Protection of HepG2 cells treated with aqueous extract of TLMI at 25 μ g/ml concentration E) Protection of HepG2 cells treated with TCW at 25 μ g/ml concentration F)Protection of HepG2 cells treated with alcoholic extract of TLMI at 25 μ g/ml concentration G) Protection of HepG2 cells treated with alcoholic extract of TLMI and TCW at 25 μ g/ml concentration H) Protection of HepG2 cells treated with aqueous extract of TLMI and TCW at 25 μ g/ml concentration.



Graph 1:-Cell viability at 6.25µg/ml concentration of different treated groups with error bars**Graph 2:-**Cell viability at 12.5µg/ml concentration of different treated groups with error bars**Graph 3:-**Cell viability at 25µg/ml concentration of different treated groups with error bars**Graph 4:-**Cell viability at 50µg/ml concentration of different treated groups with error bars



Graph 5:-Cell viability at 100µg/ml concentration of different treated groups with error bars.

P value	CCL4	Silimarin	Both CCL4 and Silimarin
<0.05	C*	S*	b*
<0.01	C**	S**	b**
<0.001	C***	S***	b***

The results showed that, alcoholic extract of tender leaves of MI ($P < 0.05$) and , alcoholic extract of combination of tender leaves of MI + tender coconut water possess more hepato-protective effect at 25 µg/ml concentration ($P < 0.01$), aqueous extract of tender leaves of MI, Tender coconut water ($P < 0.05$), aqueous extract of combination of tender leaves of MI + tender coconut water possess more hepato protective effect at 12.5 µg/ml concentration ($P < 0.01$) when compared to positive control Silymarin. All the drugs showed their maximum protective effect at 25 µg/ml concentration except aqueous extract of combination of tender leaves of MI +tender coconut water, which showed maximum viability at 12.5 µg/ml concentration when compared to each other. The percentage of viability is more in combination. It is revealed that percentage of viability is more at lower concentration of aqueous extract of the combination of tender leaves of MI +tender coconut water than that of positive control silymarin.

The main mechanism involved in the protection may be associated with its strong capability to reduce the intracellular level of reactive oxygen species. The leaves of *Mangifera indica* Linn. is reported to have antioxidant property [8]. The effective components which might act against oxidative damage are mainly alkaloids, phenolic compounds, flavonoids, saponins and tannins present in the tender leaves of M.indica. Tender coconut water is reported to have a free amino acid, L-arginine has shown significant anti oxidant activity. Also the ascorbic acid present in the TCW significantly reduces lipid peroxidation.

In *Bhavaprakasha nighantu* (An important lexicon of ayurveda) it is mentioned that *Amrapallava* (Tender leaves of *Mangifera indica* Linn) is having *kaphapitha samana* property and *Nalikerodaka* (tender coconut water) is having *vatapittahara* property. In *Kamala* (jaundice) also all these doshas are involved.

Hence it is proven by cell line (HepG2) study that, the combination of tender leaves of *Mangifera indica* Linn. and tender coconut water is a promising drug in hepato-biliary disorder and hepatotoxicity; which needs more scientific validation to develop as a valuable medicine.

Conclusion:-

The present study “Hepato protective effect of combination of tender leaves of *Mangifera indica* Linn. and Tender coconut water in Hep G2 cells” can be concluded as follows

All the test drugs , aqueous extract of tender leaves of *Mangifera indica* Linn (25µg/ml concentration, $P < 0.05$) Tender coconut water (25µg/ml concentration, $P < 0.05$), alcoholic extract of tender leaves of *Mangifera indica* Linn

(25µg/ml concentration, $P<0.01$), aqueous extract of combination of tender leaves of *Mangifera indica* Linn+ tender coconut water(12.5µg/ml concentration, $P<0.001$) and alcoholic extract of combination of tender leaves of *Mangifera indica* Linn + tender coconut (25µg/ml concentration, $P<0.01$) water possess potent hepato-protective effect.

Aqueous extract of combination of tender leaves of *Mangifera indica* Linn + tender coconut water possess strong hepato-protective effect at lower concentration than that of positive control silymarin.

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