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Antioxidant, Antibacterial and Anti-Inflammatory Activity of *Acalypha indica* and *Terminalia* chebula: An *In-Vitro* Analysis.

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ABSTRACT

Traditional medicinal plants and herbs have been a pivotal part of treatment for many centuries. *Acalypha indica* (Euphorbiaceae) and *Terminalia chebula* possess high medicinal values and are used traditionally to cure various ailments for human beings. In this study, antioxidant activity, antibacterial activity, anti-inflammatory activity, cytotoxicity and apoptosis were evaluated. The methanolic extract of *Acalypha indica* showed 54 percent inhibition when compared to *Terminalia chebula,* which showed 43 percent of inhibition. *Terminalia chebula* has a zone of inhibition of 0.9 cm against *Klebsiella pneumoniae*, *Proteus mirabilis, Lactobacillus acidophilus* and *Salmonella enterica* and hence *Terminalia chebula* has more antibacterial activity than *Acalypha indica*. The MTT assay showed percentage inhibition in dose dependant manner. Also the percentage viability decreased as concentration increased when tested for the cyotoxicity of *Acalypha indica* when compared with doxorubicin.The DAPI staining showed that apoptosis is maximum at the 24th hour.

Keywords: Medicinal plants; anti-bacterial; anti-inflammatory; cytotoxicity.

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INTRODUCTION

Inflammation is a part of the complex biological response of vascular tissues to harmful stimuli, such as pathogens, damaged cells or irritants. The classical signs of acute inflammation are pain, heat, redness, swelling, and loss of function. Inflammation is a protective attempt by the organism to remove the injurious stimuli and to initiate the healing process. Inflammation is a stereotyped response, and therefore it is considered as a mechanism of innate immunity, as compared to adaptive immunity, which is specific for each pathogen. Traditional plants have been used over conventional drugs as a cure for inflammation for many years due to their less side effects. Many traditional plants have been reported to have anti-inflammatory activity such as curcuma longa L [1].

Acalypha indica commonly known as Indian Acalypha belongs to the family Euphorbiaceae. It is a common annual herb, found mostly in the backyards of houses and waste places throughout the plains of India. *Acalypha indica* leaves have significant antibacterial activity against both gram-negative and gram-positive bacteria [2]. The plant is reported to have a post-coital antifertility effect [3] , anti-venom properties [4] , wound healing effects [5], antioxidant activities [6] , anti-inflammatory effects acaricidal effects [7] , diuretic effects [8] and anti-bacterial activities [9].

Terminalia chebula another medicinal plant which belongs to the family combretaceae is used in herbal formulations like triphala. *Terminalia chebula* is a medium to large deciduos tree found in southern Asia mostly. The dried fruits of *Terminalia chebula* has been reported to have hepatoprotective activity [10] , antifungal [11] and antispasmodic activity [12]. In the present study, we investigated the antioxidant, antibacterial and anti-inflammatory activity of *Acalypha indica* and *Terminalia chebula*. We also checked for the cytotoxicity and apoptosis of *Acalypha indica* leaf extract on raw 264 cells.

MATERIALS AND METHODS

Plant Materials:

Fresh leaves of *Acalypha indica* and *Terminalia chebula* seeds were collected from a small farm situated 3 kms from VIT university campus. The leaves and seeds were washed thoroughly with tap water to remove dirt and dust, and then shade dried for 5 days. The shade dried leaves and seeds were coarsely ground into a fine powder with the help of a mixer grinder.

Preparation of plant extract

The shade dried coarsely powdered leaves were subjected to Soxhlet extraction using hexane, acetone and methanol.

Antioxidant assay

The antioxidant activity of the extracts was determined by the DPPH radical scavenging assay with minor modifications [13]. Experiments were carried out in triplicate. Briefly, 10 mg/l solution of DPPH radical (Sigma aldrich) in methanol was prepared and then 1 ml of this solution was mixed with different extracts of sample solution (100 µl). After 30 min, the absorbance was measured at 517 nm. Decrease in the absorbance of the DPPH solution indicates an increase of the DPPH antioxidant activity. Inhibition of DPPH of the sample extract was determined by the formula:

$$\%\ inhibition = \frac{A_{blank} - A_{sample}}{A_{blank}} \times 100$$

Total polyphenol estimation

The total polyphenol content was estimated by the method of Singleton with minor modifications [14]. To 0.25 ml of the sample 0.5 ml of Folin's reagent was added followed by 0.5 ml sodium carbonate solution and 0.25 ml distilled water, and then the mixture was incubated for 30 min and absorbance was

recorded at 700 nm. Total polyphenol content was measured using Folin–Ciocalteu colorimetric method. Gallic acid was used as a reference for constructing the standard curve. The results were expressed as mg of gallic acid equivalents (GAE) / g of extract.

Antibacterial assay

Agar well diffusion method

Petriplates containing 20 ml LB medium were seeded with 24 hr culture of bacterial strains. Wells were cut and 20 µl of the plant extracts (namely hexane, methanol and acetone extracts) were added. The plates were then incubated at 37 °C for 24 h. The antibacterial activity was assayed by measuring the diameter of the inhibition zone formed around the well. Chloramphenicol disc was used as a positive control.

Minimum inhibitory concentration

Microdilution method

The minimum inhibitory concentration (MIC) was determined by micro dilution method. The methanol extracts were diluted to get series of concentrations from 0.0195 mg/ µl to 10 mg/ µl in sterile nutrient broth. The microorganism suspension of 10 µl was added to the broth dilutions. These were incubated for 24 h at 37 ºC. MIC of each extract was taken as the lowest concentration that did not give any visible bacterial growth.

Anti-inflammatory assay

Inhibition of albumin denaturation

The inhibition of albumin denaturation assay was done according to Mizushima with minor modifications [15]. The reaction mixture was consisting of test extracts at different concentrations and 1% aqueous solution of bovine albumin fraction. pH of the reaction mixture was adjusted using 1N HCl. The samples were incubated at 37 °C for 20 min and then heated at 57 °C for 20 min. After cooling the samples, the turbidity was measured spectrophotometrically at 660 nm. The experiment was performed in triplicate. Percent inhibition of protein denaturation was calculated as follows:

$$\%\ inhibition = (Abs_{control} - Abs_{sample}) \times 100 \Big/ Abs_{control}$$

Proteinase inhibition

The reaction mixture (2 ml) contains 0.06 mg trypsin, 1 ml of 20 mM Tris HCl buffer (pH 7.4) and 1 ml test sample of different concentrations. The mixture was incubated at 37 ºC for 5 min and then 1 ml of 0.8% (w/v) casein was added. The mixture was incubated for an additional 20 min. 2 ml of 70% perchloric acid was added to terminate the reaction. Cloudy suspension was centrifuged and the absorbance of the supernatant was read at 210 nm against buffer as blank. Percent inhibition of protein denaturation was calculated as follows:

$$\%\ \text{inhibition} = (Abs_{control} - Abs_{sample}) \times 100 \Big/ Abs_{control}$$

Anti-proliferative assay

The MTT assay is based on the protocol described for the first time by Mossmann with minor modifications [16]. The assay was optimized for the cell lines used in the experiments. Briefly, for the purposes of the experiments at the end of the incubation time, cells were incubated for 4 h with 0.8 mg/ml of MTT, dissolved in serum free medium (MEM or DMEM). Washing with PBS (1 ml) was followed by the addition of DMSO (1 ml), gentle shaking for 10 min, so that complete dissolution was achieved. Aliquots (200) of the

resulting solutions were transferred in 96-well plates and absorbance was recorded at 560 nm using the microplate spectrophotometer system (Spectra max190-Molecular Devices).

Cytotoxicity assay

The cells were grown in a 96-well plate in Duelbacco Minimum Eagle's Medium (DMEM) (HiMedia) supplemented with 10% fetal bovine serum (Gibco Laboratories) and antibiotics (streptomycin, penicillin-G, kanamycin, amphotericin B). About 25 µL cell suspension (5 x 10^3 cells/well) was seeded in each 96 well and incubated at 37 ^{0}C for 48 h in 5% CO_2 for the formation of confluent monolayer. The monolayer of cells in the plate was exposed to various dilutions of the plant extract. The cell viability was measured using MTT assay with MTT (5 mg/mL) and 10% DMSO.

This tetrazolium salt is metabolically reduced by viable cells to yield a purple insoluble formazan product measured at 570nm spectrophotometerically. Controls were maintained throughout the experiment (untreated wells as cell control and diluents treated wells as diluent control). The assay was performed in triplicate for each of the extracts. The mean of the cell viability values was compared to the control to determine the effect of the extract on cells and percentage cell viability was plotted against concentration of the plant extract.

RESULTS

The methanolic extract of *Acalypha indica* showed 54 percent inhibition whereas *Terminalia chebula* which showed 43 percent inhibition (fig.1). The amount of polyphenols in *Acalypha indica* was found to be 0.33 mg and in *Terminalia chebula* it was found to be 0.23 mg (fig. 2). *Terminalia chebula* has a zone of inhibition of 0.9 cm against *Klebsiella pneumoniae*, *Proteus mirabilis*, *Lactobacillus acidophilus* and *Salmonella enterica* and 0.8 cm against *Staphylococcus aureus* and *E.coli* whereas *Acalypha indica* showed a zone of inhibition of 0.6 cm against *Klebsiella pneumonia*, 0.4 cm against *Staphylococcus aureus* ,0.7 cm against *Proteus mirabilis* ,0.3cm against *E.coli* and little or no inhibition zone against *Lactobacillus acidophilus* and *Salmonella enterica* (Table 1).

Table 1: Measurement of zone of inhibition in cm for various plant extracts with different microorganisms

Strain name	*Acalypha indica*	*Terminalia chebula*
Klebsiella pneumoniae	0.6	0.9
Staphylococcus aureus	0.4	0.8
Proteus mirabilis	0.7	0.9
Escherichia coli	0.3	0.8
Lactobacillus acidophilus	0.1	0.9
Salmonella enterica	0.12	0.9

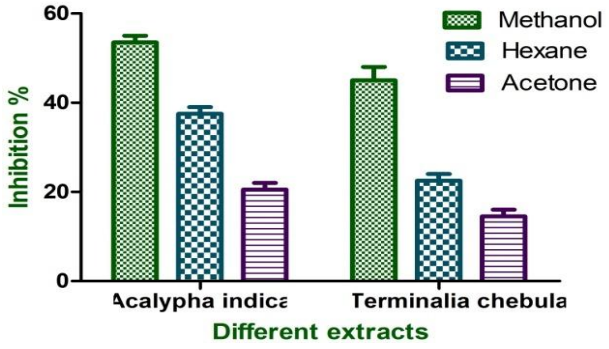

Figure 1: Antioxidant activity for different plants with different extracts

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Fig. 1 shows the antioxidant activity of methanol, hexane and acetone extracts of Acalypha indica and *Terminalia chebula*. The methanolic extract of *Acalypha indica* shows 54 percent inhibition whereas *Terminalia chebula* shows 43 percent inhibition.

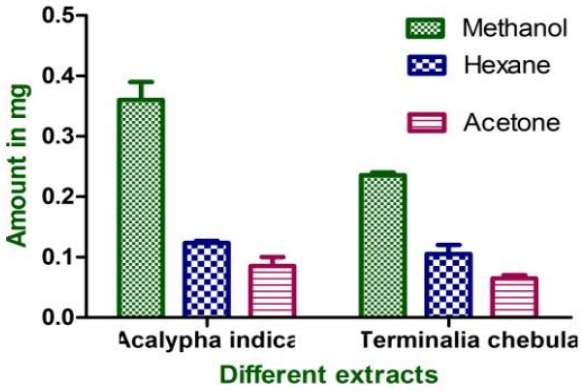

Figure 2: Total polyphenol estimation for different plants with different extracts

Fig. 2 shows the methanol, hexane and acetone extracts of *Acalypha indica* and *Terminalia chebula* and polyphenol amount in mg was plotted. The amount of polyphenols in *Acalypha indica* was found to be 0.33 mg and in *Terminalia chebula* it was found to be 0.23 mg.

The minimum inhibitory concentration was also determined using microdilution method. The methanol extract of *Acalypha indica* showed MIC at 7.625 µg/ml for *Klebsiella pneumoniae,* MIC at 17.45 µg/ml for *Salmonella enterica*, MIC at 1.64 µg/ml for *E.coli* and MIC at 31.033 µg/ml for *Lactobacillus acidophilus*. The methanol extract of *Terminalia chebula* showed MIC at 13.25 µg/ml for *Klebsiella pneumoniae*, MIC at 9.61 µg/ml for *Salmonella enterica* , MIC at 3.82 µg/ml for *E.coli* and MIC at 4.68 µg/ml for *Lactobacillus acidophilus*. All the extracts exhibited antibacterial activity against gram-positive and gram negative organisms with minimum inhibitory concentrations (MIC) between 1.64 to 31.033 µg/ml (Table 2). In the inhibition of albumin denaturation assay, the methanolic extract of *Acalypha indica* shows 79 percentage of inhibition at a concentration of 125 mg/ml whereas *Terminalia chebula* shows 89 percentage of inhibition (fig. 3).

Table 2: Minimum inhibitory concentration for different bacterial stains

Bacterial strains	*Acalypha indica* (in µg/ml)	*Terminalia chebula* (in µg/ml)
Klebsiella pneumoniae	7.624	13.254
Salmonella enterica	17.459	9.613
E.coli	1.64	3.823
Lactobacillus acidophilus	31.033	4.689

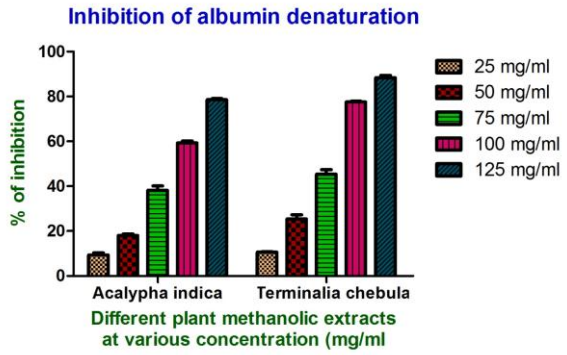

Figure 3: Inhibition of albumin denaturation for different methanolic plant extracts at various concentrations

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Fig. 3 shows the methanolic extract of *Acalypha indica* and *Terminalia chebula* at different concentrations and the % of inhibition of albumin. The methanolic extract of *Terminalia chebula* at 125 mg/ml shows 79 % inhibition when compared to methanolic extract of *Acalypha indica* at 125 mg/ml which shows 89% of inhibition

In case of proteinase inhibition assay, the methanolic extract of *Acalypha indica* shows maximum % of inhibition with 79 % when compared to *Terminalia chebula* which shows 75 % **(fig. 4)**. In the anti-proliferative assay, Acalypha *indica* showed 53 % of inhibition at 4th h with concentration of 500 µg/ml whereas *Terminalia chebula* showed 48 %. At 8th h *Acalypha indica* showed 57 % of inhibition at 500 µg/ml and *Terminalia chebula* 50 %. At 12th h also, *Acalypha indica* showed a higher % of inhibition of 59 % whereas *Terminalia chebula* showed 52 % **(Fig. 5)**.

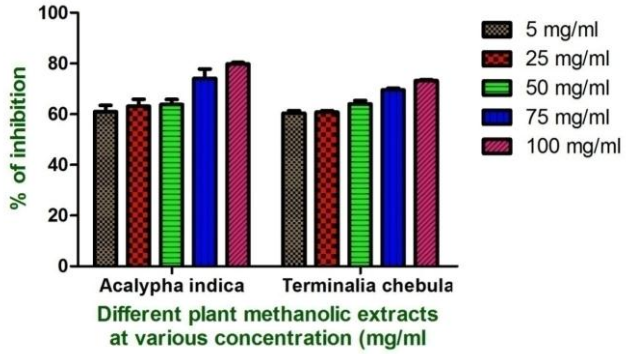

Figure 4: Proteinase inhibition for different methanolic plant extracts at various concentrations

Fig. 4 shows the methanolic extract of *Acalypha indica* and *Terminalia chebula* at different concentrations and the % inhibition of proteinase. The methanolic extract of *Acalypha indica* shows 79 % inhibition at 100mg/ml whereas the methanolic extract of *Terminalia chebula* shows 75% inhibition at 100 mg/ml.

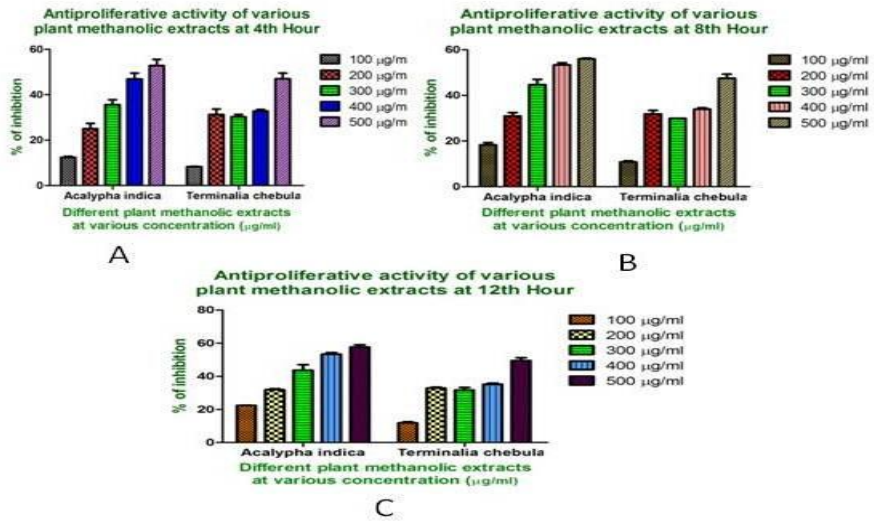

Figure 5: Antiproliferative activity of different methanolic extracts at various time interval

Fig. 5A shows the methanolic extract of *Acalypha indica* and *Terminalia chebula* at different concentrations and % inhibition at 4th hour. The methanolic extract of *Acalypha indica* shows 53 % inhibition at 500ug/ml whereas the methanolic extract of *Terminalia chebula* at 500 µg/ml shows 48 % inhibition. Fig. 5 B shows the inhibition at 8th hour. The methanolic extract of acalypha shows 57 % inhibition at 500ug/ml and the methanolic extract of terminalia chebula show 50% inhibition at 500 µg/ml. Fig. 5 C show the inhibition at 12th

hour. The methanolic extract of acalypha shows 59 % inhibition at 500ug/ml whereas the methanolic extract of *Terminalia chebula* shows 52% inhibition at 500 µg/ml.

The cytotoxicity of the plant extract *Acalypha indica* on Raw 264 cells of 5 concentrations was tested. The percentage of viability was 92%, 73%, 64% and 36% for 25µg/well, 50 µg/well, 100 µg/well and 200 µg/well after 24 h of treatment respectively, whereas in case of control cells (untreated) shows 75% viability even after 24 h of incubation. Since, we were intended to test the antitumor activity of same plant by comparing with commercially available drug "doxorubicin", we found considerable percentage of growth inhibition by the plant extract (Fig. 6).

Figure 6: Comparison of cytotoxcity of *Acalypha Indica* with standard drug at different concentrations

Fig. 6 shows the cytotoxicity of the plant extract *A. indica* on Raw 264 cells of 4 concentrations was tested. The percentage of viability was 92%, 73%, 64% and 36% for 25µg/well, 50 µg/well, 100 µg/well and 200 µg/well after 24 hours of treatment respectively. Where as in case of control cells (untreated) were shown more 75% viability even after 24 hours of incubation.

The acridine orange staining showed that apoptosis is maximum at the 24th h. The cells treated with *Acalypha indica* plant extract and stained with acridine orange were observed at various h (6th h, 12th h and 24th h). As time increases apoptosis happens and at the 24th h apoptosis can be observed at its maximum with chromosomal condensation and fragmentation (fig. 7).

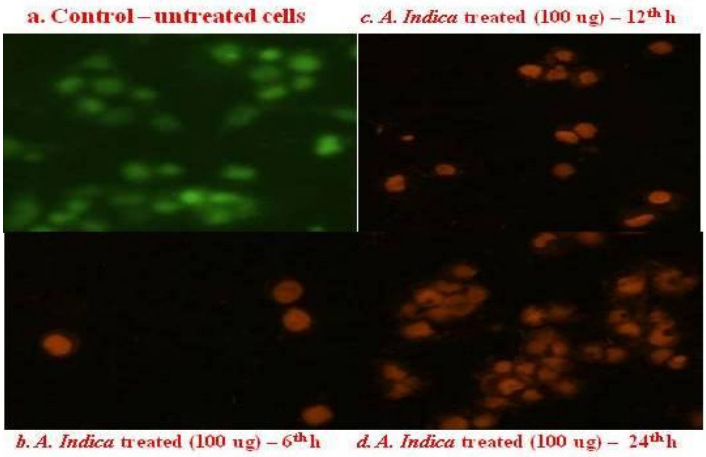

Figure 7: Apoptosis induction of A. Indica at different time interval

DISCUSSIONS

The present study revealed that *Acalypha indica* has more radical scavenging activity than *Terminalia chebula* from fig 1 which indicate more amounts of antioxidants present in *Acalypha indica*. All three extracts of *Acalypha indica* and T*erminalia chebula* showed varying degrees of antibacterial activity against all microorganisms tested. The gram positive bacteria are more susceptible than the gram negative bacteria.

These different antibacterial activities could be due to the nature and concentration of antibacterial compounds plus it's their mode of action [17].

Denaturation of proteins is a well-documented cause of inflammation. Phenylbutazone, salicylic acid, flufenamic acid (anti-inflammatorydrugs), have shown dose dependent ability to thermally induced protein denaturation[18]. As a part of the investigation on the mechanism of the anti-inflammatory activity, ability of extract to inhibit protein denaturation was studied. The in-vitro study of anti-inflammatory activity indicates that the inhibition percentage of albumin denaturation by *Terminalia chebula* is higher than *Acalypha indica*.

Proteinases have been implicated in arthritic reactions. Neutrophils are known to be a rich source of proteinase which carries in their lysosomal granules many serine proteinases. It was previously reported that leukocytes proteinase play an important role in the development of tissue damage during inflammatory reactions and significant level of protection was provided byproteinase inhibitors [19]. The methanol extract of *Acalypha indica* exhibited significant antiproteinase activity at a concentration of 100 mg/ml.

The MTT assay is broadly used for measuring cell viability, proliferation of living cells, and cytotoxicity of new drug candidates. The anti-proliferative activity revealed that *Acalypha indica* has more anti-proliferative activity than *Terminalia chebula*. MTT reduction is attributed to mitochondrial activity, although it is related both to non−mitochondrial enzymes and to lysosomes and endosomes [20].

The cytotoxicity of *Acalypha indica* on Hela cell lines showed that the IC_{50} value is 0.01[21]. Also it was reported that the aerial parts of *Acalypha indica.* Linn extracts showed non-cytotoxic response against vero cells [22]**.** The cytotoxicity of *Acalypha indica* extract on raw 264 cell lines was tested with the MTT assay and the anti-tumor activity of *Acalypha indica* plant extract was tested by comparing with the commercially available drug doxorubicin and we found that the plant extract had significant percentage of growth inhibition. Till more work has to be done to confirm the anti-tumor activity of the plant extract.

The cells treated with *Acalypha indica* plant extract and acridine orange stain was observed at various h (6^{th} h, 12^{th} h and 24^{th} h). As time increases apoptosis happens and at the 24^{th} h apoptosis can be observed at its maximum with chromosomal condensation and fragmentation.

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