Original Research Article

Assessment of Cytotoxic, Antioxidant, Thrombolytic, Anti inflammatory and Antimicrobial activity of *Curcuma longa Linn*, *Cissus quadrangularis* and *Boerhaavia diffusa* herbal formulation - an invitro study

ABSTRACT

Background & Aim:

Medicinal plants have long played an important role in traditional,cultural, meditative, and spiritual lives in India, and such plants have long played an important role in tribal people's spiritual, meditative, and spiritual lives. Curcumin, a naturally occurring compound found in the rhizomes of the plant Curcuma longa Linn., has been shown to have antioxidant and cytotoxic properties in laboratory studies. Anti-inflammatory, antihelminthic, antifungal, antihemorrhoidal, analgesic, and antibacterial properties are also found in C. quadrangularis. An ethanol extract of B. diffusa was found to have antiproliferative, immunomodulatory, and anti-inflammatory properties. The aim of the study is to assess the cytotoxic, antioxidant and thrombolytic, antiinflammatory and antimicrobial properties of the aqua-alcoholic extract of the herbal formulation (*Curcuma longa Linn* +*Cissus quadrangularis* +*Boerhaavia diffusa)*

Methods:

Curcuma longa Linn, *Cissus quadrangularis and Boerhaavia diffusa* were washed thoroughly and shade dried. The dried plant material was finely powdered and grounded using a slender blender. In-Vitro studies cytotoxic, antioxidant, thrombolytic activity was done by Brine shrimp lethality assay, DPPH method and clot lysis method respectively.

Results:

The herbal formulation showed good cytotoxic activity, moderate antioxidant activity with increasing concentrations highest at 50µL and good thrombolytic activity at 1000µL**.**

Interpretation & Conclusion: This herbal formulation on in vitro study shows promising results further studies on animals and human clinical trials needs to be done.

Key words: In-vitro study, cytotoxic, Antioxidant, thrombolytic,herbal study

1.INTRODUCTION

Plants play an important role in drug production, and the pharmaceutical industry relies heavily on natural products to produce new drugs (1)(2). According to a WHO survey, folk medicine is used by 80% of the world's population for primary health care (3). Clinical microbiologists enjoy experimenting with medicinal plants in search of new medicines to develop(4,5). Due to its legacy and heritage, India has a wealth of experience in the fields of health care, including Siddha, Homeopathy, Unani, and Ayurveda(6,7) . Medicinal plants have long played a significant role in the spiritual, meditative, and spiritual lives of tribal people in India, and such plants have long played a significant role in the spiritual, meditative, and spiritual lives of tribal people (Princeton et al. 2020),(Mathew et al. 2020).

Curcumin, a natural compound found in the rhizomes of the plant Curcuma longa Linn., has been shown in experimental studies to have anti-inflammatory properties. Curcuma longa has been clinically validated for its anti-inflammatory properties in traditional medical systems for decades(Sridharan et al. 2019),(R et al. 2020). Curcumin inhibited the development of arachidonic acid, cyclooxygenase, lipoxygenase, cytokines (interleukins and tumor necrosis factor), nuclear factor-B, and steroidal hormones. Curcumin has been shown to stabilize the lysosomal membrane and cause oxidative phosphorylation uncoupling, as well as possessing a high oxygen radical scavenging activity, which accounts for its antiinflammatory properties. Since ancient times, turmeric root has been used as a spice in India and other Asian countries. It's also commonly used as a medicine, particularly for the treatment of inflammatory conditions(8). Over the years, curcumin has been studied for a number of medicinal purposes. It has been shown to help cure a host of illnesses, including diabetes, autoimmune disorders, and cancers (9–11). Curcumin is a pleiotropic molecule that interacts with a wide range of inflammatory targets, including TNF and interleukins (ILs) (12)(Antony et al. 2021),(Sarode et al. 2021). Curcumin has also been shown to have antimicrobial action against fungi(13) and a wide range of Gram-positive and Gram-negative bacteria in vitro.

Cissus quadrangularis L. is a fleshy plant found throughout the world, especially in Asia, Africa, and a few other warm tropical areas. It's one of India's most famous dishes. The whole plant is used as a digestive aid (Pachana) as well as a palliative and roborant in Ayurveda. C. quadrangularis contains a lot of ascorbic acid, carotenoids, flavonoids, and steroids. C. quadrangularis also has anti-inflammatory, antihelminthic, antifungal, antihemorrhoidal, analgesic, and antibacterial effects.

Boerhaavia diffusa L. (Nyctaginaceae) is an annual herbaceous plant found in the tropical regions of India, South America, and Africa. Jaundice, dyspepsia, nephrotic disease, convulsions, spleen enlargement, stomach pain, tension, and inflammation are all conditions that B. diffusa roots are used to treat in Ayurveda (14)(Hannah R et al. 2021),(Chandrasekar et al. 2020). Despite the fact that a methanol extract of B. diffusa (whole plant) has been shown to have antiproliferative and

antiestrogenic properties (15), an ethanol extract has been shown to have hepatoprotective, immunomodulatory, and anti-inflammatory properties (15–17).

The Nyctaginaceae family includes the herbaceous B. diffusa Linn. It can be found in the tropics and subtropics all over the world. It's been used for millennia by indigenous and aboriginal cultures, as well as in Ayurvedic or natural herbal medicine (18,19). Pharmacological trials have shown that B. diffusa has diuretic, anti-inflammatory, antifibrinolytic (20), anticonvulsant (21), and hepatoprotective properties. Diabetes, tension, dyspepsia, stomach pain, inflammation, jaundice, spleen enlargement, and congestive heart failure are all treated with B. diffusa in Ayurvedic medicine in India and Unani medicine in Arab countries.The aim of the study is to assess the cytotoxic, antioxidant, thrombolytic antiinflammatory and antimicrobial properties of the herbal formulation.

2.MATERIALS AND METHODS

2.1 COLLECTION OF PLANTS

Curcuma longa Linn rhizomes, Cissus quadrangularis and Boerhaavia diffusa were washed thoroughly and shade dried. The dried plant material was finely powdered and grounded using a slender blender**.**

2.2. PREPARATION OF HERBAL FORMULATION

5 grams of turmeric, 5 grams of Cissus quadrangularis, and 5 grams of Boerhaavia diffusa are combined in a beaker of 50 milliliters of ethanol and 50 milliliters of distilled water. Placed in a shaker for 24 hours. The herbal mixture was condensed and reduced to 75 ml after heating at 25-30 degrees Celsius. The herbal formulation is then purified and boiled for 10 minutes at 25 degrees Celsius. After that, the herbal solution is moved to a bottle for further examination.

2.3. CYTOTOXIC ACTIVITY

BRINE SHRIMP LETHALITY ASSAY:

Salt water preparation

Weighing 2g of iodine-free salt and dissolving it in 200 mL of purified water

10-12 ml of saline water was poured onto 6 well ELISA pots. 10 nauplii were gradually added to each well (20L, 40L, 60L, 80L, 100L). The herbal formulations were then inserted at the appropriate concentration range. For 24 hours, the plates were incubated.

After 24 hours, the ELISA plates were examined and counted for the amount of live nauplii present, which was then measured using the formula below (Fig1).

number of dead nauplii/number of dead nauplii+number of live nauplii×100

2.4. ANTIOXIDANT ACTIVITY

DPPH METHOD:

DPPH assay was used to test the antioxidant activity of the herbal formulation. Diverse concentration (2-10ug/ml) of the herbal formulation was mixed with 1ml of 0.1mM DPPH in methanol and 450ul of 50mM Tris HCL buffer (pH 7.4) and incubated for 30 minutes (Fig2). Later, the reduction in the quantity of DPPH free radicals was assessed dependent on the absorbance at 517 nm. BHT was employed as control. The percentage of inhibition was determined from the following equation,

$$\% \text{ inhibition} = \frac{\text{Absorbance of control} - \text{Absorbance of test sample}}{\text{Absorbance of control}} \times 100$$

2.5. THROMBOLYTIC ACTIVITY

Se 5 ml of venous blood drawn from avolun , which was placed in seven separate pre-weighed sterile microcentrifuge tubes and incubated at 37 °C for 45 minutes (Fig3). After the clot had formed, the fluid from each microcentrifuge tube was fully released, and the clot weight was calculated by subtracting the weight of the clot-containing tube from the weight of the tube alone. 500 µl of streptokinase (SK) was added to the microcentrifuge tubes as a positive control, and 500 µl of distilled water was added to the microcentrifuge tubes as a negative non-thrombolytic control. The herbal formulation is added at varying concentrations. After that, all of the tubes were incubated at 37°C for 90 minutes to check for clot lysis. The released fluid was discarded during incubation, and the tubes were weighed again to see if there was a difference in weight after the clot was disrupted.

Clot lysis value = weight of clot before formulation - weight of clot after formulation.

2.6. ANTI INFLAMMATORY ACTIVITY

The herbal formulation was assessed for the anti-inflammatory activity by Albumin Denaturation Assay. The formulation was tested by the following convention proposed by Muzushima and Kabayashi with specific alterations (Pratik Das et al.,2019). The herbal formulation comprising of 0.05mL of *Curcuma longa Linn*, *Cissus quadrangularis*, *Boerhaavia diffusa* of various fixation (10µl, 20µl, 30µl, 40µl, 50µl) was added to 0.45 mL of bovine serum albumin(1% aqueous solution) and the pH of the mixture was acclimated to 6.3 utilising a modest quantity of 1N hydrochloric acid. These samples were incubated at room temperature for 20 minutes and then heated at 55 ° C in a water bath for 30 minutes (Fig 4). The samples were cooled and the absorbance was estimated spectrophotometrically at 660nm. The standard used in this study was Diclofenac Sodium utilised as a control.

Percentage of protein denaturation was determined utilising the following equation.

$$\% \text{ inhibition} = \frac{\text{Absorbance of control} - \text{Absorbance of sample}}{\text{Absorbance of control}} \times 100$$

2.7- ANTIMICROBIAL ACTIVITY- AGAR WELL DIFFUSION METHOD

ANTIBACTERIAL ACTIVITY

Antibacterial activity of the herbal formulation against Staphylococcus aureus,Streptococcus mutans and Escherichia coli strains. This activity was carried out on MHA agar to establish the zone of inhibition. Muller hinton agar was prepared and sterilized at 120 pounds for 45 minutes. The media was poured onto the sterilized plates and set aside to solidify. The test species were swabbed after the wells were cut with a well cutter. The plates were filled with herbal formulation of various concentrations and incubated for 24 hours at 37 ° C. The zone of inhibition was assessed after the incubation period. (Fig 5)

ANTIFUNGAL ACTIVITY

The agar well diffusion assay uses Candida albicans as the research pathogen. The medium was prepared with Sabouraud's Dextrose Agar. The wells were swabbed with test species and herbal formulation of various concentrations were applied to the prepared and sterilized medium. The plates were incubated for 48-72 hours at 28° C. The zone of inhibition was assessed after the incubation period. (Fig5)

3. RESULTS

Brine shrimp lethality activity of the herbal formulation showed less mortality suggesting low toxicity and the control showed no mortality. The cytotoxicity assay revealed the alive nauplii at varying concentrations from 5µl to 80 µl was 10, 7, 9, 9, 9 and all the nauplii were alive in the control in Table 1. The least number of alive nauplii was found at 10 µl in Fig 6.

A DPPH assay was done to analyse the antioxidant property which showed moderate antioxidant properties of the CCB formulation at increasing concentrations from 10µl to 50µl was 37.3, 42.6, 57.8, 77, 86.5 but the values were not more than the standard values Table 2. The significantly increasing antioxidant activity was evident as the concentration increases in Fig 7.

Clot lysis assay was done to assess to the thrombolytic activity of the herbal formulation at varying concentrations from 200 µl to 1000 µl with the clot lysis value of 0.089g, 0.087g, 0.132g, 0.128g, 0.214g. 0.211g and 0.04 g clot lysis value for positive and negative control respectively in Table 3. The clot lysis was found to be the highest at 1000 µl which was significantly similar to the positive control in Fig 8.

Albumin denaturation assay was done to determine the anti-inflammatory activity of the herbal formulation, the results showed the % inhibition at varying concentrations of the herbal formulation

from 10µl to 50µl in 660 nm wavelength 54.6, 58.3, 68.2, 77.6, 87.3 in Table 4. The highest percentage inhibition was seen in 50µl when compared to standard in Fig 9.

Agar well diffusion method was done to demonstrate antimicrobial activity. Antibacterial activity was done in mueller hinton agar for Staphylococcus aureus, Streptococcus mutans and Esherichia coli strains. There were no significant good results on all 3 bacterial strains. Antifungal activity was done in Sabouraud's Dextrose Agar for Candida albicans and moderate antifungal activity was found when compared to the control in Table 5. The zone of inhibition in the agar well is represented as a bar chart in Fig 10.

4. DISCUSSION

Many medicinal plants have been used as herbal medicines to treat a variety of infectious diseases all over the world. In developed countries, medicinal schemes continue to play an important role as therapeutic remedies for primary care.While herbal preparations are often used as self-medication for acute ailments, herbal medicine practitioners prefer to treat chronic illnesses. Patients of allergies, eczema, premenstrual syndrome, rheumatoid arthritis, migraine, menopause, and irritable bowel syndrome may be on a normal caseload. Herbalists don't normally cure acute psychiatric or musculoskeletal disorders; instead, the goal of herbal medicine is to achieve long-term changes in health. Practitioners sometimes refer to treating the disease's "underlying cause," and may administer herbs to fix dysfunctional habits rather than only treating the symptoms. (22)

Our study showed significantly good thrombolytic activity when compared to positive and negative control. The highest thrombolytic activity was evident at 1000 µl and the least at 200 µl. The activity was found increasing with increasing concentrations. Our study showed significantly good antioxidant properties when compared to the standard, At 50 µl concencentration the highest reading was noted as 86.5 using DPPH assay and further analysing in wavelength of 517 nm. The efficiency decreases with decreasing concentrations. The least efficiency was found in 10µl. The varying concentrations showed varying absorbance at 517 nm wavelength, at 10µl, 20µl, 30µl, 40µl, 50µl the respective absorbance were 37.3, 42.6, 57.8, 77, 86.5 respectively. The moderate cytotoxicity activity was evident in the herbal formulation.

The brine shrimp test is a rapid, economical and easy bioassay for determining the lethality of plant extracts, which correlates relatively well with cytotoxic and anti-tumor properties in most cases. (23) The majority of the time, a desired biological response is due to a combination of bioactive plant components, rather than a single component. (24) As a result, biological activity must be tested on

crude extracts. The brine shrimp lethality assay has proven to be a useful tool for tracking natural product biological activities. In our study the lethality of the brine shrimp nauplii was very less. The existence of antitumor compounds in herbal formulations may explain why plant extract is cytotoxic. Yogesh et al in his study demonstrated Woodfordia fruticosa kurz flowers to be moderately toxic on using brine shrimp lethality assay. (25)

The DPPH assay is a common method for evaluating antioxidant molecules' free radical scavenging abilities by quenching the stable colored DPPH radical. Plant extracts containing antioxidants scavenge the radicals produced by DPPH, providing an ambitious framework for future in vivo studies. Studies suggests that phytochemicals such as phenolics and flavonoids have the ability to donate hydrogen and quench DPPH radicals. Sugapriya et al in her study analysed the thrombolytic activity and found Cissus quadrangularis had good thrombolytic activity. (26)

Traditional anti-inflammatory and antimicrobial herbal therapies or plants range from the rapidly growing flora of woods to those grown in farm fields(Alsawalha et al. 2019),(Yu et al. 2020),(Shree et al. 2019). They've been studied, examined, and proven to have anti-inflammatory properties, or they're being checked now. All around the world, these herbs grow spontaneously or are cultivated specially for this purpose. As a result of advances in herbal medicine, new and popular herbs have arisen (27) .

In our study good anti-inflammatory properties were evident when compared to the standard. The highest antiinflammatory effect was evident at 50µl using albumin denaturation assay. Laksmitawati et al. found that A. muricata leaf extract had anti-inflammatory activity by inhibiting the inflammatory mediators TNF-, IL-1, IL-6, and nitric oxide in an in vitro trial. (28) Maroon et al. (29) compiled a list of natural anti-inflammatory substances. Sen et al (30) examined and tested the anti-inflammatory efficacy of the methanolic fraction of a chloroform extract of Pluchea indica roots. Goldberg et al. (31) used the mouse paw edema test to investigate the anti-inflammatory ability of aqueous extract Achillea millefolium and discovered a 35 percent reduction in oedema. Lee et al. (32) discovered that the leaves of Hedera rhombea bean have anti-inflammatory properties. Chandra et al. (33) detected anti-inflammatory behaviors of ethanolic root extract of Swertia chirata (Gentianaceae) in a carrageenan-induced rat paw edema model. The anti-inflammatory effects of Sida cordifolia L. were investigated by Franzotti et al. (34) Ojewole (35) discovered that Bryophyllum pinnatum (Crassulaceae) aqueous leaf extract has anti-inflammatory properties.

Bacterial infections can cause a variety of human diseases, ranging from self-limiting illnesses to potentially fatal medical conditions if left untreated. Nonetheless, antibiotic resistance is on the

increase as a result of widespread use, rendering previously treatable pathogens untreatable. In the case of other infectious agents, globalization has aided the spread of (resistant) strains (36) (Subramanyam et al. 2018),(Jeevanandan and Thomas 2018). Curcumin has been shown to suppress strains of Staphylococcus, Streptococcus, Helicobacter, and Pseudomonas, among others.

Among the most common infectious diseases are bacterial infections. As a result, over 50 years of intensive research has been conducted in order to develop new antimicrobial medicines derived from various sources(Ponnulakshmi et al. 2019),(Sundaram et al. 2019). Despite advances in antibacterial agent production, the development of multidrug-resistant bacteria has necessitated the development of new antibacterial agents (37). The antimicrobial study on aqua alcoholic extract of the herbal formulation demonstrated the zone of inhibition value of the herbal formulation.

Inhibition zone of the ethanol extract of these plants was calculated where three were found to be sensitive against E.faecalis, C.albicans, S.aureus, S.mutans. However, the ethanol extract of *Curcuma longa Linn, Cissus quadrangularis, and Boerhaavia diffusa* was found to have more effective antimicrobial activity showing its maximum efficacy for both bacteria and fungi. Data from the literature, as well as our results, reveal the great potential of plant extracts as antibacterial and antifungal agents, in spite of the fact that they have not been completely investigated, more studies need to be conducted. Therefore, our results revealed the importance of plant oils when associated with antibiotics, to control resistant bacteria, which are becoming a threat to human health.

6. CONCLUSION

Curcuma longa Linn, Cissus quadrangularis, and Boerhaavia diffusa have also been used in the diet for centuries as flavoring agents and topical agents for a number of ailments. The improved cytotoxic, antioxidant, thrombolytic, anti-inflammatory and antimicrobial activity of the CCB mixture was shown in this study. We discovered that the extract of the CCB formulation has a variety of pharmacological activities in the current research. In the current study, we discovered that the extract of the CCB herbal formulation has a range of pharmacological activities. This study has paved the way for the plant's future application in drug development and a variety of herbal formulations with less side effects.

NOTE:

The study highlights the efficacy of "HERBAL" which is an ancient tradition, used in some parts of India. This ancient concept should be carefully evaluated in the light of modern medical science and can be utilized partially if found suitable.

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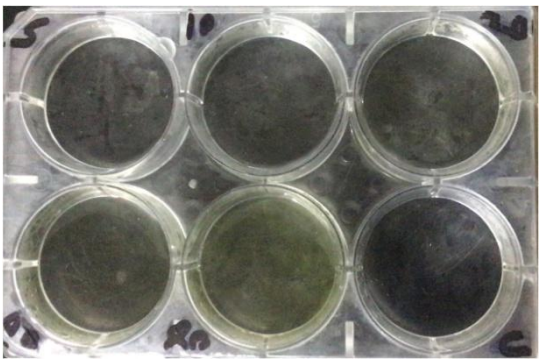

Fig 1- figure showing brine shrimp lethality assay

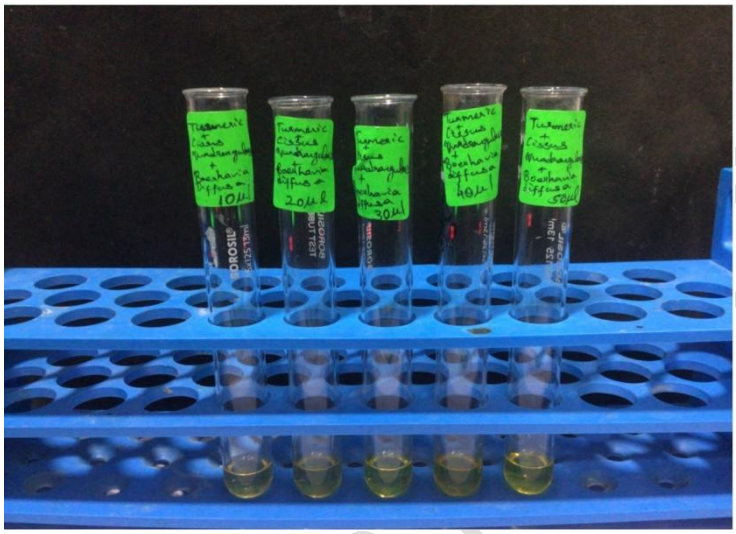

Fig 2- figure represents samples of herbal formulation for antioxidant study

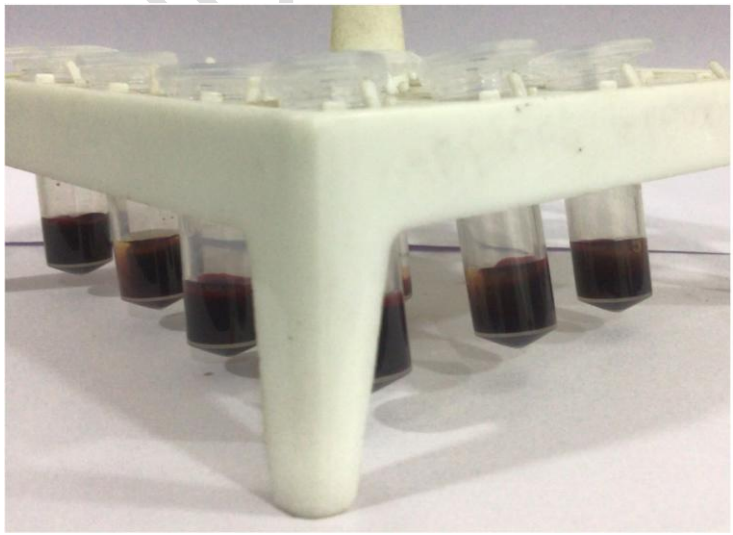

Fig 3- image represents lysis of clot by herbal formulation at different concentrations

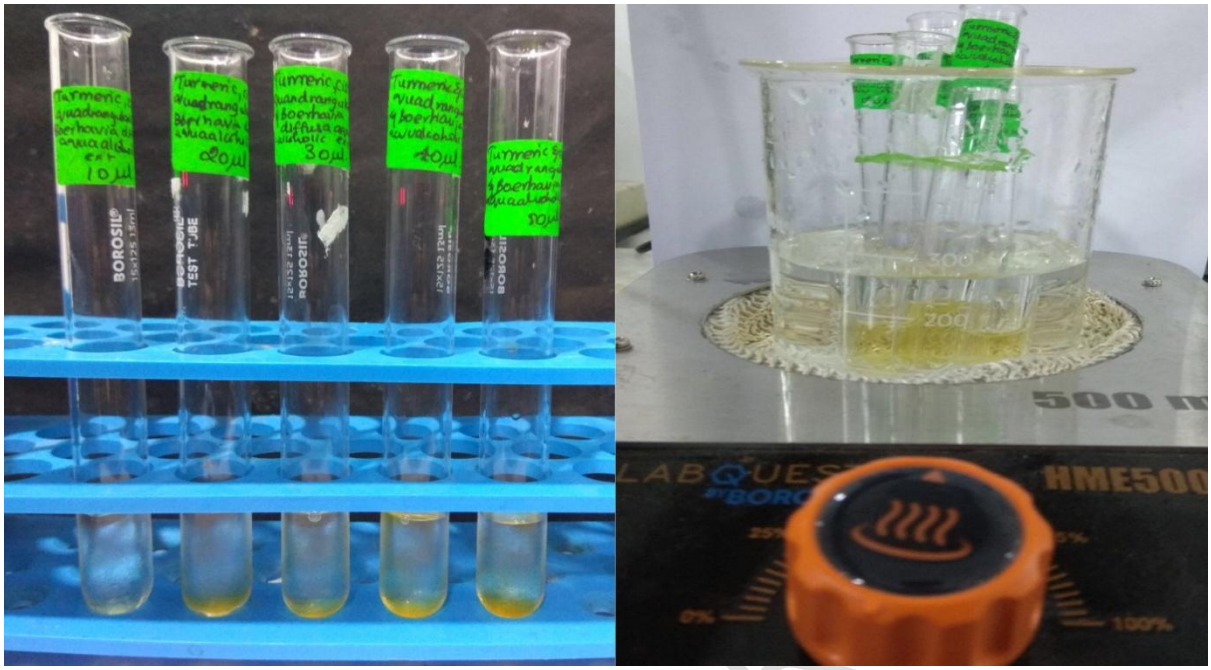

Fig 4- picture represents herbal formulation -CCB at varying concentrations in albumin denaturation assay

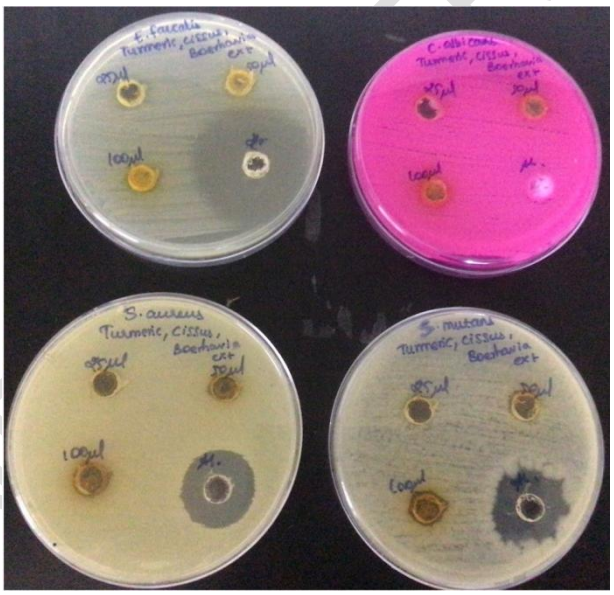

Fig 5- picture showing herbal formulation- CCB at varying concentrations for Culture method

CYTOTOXIC ACTIVITY					
5µl	10µl	20µl	40µl	80µl	Control
10	7	9	9	9	10

Table 1: table representing the brine shrimp cytotoxicity assay on the herbal formulation at varying concentration and negative control, alive nauplii in the well after incubation.

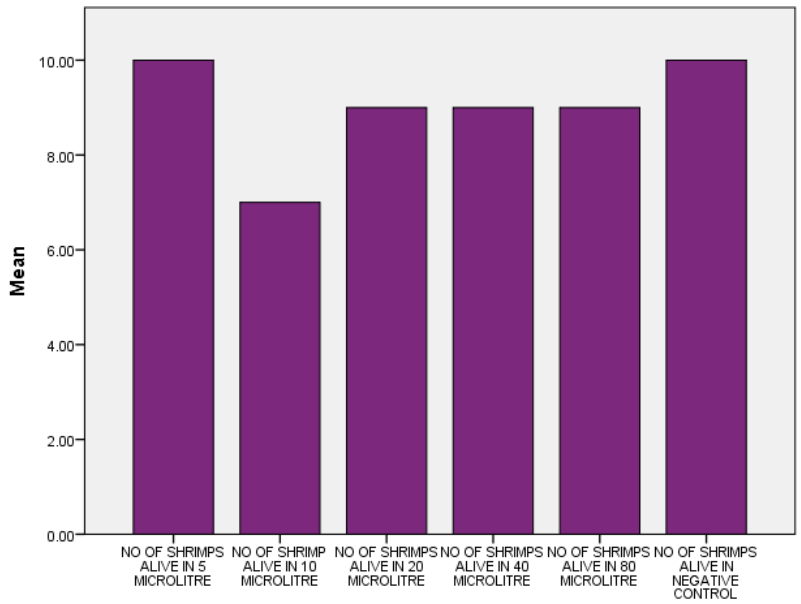

Fig 6: bar chart representing the number of nauplii alive in brine shrimp cytotoxicity assay on the herbal formulation at varying concentration and negative control.

ANTIOXIDANT ACTIVITY		
Size	Standard	CCB
10µl	76.56	37.3
20µl	78.52	42.6
30µl	85.63	57.8
40µl	88.68	77
50µl	93.15	86.5

Table 2: Table representing the antioxidant activity of the herbal formulation CCB at varying concentration under the wavelength of 517nm and compared with the standard values.

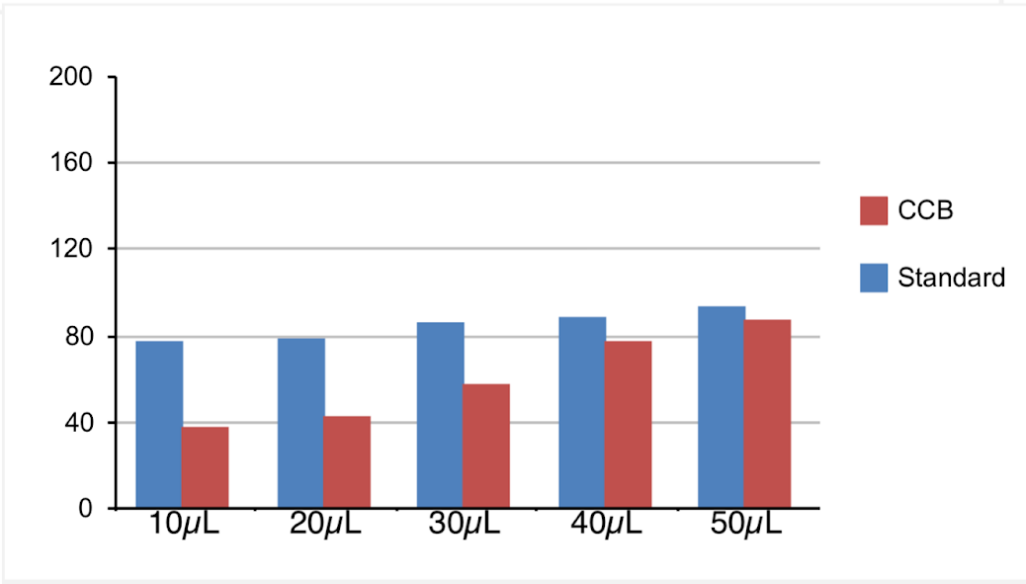

Fig 7: bar chart representing the antioxidant activity of the herbal formulation CCB at varying concentration under the wavelength of 517nm and compared with the standard values.

THROMBOLYTIC ACTIVITY				
Volume of blood	Weight of clot after 45 mins	Volume of formulation	Weight of clot after 90 mins	Clot lysis Value
200µl	1.453g	200µl	1.364g	0.089g
400µl	1.508g	400µl	1.421g	0.087g
600µl	1.422g	600µl	1.290g	0.132g
800µl	1.479g	800µl	1.351g	0.128g
1000µl	1.545g	1000µl	1.331g	0.214g
Positive control	1.548g	Positive control	1.337g	0.211g
Negative control	1.620g	Negative control	1.580g	0.04g

Table 3: table representing the thrombolytic activity of the herbal formulation at varying concentration, positive and negative control and the clot lysis values after incubation.

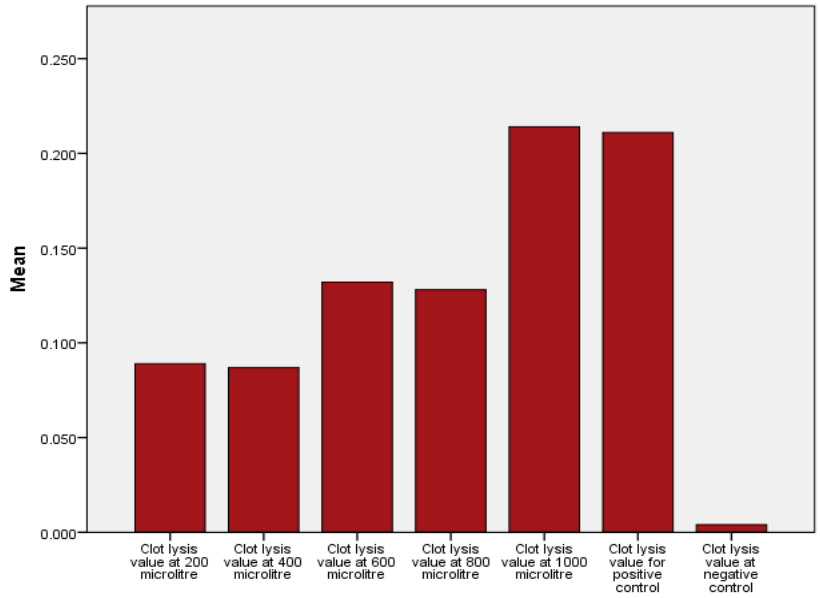

Fig 8: bar chart representing the thrombolytic activity of the herbal formulation at varying concentration, positive and negative control and the clot lysis values after incubation.

ANTI-INFLAMMATORY ACTIVITY		
Size	Standard	CCB
10µl	47	54.6
20µl	60	58.2
30µl	72	68.2
40µl	78	77.6
50µl	84	87.3

Table 4 : table represents the antiinflammatory activity of the herbal formulation-CCB at varying concentration under 660nm wavelength.

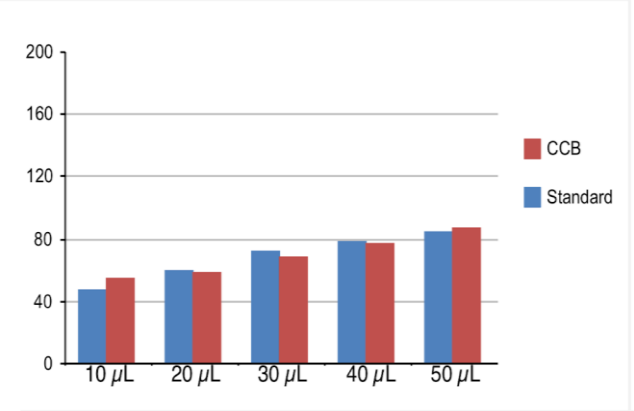

Fig 9: bar chart represents the anti-inflammatory activity of the herbal formulation - CCB at varying concentration under 660nm wavelength.

ANTIMICROBIAL ACTIVITY				
	25µl	50µl	100µl	Positive control
E. faecalis	9	10	10	38
C. albicans	9	10	10	12
S. aureus	9	11	11	26
S. mutans	9	9	11	27

Table 5: table represents antimicrobial activity of the herbal formulation - CCB at varying concentration and a positive control.

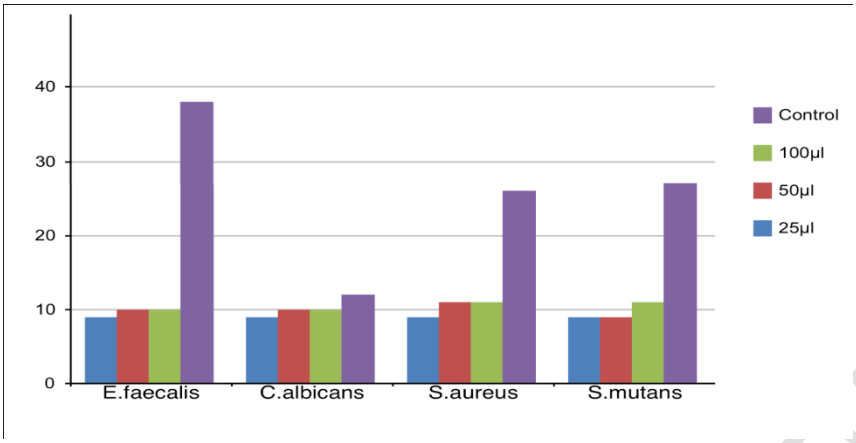

Fig 10: bar graph represents antimicrobial activity of the herbal formulation - CCB at varying concentration and a positive control.