doi.org/10.36721/PJPS.2024.37.4.REG.761-767.1

In vitro biological activities of selected medicinal plants and their synergistic effects

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Abstract: The present work aimed to use the methanol extracts of *Croton bonplandianus* (Cb) and *Tithonia diversifolia* (Td) and the synergistic activity of *Croton bonplandianus* and *Tithonia diversifolia* (CbTd) for the phytochemical screening, anti-microbial, anti-oxidant, and anti-inflammatory activities. Phytochemical screening was done by the standard protocols. *In vitro* antimicrobial, antioxidant, and anti-inflammation, were assayed by using disc diffusion, total antioxidant activity, DPPH method, HRBC (human red blood cells) membrane stabilization, and anti-protein denaturation tests. The synergistic approach of the two plants showed potent antimicrobial, antioxidant, and anti-inflammation activity. *In vitro* antibacterial activity was done against *Pseudomonas aeroginosa, Escherichia coli, Bacillus subtilis,* and *Staphylococcus aureus* at different concentrations. The maximum zone of inhibition (21 mm) was observed in the combinatorial approach. The maximum inhibition of free radicals is observed in CbTd with a low IC_{50}, i.e., 7.64 mg/ml, followed by Cb with an IC_{50} value of 11.8mg/ml and Td with an IC_{50} of 28.3 mg/ml. The percentage inhibition of hemolysis and protein denaturation is high in CbTd (93% and 69%). The experimental analysis reveals the effectiveness of the synergistic effect of these plants with anti-microbial, anti-oxidant, and anti-inflammatory activity, further it can be used in the formulations against infectious human pathogens.

Keywords: *Croton bonplandianus* (Cb), *Tithonia diversifolia* (Td), *Croton bonplandianus* and *Tithonia diversifolia* (CbTd), phytochemical screening, antimicrobial activity, antioxidant activity, anti-inflammatory activity.

INTRODUCTION

Medicinal plants are important for treating serious diseases (Rakotoarivelo *et al*., 2015). Each medicinal plant has various phytochemicals that can be used in the medical field for the development of new drugs (Yuan *et al*., 2016). Natural items have been employed since the Vedic period as a key source for the finding of new drugs. Semi-synthetic derivatives and natural products play a crucial role in the development of innovative medications in current pharmaceutical sectors (Sukanya *et al*., 2009). Alkaloids, tannins, phenols, flavonoids, and other phytochemicals are found in plants. Medicinal plants can have significant antibacterial, anticancer, antifungal, and anti-oxidant activity due to the existence of these phytochemicals, which have been traditionally utilized for treating infectious diseases.

Tithonia diversifolia (Hemsley) Gray is a shrub native to Central America and widely planted in many tropical regions, including South India. It is integrated into the Asteraceae family and is also known as the Mexican sunflower. Despite its reputation as a dangerous weed, it is useful in the treatment of a variety of ailments, including sore throats, stomach problems, indigestion, pains, and liver illnesses (Orwa *et al.,* 2009). Antibacterial and antifungal (John-Dewole and Oni, 2013), anti-pesticidal (Schuster *et al.,* 1999), anti-malaria (Madureira *et al.,* 2002), anti-proliferation (Gu *et al.,* 2002) and anti-inflammatory characteristics (Rungeur *et al.,* 1998).

Croton bonplandianus, also known as ban tulasi, jungle tulasi, kukka mirapa and other names, is a perennial herb that can be found in wastelands and roadside locations. It is indigenous to Argentina's northern region (Chakrabarty *and Balakrishnan,* 1992). Croton is high in secondary metabolites like terpenoids (Bapuji and Ratnam Ethnobotany, 2009). The plant has the potential to treat liver illnesses, bodily edema and skin diseases. This plant's leaves are used to cure wounds and cuts, cholera, and venereal sores (Asolkar *et al.,* 1992). This plant has hepatotoxic and cardiotoxic (Ahmad and Beg, 2001), antibacterial and antifungal (Saggoo *et al.,* 2010), anti-tubercular, cytotoxic, antioxidant (Quaiser *et al.,* 2013), and antitumor activities (Islam *et al.,* 2010).

Natural antioxidants from medicinal plants fight against free radicals and maintain good health (Khan *et al*., 2012). Natural anti-inflammatory agents from medicinal plants achieve more pharmacological responses with fewer side effects (Bagad *et al.,* 2013). Due to their natural origin, antimicrobials derived from medicinal plants are thought to be safer than synthetic substances (Upadhyay *et al*., 2013). Secondary metabolites in medicinal plants showed their antimicrobial properties (Savoia, 2012). Due to the pharmacological significance and wide availability of these plants, research was done to investigate their *in vitro* biological activities.

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MATERIALS AND METHODS

Plants source

Leaf samples of *Croton bonplandianus* were collected from nearby surroundings in Tirupati, and those of *Tithonia diversifolia* were collected from Sri Padmavati Visvavidyalayam (Tirupati, Andhrapradesh, India).

Fig. 1: Gprs of *Croton bonplandianus*, *Tithonia diversifolia*

Extraction procedure

The shade-dried leaves of each plant (at 20°C) were powdered in a mixer grinder (Philips from India). The 10 grammes of ground leaf powder were dissolved in 100 ml of methanol solvent. The extracts were then filtered and further dried using a rotary evaporator (Heidolph Hei-VAP from Japan) at 55-80°C.

Qualitative phytochemical screening

Using conventional methods, leaf methanol extracts of *C. bonplandianum, T. diversifolia* and amalgamated powder of were subjected to qualitative phytochemical screening for the detection of secondary metabolites (Madhu *et al*., 2020).

At the point when two millilitres of concentrate were treated with one millilitre of Molisch's reagent and a couple of drops of conc. sulfuric acid, the presence of sugars was affirmed by the creation of a purple or ruddy color. 2 ml of 5% ferric chloride were added to 1 ml of water, tannins are available when a dim blue or greenish-dark arrangement happens. Shaking a graduated chamber with 2 ml of concentrate and 2 ml of refined water for 15 minutes the long way caused a 1 cm layer of froth, which was an indication that saponins were available. 2 drops of hydrochloric acid were added to 2 ml of the concentrate and Mayer's reagent was then included to the acidic portion of the concentrate. Alkaloids can be recognised by the presence of white or green hasten. The presence of flavonoids is indicated by their yellow color by adding 1ml of 2N sodium hydroxide to 2 ml of concentrate. 3 ml of chloroform and 10% smelling salt arrangements were added to 2 ml of concentrate.

The improvement of a pink colour means the presence of glycosides. 1 ml of the concentrate, 2 ml of refined water, and a couple of drops of 10% ferric chloride were added. The presence of phenols is shown by the development of a blue or green color. 2ml of chloroform and con. sulphuric corrosive were added to 0.5 ml of the concentrate for treatment. Terpenoids are available when the contact fosters a rosy, earthy color. A modest quantity of 10% smelling salt arrangement was added to 1 ml of leaf extract. Hasten, which is pink in variety, recommends the presence of anthraquinones by adding iml of leaf extract to 10% ammonia solution by the appearance of pink colour. The presence of an earthy-colored ring demonstrates the presence of steroids, while the presence of a somewhat blue earthy-colored ring shows the presence of phytosteroid. To 1ml of leaf extract a comparable volume of chloroform and a couple of drops of concentrated sulfuric acid are added.

Quantitative phytochemical screening

Quantification of flavonoids

Garg measured the flavonoid concentration (Garg and Garg, 2019). By mixing 0.5 ml of plant extract with 0.1 ml of 10% aluminium nitrate, 0.1 ml of 1M potassium acetate, and 4.3 ml of 80% ethanol, a total volume of up to 5 ml was produced. The combined mixture was left to stand at room temperature for 40 minutes (28°C). The absorbance was spectrophotometrically determined at 415 nm. In terms of quercetin equivalents (QE) per gram, the total flavonoid content of plant extracts was calculated.

Quantification of phenols

The quantification of total phenol content was assessed using the Folin-Ciocalteu approach (Sembiring *et al.,* 2018) with slight modifications. Each extract was combined with 50µl of phenol reagent in an amount of 100µl. After adding 1.85 ml of distilled water, the mixture was left to stand for 3 minutes. Further vortex the mixture and add 300µl Na2CO3 and the final volume was made to 4ml by adding deionized water.

Incubate the whole mixture in a dark place at room temperature for approximately 1 hour and read absorbance at 725nm. Total phenolic contents were expressed in terms of gallic acid equivalents in mg per gramme of plant extract.

Antimicrobial activity

The antibacterial activity was tested using the agar disc diffusion method (Hussein *et al*., 2019). The bacterial cultures were collected from Sri Venkateswara Institute of Medical Sciences, Tirupati. The 24hrs fresh bacterial cultures were used for the susceptibility test.

This was carried out by dispensing sterilised nutritional agar medium into a Petri dish and allowing it to solidify. 0.1 ml of liquid bacterial pathogen culture was swabbed

Table 1: Phytochemical screening of methanol leaf extracts

Test	Cb	Td	CbTd
Tannins	+	+	++
Phenols	+	+	++
Flavonoid	+	+	++
Carbohydrates	+	+	++
Glycosides	-	-	-
Terpenoids	-	+	+
Coumarins	+	-	+
Steroids	-	+	+
Anthraquinones	-	-	-
Alkaloid	-	+	+
Saponins	+	+	++

The symbol + refers to the presence, - refers to the absence and ++ indicates the more presence

Table 2: Antibacterial Activity of Cb, Td and CbTd

	T. diversifolia			*C. bonplandianus* Concentrations in μg/ml			CbTd			Standard Gentamycin
	25	50	75	25	50	75	25	50	75	30mcg
	Zone of inhibition in mm									
E. coli	-	4±0.02	6±0.04	4±0.04	6±0.06	10±0.05	7±0.05	12±0.04	20±0.03	22±0.05
P. aeruginosa	-	4±0.04	8±0.03	3±0.06	7±0.03	15±0.08	8±0.04	13±0.05	21±0.04	26±0.05
S. aureus	-	3±0.5	7±0.05	4±0.07	5±0.05	10±0.03	9±0.06	12±0.09	19±0.05	23±0.06
B. subtillis	-	4±007	6±0.04	3±0.06	7±0.04	14±0.02	10±0.04	11±0.05	18±0.08	21±0.07

onto an agar plate. At the centre, sterile discs with a maximum diameter of 3mm were positioned. Different volumes of plant extracts (25, 50 and 75μl) were poured into each well and incubated at 37°C for 24 hours. Gentamysin disc 30mcg was used as standard. Antibacterial actions were examined by measuring the inhibition zone surrounding each well.

Antioxidant activity

Total antioxidant activity

The total antioxidant activity was evaluated by the method of Deepak (Deepak and Anurekha, *(*2018). Add 1 ml of reagent solution containing 0.6 M sulfuric acid, 28 M sodium phosphate, and 4 M ammonium molybdate to 0.1ml of plant extract. Afterward, it was incubated at 95°C for about 90 minutes. At 695 nm, the absorbance was measured against a blank. The total amount of antioxidant activity was measured in ascorbic acid equivalents.

DPPH radical scavenging activity

Different concentrations of 0.01ml of plant extracts are mixed with 3 ml of a 0.1mM solution of DPPH in methanol. After 15 minutes of incubation time in the dark, the absorbance was read at 517nm. The proportion of free radical scavenging actions was evaluated by Siddartha Baliyan., 2022.

% of Scavenging Effect = (Control Abs - Sample Abs) / (Control Abs) X 100

Anti-inflammatory activity

Measurement of HRBC membrane stability

The effectiveness of anti-inflammatory drugs was assessed using HRBC membrane defence (Adek Zamrud Adnan *et al*., 2019). Equal parts of sterile Alsever's solution (2% dextrose, 0.8% sodium citrate, 0.42% sodium chloride and 0.5% citric acid in sterile water) and blood are mixed and centrifuged for 10 minutes at 3000 rpm. After another 0.85% isosaline wash, the packed cells were suspended in a 10% isosaline concentration. The test mixture contains 2 ml of plant extract, 0.5 ml of HRBC solution, 1ml of phosphate buffer (pH 7.4), 2ml of hyposaline (0.36%), and 1 ml of phosphate buffer (pH 7.4). In addition, the reference medication diclofenac was used as a comparison to the hyposaline control.

After 30 minutes of incubation at 37°C, the assay mixture is centrifuged for 10 minutes at 3000 rpm. To determine the precise concentration of haemoglobin in the supernatant, absorbance at 560nm in the UV spectrophotometer was measured. The percentage of hemolysis is calculated using the formula below.

% of hemolysis inhibition = (Abs of control-Abs of the sample) / Abs of control X 10

Protein denaturation test

With a few minor modifications, the Sharmila Dharmadeva *et al*., (2018) described protein denaturation assay was used. Bovine albumin, phosphate buffered saline (PBS, pH 6.4) and plant extract were all included in the reaction mixture, which had a volume of 4.78ml. The entire mixture underwent a vortex and 15 minutes of incubation in a water bath at 37°C. The mixture was then

heated at 70°C for 5 minutes. Following cooling, absorbance at 660nm was measured using phosphate buffer as the reference.

% inhibition of denaturation = 100 (1-A2/A1). Where A1 is the absorbance of the control and A2 is the absorbance of the test sample.

STATISTICAL ANALYSIS

All data were displayed with their corresponding means and standard deviations. Significant values were determined by two-way Anova using GraphPad Prism Software version 9.0 to compare the average of each plant. p<0.001 was the statistical significance.

RESULTS

Qualitative phytochemical studies

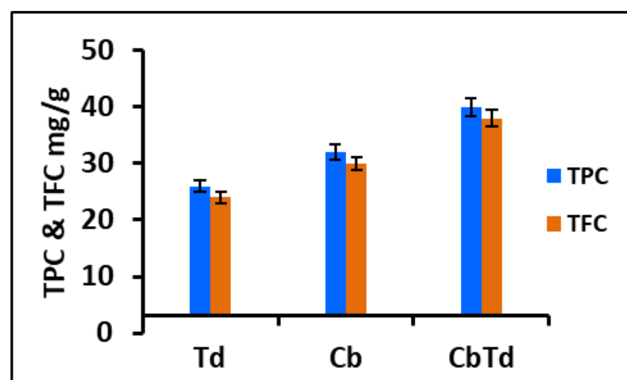
The methanol extracts of Cb, Td, and CbTd showed the presence of secondary metabolites like tannins, carbohydrates, saponins, flavonoids, alkaloids, terpenoids, phenols, coumarins, and steroids (table 1). Due to their richness in phenols, flavonoids and saponins, the methanol plant extracts showed potential for the discovery of new drugs.

Quantification of total phenols and flavonoids

Total phenolic and flavonoid contents are reported in three different methanol extracts. Among these CbTd methanol extracts, some are rich in phenols and flavonoids compared to methanol extracts of *C. bonplandianus* and *T. diversifolia,* as shown in fig 2.

Antimicrobial activity

Antibacterial activity of methanolic leaf extracts involving Cb, Td and CbTd was compared with the tested bacteria strains. Further, assessed for the presence or absence of a zone of inhibition in the agar well diffusion procedure. The zone of inhibition fashioned by the crude extracts on diverse bacterial strains was between 3 mm and 21 mm. However, CbTd methanolic extracts displayed the greatest inhibitory activity on E. *coli, P. aeruginosa, S. aureus* and *B. subtilis,* which are represented in table 2.



Values are expressed as mean±SD, statistical significance at 1% level (p≤0.001)

Fig. 2: Total phenolic content (TPC); milligrams gallic acid equivalent/gram of plant extract. Total flavonoid content (TFC); milligrams of quercetin equivalent/gram of plant extract.

Antioxidant activity

Total antioxidant and DPPH free radical scavenging activity were observed in all extracts. The predominant activity was examined in CbTd, followed by Cb and Td (shown in fig. 3 and 4).

Total antioxidant activity

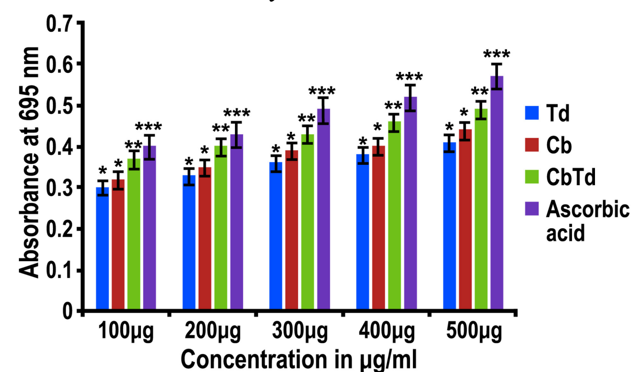


Fig. 3: Total antioxidant activity by methanol leaf extracts of Cb, Td and CbTd.

DPPH free radical scavenging activity

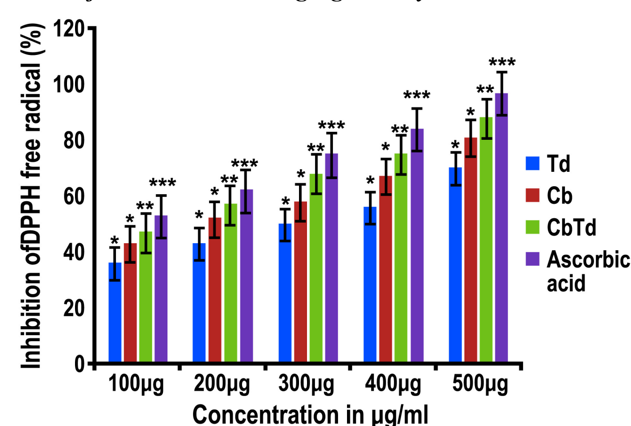


Fig. 4: DPPH free radical scavenging activity by methanol leaf extracts of Cb, Td and CbTd.

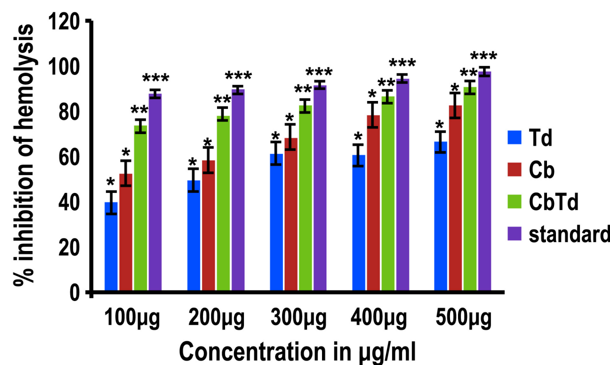


Fig. 5: Inhibition of hemolysis by methanol leaf extracts of Cb, Td and CbTd.

Anti-inflammatory activity

The three methanolic extracts of Cb, Td and CbTd are evaluated for anti-inflammatory activity by two methods. Primarily the % inhibition of hemolysis was studied and maximum inhibition was found in CbTd while minimum in Td. Secondarily inhibition of protein denaturation was studied and found maximum in CbTd while minimum in Td shown in fig 5. Methanol leaf extracts can inhibit hemolysis and protein denaturation in a concentration-dependent manner (100-500µg/ml). The order of percentage inhibition of hemolysis and protein denaturation was in increased order i.e Td, Cb and CbTd (fig. 6).

Anti-Protein denaturation test

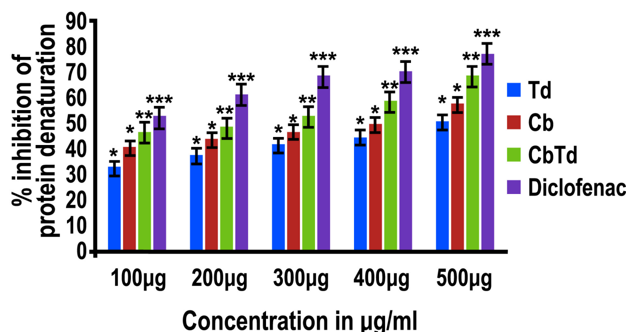


Fig.6: Inhibition of protein denaturation by methanol leaf extracts of Cb, Td and CbTd.

DISCUSSION

Medicinal flora has been examined in a broad range globally due to their potential anti-oxidant actions with no adverse consequences and cost-effectivity (Auddy *et al*., 2003). It is acknowledged the importance of discovering naturally occurring antioxidants that can reinstate the artificial antioxidants since they revealed toxicity and carcinogenicity (Velioglu *et al.,* 1998). It is acknowledged that phenolic compoundsfrom plant extracts possess absolute anti-oxidant activity (Elzaawely *et al.,* 2007). Therefore, to assess the total phenolics, flavonoids contents from plant extracts were carried out through the Folin-Ciocalteu's Reagent (FCR) approach, which is considered a robust and functional approach for assessment (Luximon-Ramma *et al.,* 2003). Results pointed out that there was a comparatively high methanolic extract yield (32%) from CbTd, which is rich in total phenolics and flavonoids.

Results pointed out that there was a comparatively high amount of phenolics and flavonoids in the methanolic extract of CbTd. Due to the redox potential of phenolic compounds, they can engage in the predominant function of neutralising and absorbing free radicals, reducing singlet as well as triplet oxygen by disintegrating peroxides (Osawa *et al.,* 1994). Consequently, phenolic compounds are regarded as good antioxidants (Rice-Evans *et al*., 1995). The ability of natural products to donate electrons can be assessed by the 2, 2′-diphenyl-1-picrylhydrazyl radical (DPPH) solution bleaching method (Nunes *et al*., 2012). The technique relies on the DPPH being scavenged by the addition of an antioxidant or radical species that makes the DPPH solution less colorful. The concentration and potency of the antioxidants are inversely correlated with the degree of colour change. Significant free radical scavenging activity of the test compound is indicated by a significant decrease in the absorbance of the reaction mixture (Krishnaiah *et al*., 2011).

With low IC50 values, the CbTd methanol extracts exhibit strong free radical scavenging activity. Moreover, resistance to antibiotics is a problem that persists in the medical care area in both developing and developed countries. The materialisation of broad spread from multi-drug resistant pathogens has consequently intimidated the present antibacterial remedy. In turn, this has demanded an exploration of the latest source of anti-microbial substances from floras, which produce diverse bioactive agents with therapeutic properties. Hence, this study has been implemented to assess the anti-microbial actions of diverse medicinal plant extracts against human pathogens involving reference strains (Umapathy *et al.,* 2010).

On the other hand, these plant extracts have revealed anti-inflammatory properties too. This test can be demonstrated by the HRBC membrane stabilisation method and the anti-protein denaturation method. The results are dependent on concentration and protection increases with an increase in the concentration of the sample. An examination of the red blood cell membrane, which is identical to that of the lysosomal membrane, hampers hemolysis of red blood cells and gives broad insights into the inflammatory process (Umapathy *et al.,* 2010). Stabilization of these cell membranes might refrain or limit the lyses and discharge of the cytoplasm contents and further mitigate the damage to tissues (Okali *et al.,* 2008).

The review states that the denaturation of protein molecules leads to inflammation. Inhibition of protein denaturation plays a major role in anti-inflammatory activity. Therefore, combinatorial plant extracts possess prominent protection of cell membranes against injurious agents and further mitigate the advancement of inflammation. Therefore, our current examination supports previous literature reports that phytochemical compounds like phenols as well as flavonoids display several biological effects like anti-oxidant activity, anti-microbial activity and anti-inflammatory activity (Venkatanarayana *et al.,* 2010). Based on our results, we can confirm that the metabolites from synergistic plant extracts have significant role in antibacterial, antioxidant and antiinflammation and other properties.

CONCLUSION

According to the findings, phenols, flavonoids, alkaloids, saponins, steroids, tannins, and carbohydrates are readily found in methanol CbTd extracts. The quantitative phytochemical screening reveals the highest levels of phenols and flavonoids and even a link to antiradicals. The methanol leaf extracts exhibited substantial action against a variety of bacteria, indicating that the synergetic interactions between the two plants could be exploited to isolate new medicines with high efficacy for treating a variety of ailments. CbTd methanol leaf extracts have robust antioxidant, antibacterial and anti-inflammatory properties, which are good for chemical and biological analysis that can be used for pharmacological formulations in future. The synergistic effect of two medicinal plant extracts against human infections is demonstrated in this study.

ACKNOWLEDGEMENT

The authors are thankful to the DST-FIST, Dept. of Applied Microbiology and DST-CURIE of Sri Padmavati Mahila Visvavidyalam, Tirupati for providing lab facilities.

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