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RESEARCH ARTICLE

ANTIBACTERIAL AND CYTOTOXICITY STUDIES OF GOSSYPOL ISOLATED FROM FRUITS OF THESPESIA POPULNEA (L.) SOL. EX CORREA- A REMEDY FOR SKIN DISEASES

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Abstract

Skin infections caused by *Staphylococcus aureus* and *Streptococcus pyogenes* and dermatophytoses are becoming severe nowadays. In a previous study, gossypol from fruits of Thespesia populnea (L.) Sol. ex Correa was found to possess growth-inhibiting activity against dermatophytes, causing dermatophytoses, the major fungal disease of the skin (Anuthara et al., 2021). There may be a chance of secondary bacterial infection in the case of dermatophytoses. Hence the present study was designed to evaluate the activity of gossypol against *Staphylococcus aureus* and *Streptococcus pyogenes*, the major bacterial pathogens of the skin. Gossypol (GP) was isolated from the fruits of T. populnea and was identified and characterised by HPLC, HPTLC, FTIR, LCMS and NMR spectroscopy in a previous study (Anuthara et al., 2021). Then checked its antibacterial activity against *Staphylococcus aureus* and *Streptococcus pyogenes*. The MIC and MBC of gossypol were 31.25 µg/mL and 62.50 µg/mL for *Staphylococcus aureus*. The MIC of gossypol against *Streptococcus pyogenes* was 125 µg/mL. It was not bactericidal at the tested concentration. The antibacterial study was done by the disc diffusion method, and it showed inhibition against the growth of two pathogens tested. The minimum inhibitory concentration (MIC) was determined according to the CLSI (clinical and laboratory standards Institute) method- M07-A9 with slight modification (CLSI, 2012b). Since GP showed good inhibition activity against bacterial pathogens, the cytotoxicity of the compound was also tested towards the goal of developing a new antibacterial preparation. Cytotoxicity of gossypol has been evaluated by direct microscopic observation and MTT assay. The apoptosis was detected by acridine orange (AO) and ethidium bromide (EtBr) double staining method.

Introduction:-

Plants having a recognised medical use are referred to as medicinal plants. Various extracts of these plants are employed in healthcare and treating multiple illnesses. The phytochemical constituents present in these plants are responsible for their curative property. They are generally considered safe for human beings and with less toxicity and side effects. Hence, natural compounds have gained immense attention recently since synthetic drugs contribute to severe side effects. Although some researches present the antibacterial and antifungal activity of crude extracts of

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various plant parts, it is highly demanded to isolate the active principle behind this action. Thespesia populnea (L.) Sol. ex Correa. (shortly T.populnea) belongs to the family Malvaceae. Various parts of T.populnea is known for their properties like antibacterial, antifungal, antioxidant, antipsoriatic activities (Shrivastav et al .,2009, Siju et al., 2011). The leaves are used for the anti-inflammatory treatment of inflamed and swollen joints (Nadkarni, 1976). The tribal people use the yellow sap from the cut surface of fruits of T.populnea for curing various skin diseases. The yellow sap has been identified as gossypol (Srivastva et al.,1963).

Thespesia populnea (L.) Sol. ex Correa commonly known as 'Indian tulip tree' or 'Portia tree' of the Malvaceae family, is a small to medium-sized tree with a pantropical distribution, generally found along the coastal stretches. It is a mangrove associate, which provides shelter and food to many creatures of the mangroves (Peter and Sivasothi, 1999; Corner, 1997). The bark, fruits, leaves, and flowers are helpful in cutaneous infections, such as scabies, eczema, psoriasis, ringworm, guinea worm and inflammation. In South India, the yellow sticky sap secreted from the cut surface of young fruits is traditionally used to treat ringworm and other skin diseases.

The leaves are used for the anti-inflammatory treatment of inflamed and swollen joints (Nadkarni, 1976). Lukefahr and Fryxell (1967) reported the occurrence of gossypol in genera related to cotton and concluded a correlation between pigment glands and gossypol. In this context, Thespesia populnea was also reported containing pigment glands and gossypol. King and de-Silva reported the presence of +gossypol along with + gossypol in the bark and flowers of Thespesia populnea. The fruit kernels were reported to contain a yellow pigment thespesin, which was later reported as gossypol by Srivastva et al.,1963. The common genera that cause skin infection in humans are *Staphylococcus aureus* and *Streptococcus pyogenes*. The diseases caused by these bacteria include folliculitis, cellulitis, furuncles, carbuncles, ecthyma, erysipelas- a severe form of cellulitis, and staphylococcal scalded skin syndrome. They cause necrotising fasciitis or flesh-eating disease. It starts as a small wound in the skin and spreads rapidly into the surrounding tissues causing severe disfigurement and sometimes even death. The present study has been designed to isolate gossypol from the fruits of Thespesia populnea and evaluate its antibacterial and cytotoxic activity.

Material And Methods:-

Preparation of plant material

The plant Thespesia populnea was collected from Marayoor, Idukki, Kerala, from December to April. Thespesia populnea has been authenticated by the plant taxonomist Dr Jomy Augustine, St.Thomas College, Palai, Kottayam, Kerala, and a voucher specimen AR 4752 is maintained at the Institute. The fruits are washed thoroughly in running tap water, shade dried, powdered in a blender and sieved separately. This powder has been used for the isolation of gossypol.

Isolation, purification and characterisation of gossypol from T. Populena was in a previous study in our lab (Anuthara et al. 2021)

Antibacterial activity of gossypol

The antibacterial activity of gossypol was performed by disc diffusion method CLSI M02-A11 (CLSI, 2012a) on Mueller- Hinton agar. Gossypol was diluted in DMSO (0.5mg/mL). Sterile discs (HiMedia) were impregnated with 20 µl of diluted gossypol and DMSO. Streptomycin was used as a positive control. The bacterial inocula were prepared in nutrient broth and Brain-Heart Infusion broth for *Staphylococcus aureus* (MTCC 1430) and *Streptococcus pyogenes* (MTCC 3160). They were incubated for 6 h at 37^0C, and the turbidity was adjusted to 0.5 McFarland turbidity standard. Lawn cultures of the bacteria were prepared on Mueller- Hinton agar media (HiMedia). Sterile discs impregnated with test material, antibiotic streptomycin, and DMSO were dispensed onto the inoculated plates at an appropriate distance and incubated for 24h at 37^0C. The zones of inhibition were observed, measured and compared with that of standard drug. The interpretation was made as follows

The diameter of the zone of inhibition is ≥ 20mm susceptible, 15-19 mm intermediate sensitive, and ≤ 14mm Resistant.

Determination of minimum inhibitory concentration

MIC (minimum inhibitory concentration) is the lowest concentration of an antimicrobial agent that prevents the visible growth of a microorganism in the broth dilution susceptibility test. It is determined by the broth microdilution method.

The inoculum was prepared according to the CLSI (clinical and laboratory standards Institute) method- M07-A9 with slight modification (CLSI, 2012b). The medium used was Mueller-Hinton Broth. The test was done within the concentration range of 1.953µg/mL to 1000 µg/mL of medium (doubling dilutions). Gossypol was dissolved in DMSO and Streptomycin in sterile distilled water, and dilutions were prepared. MIC was determined by transferring 10 µl from the well with no growth and subculturing on respective agar plates and incubated at 37^0C overnight.

Statistical analysis
All the data were expressed as mean + standard deviation (n =3). The results were analysed by one way ANOVA, followed by Tukey's Post hoc analysis using GraphPad Prism version 5.03 for Windows (Graphpad Software, San Diego, CA, USA). A value of $p < 0.05$ was considered statistically significant.

Determination of in-vitro cytotoxic effects of GP.
RAW 264.7 (macrophage) cells were initially procured from National Centre for Cell Sciences (NCCS), Pune, India and maintained in Dulbecco's modified Eagles medium, DMEM (Sigma-Aldrich, USA).

The cell line was cultured in a 25 cm^2 tissue culture flask with DMEM supplemented with 10% FBS, L-glutamine, sodium bicarbonate (Merck, Germany) and an antibiotic solution containing: Penicillin (100U/ml), Streptomycin (100µg/ml), and Amphotericin B (2.5µg/ml). Cultured cell lines were kept at 37ºC in a humidified 5% CO_2 incubator (NBS Eppendorf, Germany). The viability of cells was evaluated by direct observation of cells by Inverted phase-contrast microscopy followed by the MTT assay method.

Cells seeding in 96 well plates:
Two days-old confluent monolayers of cells were trypsinised. The cells were suspended in a 10% growth medium, 100µl cell suspension ($5x10^3$ cells/well) was seeded in 96 well tissue culture plate and incubated at 37ºC in a humidified 5% CO_2 incubator.

Preparation of compound stock:
1mg of the sample was weighed and dissolved in 1mL DMEM using a cyclomixer. The sample solution was filtered through a 0.22 µm Millipore syringe filter to ensure sterility.

Cytotoxicity Evaluation:
After 24 hours, the growth medium was removed. A freshly prepared compound in DMEM was five times serially diluted by two-fold dilution (100µg, 50µg, 25µg, 12.5µg, 6.25µg in 500µl of DMEM) and 100mL of each concentration was added in triplicates to the respective wells and incubated at 37ºC in a humidified 5% CO_2 incubator. Non treated control cells were also maintained.

Cytotoxicity Assay by Direct Microscopic observation:
The entire plate was observed after 24 hours of treatment in an inverted phase-contrast tissue culture microscope (Olympus CKX41 with Optika Pro5 CCD camera), and microscopic observation was recorded as images. Changes in the morphology of the cells (such as rounding or shrinking of cells, granulation and vacuolisation in the cells' cytoplasm) are considered an indication of cytotoxicity.

Cytotoxicity Assay by MTT Method:
Fifteen mg of MTT (Sigma, M-5655) was reconstituted in 3 ml PBS until completely dissolved and sterilised by filter sterilisation. After 24 hours of incubation, the sample content in wells was removed, and 30µl of reconstituted MTT solution was added to all test and cell control wells. The plate was gently shaken well, then incubated at 37ºC in a humidified 5% CO_2incubator for 4 hours. After the incubation period, the supernatant was removed, and 100µl of MTT Solubilisation Solution (Dimethyl sulphoxide: DMSO, Sigma Aldrich, USA) was added, and the wells were mixed gently by pipetting up and down to solubilise the formazan crystals. The absorbance values were measured using a microplate reader at a wavelength of 540 nm (Laura, 2013).

LC50 value was calculated using ED50 PLUS V1.0 Software as 102.73005µg/mL.
The percentage of growth inhibition was calculated using the formula:

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

Detection of apoptosis by acridine orange (AO) and ethidium bromide (EtBr)double staining.
principle
DNA-binding dyes Acridine Orange (AO) and Ethidium Bromide (EtBr) (Sigma, USA) were used for the morphological detection of apoptotic and necrotic cells (Zhang et al., 1998). AO is taken up by both viable and non-viable cells and emits green fluorescence if intercalated into a double-stranded nucleic acid (DNA). EtBr is taken up only by non-viable cells and emits red fluorescence intercalation into DNA.

The effect of GP on LPS stimulated RAW 264.7 cells was determined by dual staining with Acridine orange and ethidium bromide (A.O./EtBr). After attaining confluence, the cells were stimulated with lipopolysaccharide (LPS) for 1 hour and added the LC50 concentration of the sample(102.73005µg/mL). LPS stimulated cells and untreated control cells were also maintained and incubated for 24 hours; the cells were washed by cold PBS and then stained with a mixture of AO (100 µg/ml) and EtBr (100 µg/ml) at room temperature for 10min. The stained cells were washed twice with 1X PBS and observed by a fluorescence microscope in the blue filter of the fluorescent microscope (Olympus CKX41 with Optika Pro5 camera).

The cells were divided into four categories as follows: living cells (normal green nucleus), early apoptotic (bright green nucleus with condensed or fragmented chromatin), late apoptotic (orange-stained nuclei with chromatin condensation or fragmentation) and necrotic cells (uniformly orange-stained cell nuclei) (Zhang et al., 1998).

Results:-

Antibacterial activity of gossypol
The zones of inhibition exhibited by gossypol were 27mm for *Staphylococcus aureus* and 26 mm for *Streptococcus pyogenes*, respectively. At the same time, the standard drug streptomycin showed 24mm for *Staphylococcus aureus* and 20mm for *Streptococcus pyogenes*. The MIC and MBC of GP were 31.25µg/mL and 62.50 µg/mL for *Staphylococcus aureus*. The MIC of GP against *Streptococcus pyogenes* was 125µg/mL. It was not bactericidal to *Streptococcus pyogenes* at the tested concentration. There was no growth in the wells inoculated with streptomycin.

A B

Figure 1:- Antibacterial activity of GP against *Staphylococcus aureus* and *Streptococcus pyogenes* by Disc diffusion method.
A: *Staphylococcus aureus* B: *Streptococcus pyogenes*,
G: Gossypol, S: Streptomycin, D: DMSO.

Table 1:- Antibacterial activity of GP by disc diffusion method.

Test material	Zone of inhibition in mm	
	Staphylococcus aureus	*Streptococcus pyogenes*
Gossypol	27 (S*)	26(S*)
Streptomycin	24(S*)	20(S*)
DMSO	0(R*)	0(R*)

* (R): Resistant, (S): Sensitive

Table 2:- Inhibition of growth of bacteria by different concentrations of the test material.

Bacteria	Test Material	The concentration of test material in µg/mL									
Staphylococcus aureus	G.P.	-	-	-	-	-	+	+	+	+	+
	Streptomycin	-	-	-	-	-	-	-	-	-	-
Streptococcus	GP.	-	-	-	+	+	+	+	+	+	+

pyogenes	Streptomycin	-	-	-	-	-	-	-	-	-	-

Table 3:- MIC and MBC of test materials against S. aureus and S.pyogenes.

Bacteria	MIC& MBC value in µg/mL	
	Gossypol	Streptomycin
Staphylococcus aureus	31.25/ 62.50	No growth
Streptococcus pyogenes	125 /ND*	No growth

N.D.* - Not determined

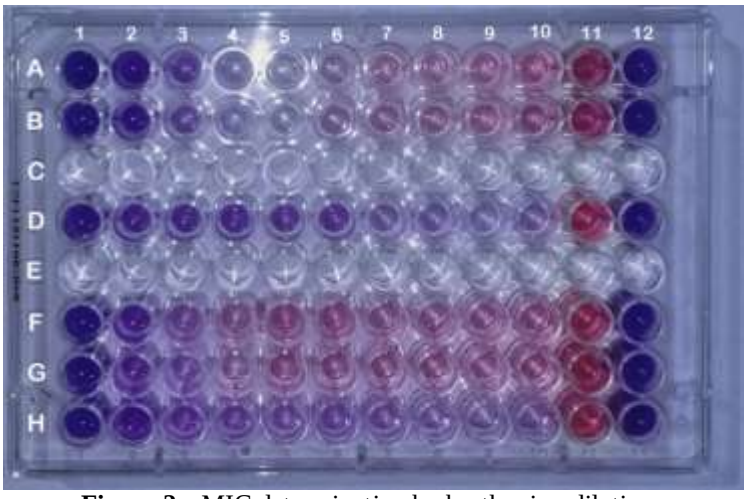


Figure 2:- MIC determination by broth microdilution.

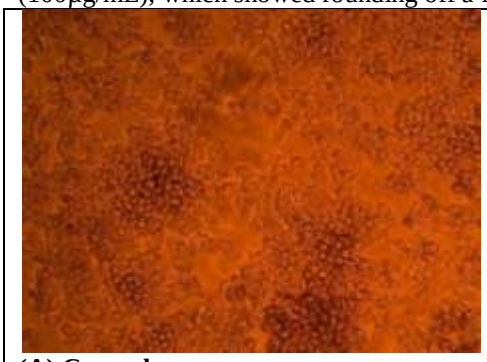
A: *Staphylococcus aureus*, B: *Streptococcus pyogenes*

Rows A and B: *Staphylococcus aureus* with GP, Row D: *Staphylococcus aureus* with streptomycin, Rows F and G: *Streptococcus pyogenes* with GP, Row H: *Streptococcus pyogenes* with streptomycin, Column 11: positive growth control without drug/GP, Column 12: only MHB and indicator, no organism.

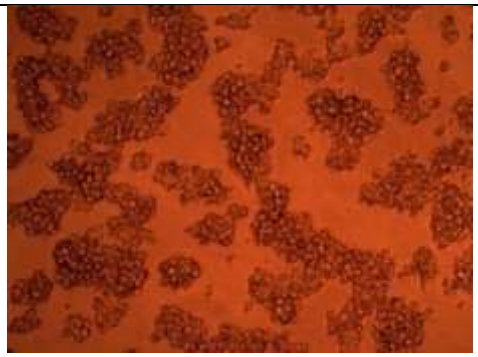
Determination of cytotoxicity of GP.

Direct Microscopic observation:

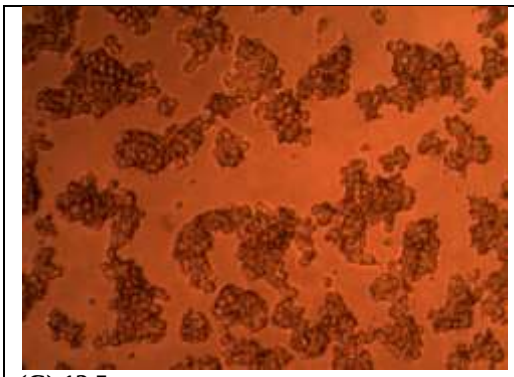
The entire plate was observed after 24 hours of treatment in an inverted phase-contrast tissue culture microscope (Olympus CKX41 with Optika Pro5 CCD camera), and microscopic observation was recorded as images (Figure 3). Upon exposure to different concentrations of the test compound GP, the cells did not show any morphological changes such as rounding or shrinking of cells, granulation and vacuolisation in the cytoplasm of the cells. The cells appeared the same as the untreated control cells at concentrations except at the highest concentration of GP (100µg/mL), which showed rounding off a few cells.



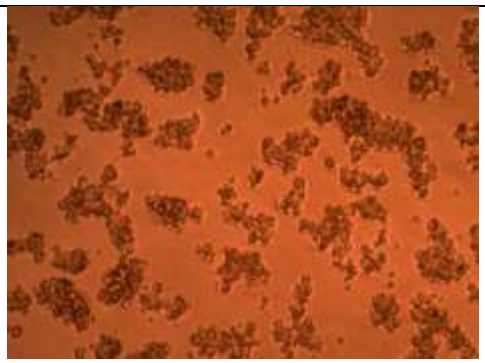
(A) Control



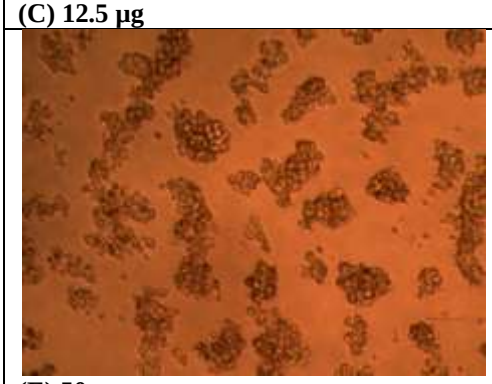
(B) 6.25 µg



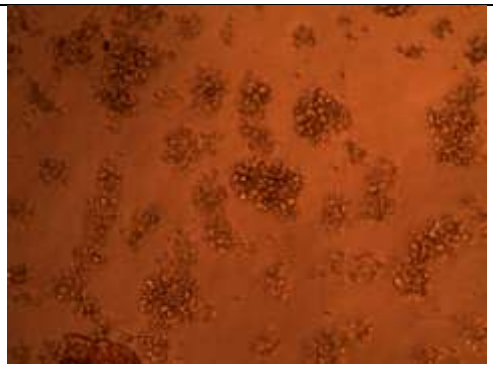
(C) 12.5 µg



(D) 25 µg



(E) 50 µg



(F) 100 µg

Figure 3:- (B) to (F): Phase contrast tissue culture microscopic image of RAW 264.7macrophage cells treated with GP and untreated control cells (A).

MTT Method:

The cytotoxic effect of gossypol on RAW264.7 cells was determined by MTT assay.
The following formula calculated the percentage viability of cells

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

The test compound presented no significant effect on the viability of RAW 264.7macrophage cells except in the higher concentration. The cells showed a percentage viability of 87.96 at the lowest tested concentration of GP (6.25µg/mL). At concentration 12.5 µg/mL, the percentage viability was 75.9. At the highest concentration (100 µg/mL), GP was reduced to 54.57. The control and tests were done in triplicate, and the results are expressed as mean + SD, where n = 3. LC50 value was 102.73005µg/mL (Calculated using ED50 PLUS V1.0 Software).

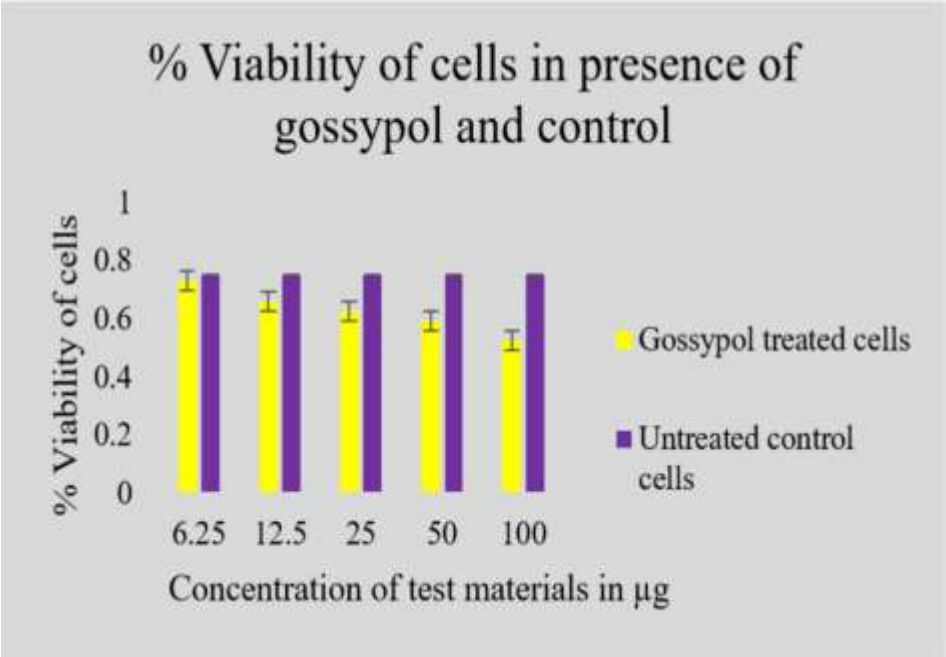
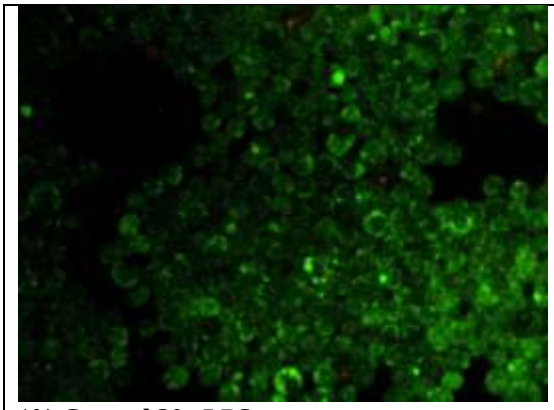


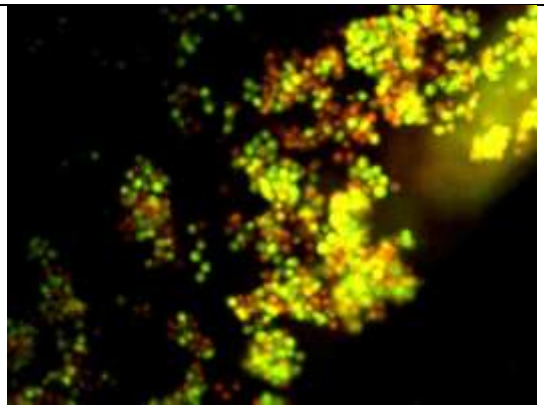
Figure 4:- % Viability of cells in the presence of GP and control.

Determination of apoptosis by acridine orange (AO) and ethidium bromide (EtBr) double staining.
A fluorescence microscope observed the stained cells in the blue filter of the fluorescent microscope (Olympus CKX41 with Optika Pro5 camera), and the following observations were made.

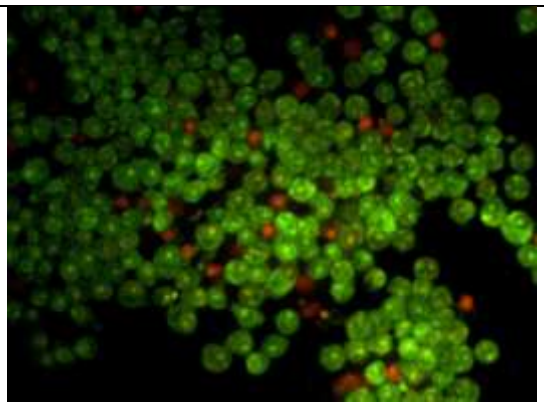
The control cells showed a normal green nucleus, which indicates the presence of living cells. The cells treated with LPS and sample GP showed a few bright green nuclei, which indicate early apoptotic cells and very few numbers of orange-stained nuclei, which indicate apoptotic cells. This result explains minimum apoptosis with the tested compound.



(A) Control 20x LPS



(B)LPS



(B) LPS + S 20x

Figure 5:- Fluorescence microscope image of untreated control cells, (B): cells treated with LPS, and (C): Cells treated with LPS and samples showing intact live cells, early apoptotic cells, and significantly reduced the number of dead cells.

Discussion:-

The common skin infections caused by *Staphylococcus aureus* include wound infection, cellulitis, impetigo, and SSSS (Staphylococcal Scalded Skin Syndrome). Usually, these bacteria are present on the surface of the skin or inside the nose of many people. Nevertheless, those do not cause any illness. However, if the bacteria get a chance to get inside the body, it may affect the skin and cause infection. Low p^H, high salt content, Keratinized surface and presence of lysozyme make the skin favourable for the growth of bacteria.

Streptococcus can cause infections like impetigo, cellulitis, erysipelas, necrotising fascitis, ecthyma, secondary skin infections of wounds, dermatitis, blistering distal dactylitis, diabetic ulcers, scabies, streptococcal perianal or vulval dermatitis. Margalith (1967) has reported the inhibitory effect of gossypol from cottonseed meal against many bacteria and some fungi. they include *Staphylococcus aureus*, Bacillus megaterium, B.cereus, B. Licheniformis, B. polymyxa, B. Thermoacidurans, E coli, Proteus mirabilis, Leuconostoc mesenteroide, Pseudomonas aeruginosa and Sarcina lutea. It can cause severe complicated infections in patients with impaired immunity and progress into a systemic infection. Vadehra et al. (1985) has also reported that gossypol is a potent antibacterial agent which inhibits many gram-positive bacteria such as *Staphylococcus aureus*, *Streptococcus* spp., and Bacillus spp.

Cytotoxicity of GP was determined by direct microscopy and MTT assay. It is the most suitable method used to determine mitochondrial dehydrogenase activity in the living cells, where the chemical MTT is reduced to formazan, a purple coloured product by NADPH. In this study, a dose-dependent approach was used to evaluate the cytotoxicity of GP on RAW-264.7 macrophage cells at different concentrations; 6.25, 12.5, 25, 50, and 100µg/mL. GP showed the highest percentage of viability (87.95%) at 6.25µg/mL concentration, and the cells did not show any morphological changes. In the highest concentration (100 µg/mL), the percentage viability was reduced to 54.57%. Effect of GP on cell apoptosis was conducted by acridine orange (AO) and ethidium bromide (EtBr) double staining under a fluorescence microscope. The result showed most live cells and only a few apoptotic cells. Huo et al. (2013) have tested the effect of GP on cell viability by MTT assay in the presence and absence of (lipopolysaccharide) LPS. They found that the cell viability was not affected by GP at concentrations 15, 30, and 45 µg/mL. The secondary bacterial infections associated with other skin diseases produce inflammation. Gossypol has been reported for its anti-inflammatory activity (Chen et al.,2018). Hence, these cumulative properties of gossypol make it a promising candidate for developing a new drug.

Conclusion:-

The fruits of Tp may contain an enormous number of compounds other than GP However; there may be a lesser chance for cytotoxicity due to the presence of these components since the traditional use of the yellow exudate from fruits of the same by the tribal people is popular. For the formulation of a topical preparation, local toxicity tests like dermal toxicity study (in rabbit/rat), photo-allergy/ dermal phototoxicity (in guinea pig), vaginal toxicity test, rectal tolerance test (in rabbit/dog), allergenicity/hypersensitivity tests (in guinea pig/mouse) must be performed. According to European Medicines Agency, in vitro release test (IVRT) for semi-solid drug products like creams,

832 gels or ointments and liquid suspensions, in vitro skin permeation studies (IVPT), pharmacokinetic bioequivalent studies, in vivo stratum corneum sampling (or tape stripping (TS) for the semi-solid formulation and vasoconstriction assay for corticosteroids must be followed. Clinical trial formulation and batches used in the comparative study must be described in detail. This procedure should be followed by an in vivo pilot dose duration-response study with relevant human volunteers for pilot and pivotal studies. All the reactions of all tests should be determined at baseline (before drug application), time of drug product removal, and several times after drug product removal (e.g. 2,4,6,19,24 hours) followed by a secondary clinical assessment has to be done (European Medicines Agency, 2018).

The present study revealed the active principle of the yellow exudate from fruits, which cure skin infection, especially dermatophytoses. Even though the in vitro cytotoxicity studies of GP showed not much toxicity, further in vivo studies have to be conducted for determination of its suitability to be used as a topical agent or an oral drug. So, GP can be recommended to be developed as an agent for bacterial skin diseases.

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