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SYNERGISTIC EFFECT OF *AZADIRACHTA INDICA* AND *CURCUMA LONGA* WITH FLUCONAZOLE GEL AGAINST *CANDIDA ALBICANS*

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Keywords:

Candida albicans, Fluconazole, Zone of Inhibition, *Azadirachta indica, Curcuma longa*

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ABSTRACT: In this study, the synergistic effect of an extract of *Azadirachta indica* and *Curcuma longa* were investigated by the agar diffusion method. Different dilutions of the extract were prepared, and antifungal activity of the extracts of *Azadirachta indica* and *Curcuma longa* against *Candida albicans* was measured alone and in combination with fluconazole gel. The zone of inhibition of *Azadirachta indica* and *Curcuma longa* was found to be 9 mm to 14 mm and 8 mm to 13 mm respectively in the concentration range of 10-60 µL against *Candida albicans*. The fluconazole gel was prepared, and various evaluation parameters like; spreadability, pH, viscosity, and drug content were determined. Extracts of *Azadirachta indica* and *Curcuma longa* were taken in different strength (10-60 mg/ml) however the concentration of fluconazole gel was kept constant (1%) and zone of inhibition was determined. The maximum zone of inhibition was found with *Azadirachta indica* in the combination of 60mg/ml and *Curcuma longa* 20 mg/ml.

INTRODUCTION: As per an examination performed by the WHO (World Health Organization), at the very least 80% population relies upon the traditional remedy for their key healthcare needs. The old therapeutic techniques, mostly the utilization of restorative herbs, still have a lively influence to protect the essential wellbeing wants in the developing nations [1-3]. The therapeutic estimation of these plants relies upon the chemical constituent that creates a known practical activity on the metabolism of human body.

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Inside the ongoing years, regularly expanding helpful issues are the expanded level of diseases and antibiotic resistance. The utilization of plant and its products have been utilized for numerous long time, that started with conventional drug and throughout the years has been joined into allopathic prescription. Since, the distant past, numerous plants classes affirmed having pharmacological impacts as they are known to have different 2° metabolites like glycosides, saponins, flavonoids, steroids, tannins, alkaloids, terpenes which are, in this way, ought to be utilized to experience the organisms [4-7].

Herbs used in conventional remedy contain an extensive variety of chemical constituents that can be used to treat and control continuing as well as infectious diseases. Natural products obtained from higher plants may have a new foundation of

antimicrobial medication with the possibly innovative mode of action [8]. They are useful in the treatment of infectious diseases while at the same time reducing many of the side effects that are often related to chemically produced antimicrobials. Therefore, it is of great attention to carry out a study of these herbs to quantify their use in popular medicine and to disclose the mechanism of action by segregation and description of their constituents [9].

MATERIAL AND METHODS:

Material: Crude plant material was collected from the local area and authenticated by National Institute of Pharmaceutical Education and Research (Mohali), Fluconazole was purchased from Yarrow chemicals, Mumbai.

Drug Profile: [10]

Name: Fluconazole

Molecular Formula: $C_{13}H_{12}F_2N_6O$

Molecular Weight: 306.27

Drug Profile: Triazole antifungal agent that is used to treat candidiasis infection and cryptococcal meningitis.

Structure:

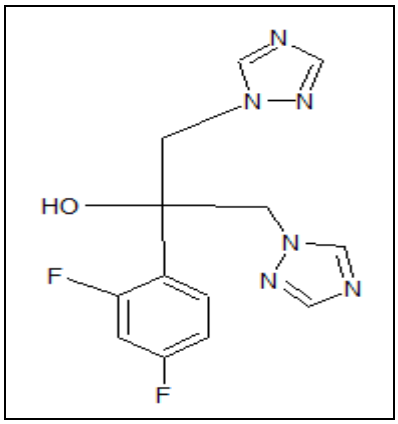

FIG. 1: CHEMICAL STRUCTURE OF FLUCONAZOLE

Synonyms:

- 2-(2,4-difluorophenyl)-1,3-di(1H-1,2,4-triazole-1-yl) propane-2-ol

- 2,4-Difluoro-alpha, alpha-bis(1H-1,2,4-triazole-1-ylmethyl) benzyl alcohol

Mechanism of Action: Fluconazole cooperates with 14-α demethylase, which is a cytochrome P-450 protein important to change over lanosterol to ergosterol. As ergosterol is a fundamental segment of the cell membrane *candida albicans*, restraint of its union outcomes in expanded cell penetrability causing leakage of the cell membrane. Fluconazole may likewise hinder endogenous respiration, cooperate with film phospholipids, restrain the change of yeasts to mycelial shapes, repress purine take-up, and weaken triglyceride and additionally phospholipid biosynthesis.

The Microorganism:

***Candida albicans*:** *Candida albicans* is dependent pathogenic yeast that is a common part of human microbial flora. It does not multiply outside human gut flora. Almost 40-60% of healthy adult's population has candida albicans in their GIT and mouth. More than 20 species of candida can cause candidiasis in humans.

Candida yeast mainly resides in the intestinal tract and can be found on the mucous membrane without causing any infection; mainly overgrowth is responsible for the infection [11]. Candida can be found mainly in two physical forms, the yeast can survive in acidic conditions, although the fungal form flourishes in a neutral or alkaline ph. Therefore, it is difficult to treat with a single therapy **Fig. 2** & **3**.

Fluconazole Gel Preparation: The composition of fluconazole topical gel was shown in **Table 1**. Fluconazole (1% w/w) was dissolved in a heated mixture of propylene glycol (20% w/w) and glycerine (10% w/w) used as the moistening agent. Cellulose polymer (HPMC, MC), polyacrylic acid polymer (Carbopol 940) gel was prepared by adding the calculated amount of in warm water. The previous mixture of the drug was added in the polymer mixture [12]. Triethanolamine (TEA) was used as a pH regulator, for the proper mixing of ingredients agitation should be continuous.

Finally, propylparaben and methylparaben were added with continuous slow shaking as a preservative. The prepared gel was placed in a round neck closed container in a dark room at room temperature **Table 1**.

FIG. 2: GROWTH OF *CANDIDA ALBICANS* ON SUBOURAUD DEXTROSE AGAR MEDIA

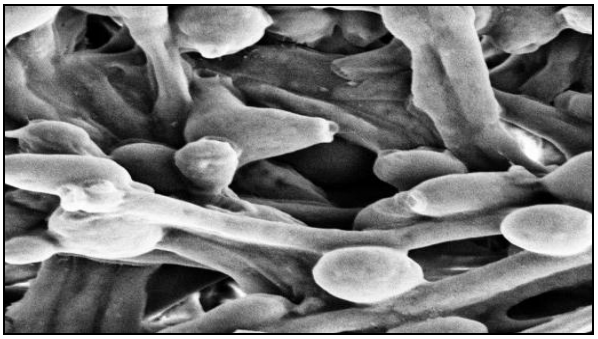

FIG. 3: MICROSCOPIC STRUCTURE OF *CANDIDA ALBICANS*

TABLE 1: COMPOSITION OF FLUCONAZOLE TOPICAL GEL (% w/w)

Ingredients	F1	F2	F3	F4	F5
Fluconazole	1	1	1	1	1
Carbopol 940	0.5	0	1	1.5	0.5
Carbopol 934	0.5	1.5	0	1	0.5
HPMC	1	1	1.5	0	1.5
Glycerine	10	10	10	10	10
Propylene glycol	20	20	20	20	20
Methyl paraben	0.3	0.3	0.3	0.3	0.3
Propyl paraben	0.1	0.1	0.1	0.1	0.1
Purified water to	100	100	100	100	100

Neem Leaves Extract Preparation: Neem leaves extract has been prepared to dry 100 gm of dried plant leaves and ground into powder for the better mixing with hexane **Fig. 4**. After filtration by muslin cloth, the filtrate was evaporated using rotary evaporator below 30 °C and extract stored at 4 °C [13].

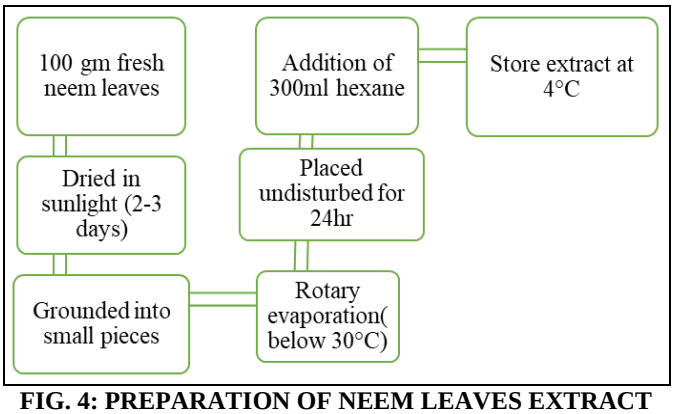

FIG. 4: PREPARATION OF NEEM LEAVES EXTRACT

***Curcuma longa* Extract:** Fresh rhizomes of *Curcuma longa* were taken and grounded finely in mortar pestle with the addition of water and then exposed to steam distillation. The oily fraction was collected, and residue in water was filtered in a conical flask [14, 29]. The water fraction was evaporated using vacuum condition overnight to give water extract. The residue was air dried and left overnight in chloroform (200 ml) and filtered, after that it is re-extracted twice with chloroform. All the chloroform fractions were combined, and solvent was evaporated to get chloroform extracts **Fig. 5**. The residue obtained after chloroform extract was again extracted with methanol to give the methanol extract [15, 30].

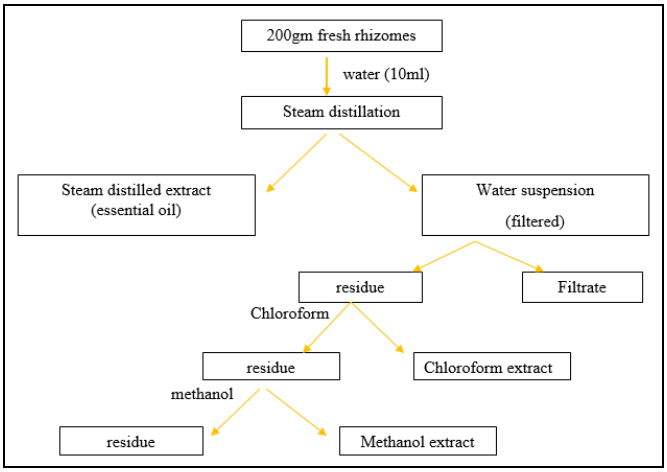

FIG. 5: *CURCUMA LONGA* EXTRACTION PROCEDURE

Evaluation of Fluconazole Gel:

Visual Examination: Visual inspection of the prepared gel has been carried out for color and appearance. The observations reported that the color of gel formulations was white and was free from any gritty particles.

Spreadability Test: Spreadability test of the prepared gel have been carried out with "parallel plate method." The gel is being placed between two

glass slides having a specific length. Weight has been placed on the upper glass slide for complete distribution of the gel in between glass slides. After some time, weight was removed. The plates have been fixed in such a way that the upper plates slide down from lower plate with the help of weight tied to it, the time is taken for the upper slide to slid from lower slide been noted down.

The steps are followed three times and mean were taken.

Value of Spreadability have been determined with the help of following formula given below-

$$S = M \times L / T$$

Where: S= Spreadability; M= mass placed on the upper slide; L= Length of the glass slides; T= Time taken for sliding by the upper slide.

pH Determination: The pH of final formulation was determined by dipping pH- electrode of digital pH meter.

Drug Content Uniformity Determination: Drug content determination has been carried out by taking 10 gm prepared gel in a conical flask containing 50 ml phosphate buffer having pH of 7.4, with continuous shaking for the complete mixing of the drug. The resultant mixer was then filtered through a 0.45 mm filtration membrane. The solution is then stored for 24 h absorbance of the different dilutions had been determined at the 266 nm wavelength.

$$\text{Content uniformity} = \text{Sample abs.} / \text{std. abs.} \times 100$$

Rheological Studies: The viscosity of prepared formulation was determined at 25 °C using Brookfield viscometer. The spindle (no. 62) was rotated at specific rpm dial reading was noted down for further calculations.

Value of viscosity have been determined with the help of following formula given below-

$$\text{Viscosity in cps} = \text{Dial reading (\% torque)} \times \text{Factor}$$

***In-vitro* Drug Release Study:** Apparatus contains a glass cylinder (10 cm in height and 3.7 cm diameter) having both the sides open a cellophane membrane (previously soaked in distilled water) were tied at the end of the tube. 10 gm of gel was placed on the cellophane membrane in donor compartment, and the cell was immersed in a beaker (recipient compartment) having 100 ml phosphate buffer (pH 4.6) lower end of the test-tube should be placed like this it touches the surface of diffusion medium in recipient compartment.

Diffusion medium was agitated using magnetic stirrer at 37 °C temperature. Sink conditions were maintained throughout the experiment. Samples were analyzed of different time intervals (0 min-240 min) using UV visible spectrophotometer.

Inoculation Procedure for *Candida albicans*:

- Agar slants are-readied to inoculate microbial culture. To get ready agar slant, a required number of culture tubes are taken, and around 12 to 15 ml of liquified agar medium is poured in each of them. The tubes are presently cotton-plugged and disinfected in an autoclave.
- After the cleansing is over the tubes are taken out and are set in inclining (slanting) position for here and there; the tubes get cooled, and the medium in them is set bringing about a sloppy surface [16, 32].
- Both the tubes are opened by removing the cotton-plug with fingers of the correct hand, and the open mouth of the tubes is disinfected by going through the fire twice.
- The inoculum containing circle/needle is taken out and gotten inside the agar incline containing tube where the inoculum is simply rubbed on the surface of the agar incline.
- Every one of the means beginning from plug expulsion from the mouth of the tubes to the rubbing of the inoculum on the surface of the agar inclination ought to be taken rapidly to maintain a strategic distance from defilement.
- At the point when inoculation is finished, the open mouths of tubes and the cotton plugs are cleaned by fire, and the cotton plugs are supplanted. The growth of microorganism observed.

Plants Extracts Activity Assay:

Well Diffusion Assay: Subouraud dextrose agar media (SDA) is used for the determination of antifungal activity of plants extracts. Micro-

organisms were spread over the media with the help of cotton plugs. Wells with a diameter of 8-10 mm have punched aseptically with the cork borer or a tip. Concentration ranges of plant extracts in 20 µl - 100 µl were placed in wells using a suitable solvent (DMSO). Then agar plates were incubated at 37 °C for 24-48 h Results were noted down in diameter inhibited growth of Candida albicans by extracts [17].

RESULTS AND DISCUSSION:

Visual Examination: Visual inspection of the prepared gel has been carried out for color and appearance. The observations reported that the color of gel formulations was white and was free from any gritty particles.

Spreadability: Spreadability test of the prepared gel have been carried out with "parallel plate method." The gel is being placed between two glass slides having a specific length. Weights have been placed on the upper glass slide for complete distribution of the gel in between glass slides. After some time, weight was removed. The plates have been fixed in such a way that the upper plates slide down from lower plate with the help of weight tied to it, the time is taken for the upper slide to slid from lower slide been noted down **Table 1**.

TABLE 2: SPREADABILITY EVALUATION OF GEL FORMULATION

Formulation code	Time took (min)	Spreadability (S) (cm)
F1	3	4.5
F2	4	4.3
F3	4.5	5.1
F4	3.9	4.6
F5	3.5	4

TABLE 3: pH OF DIFFERENT FORMULATIONS MEASURED

Formulation code	pH
F1	6.5
F2	6.1
F3	5.8
F4	6.3
F5	7.1

pH Determination of Various Prepared Formulations: The pH range of all formulation comes under the range of 5-7, which is acceptable for the avoiding of reduction risk of irritation during application. Results are tabulated in **Table 2**.

Drug Content Determination of Various Prepared Formulations: The drug content is shown in the **Table 4**. After evaluation of the entire prepared formulations, drug content was determined and comes under the range of 80% - 86%. The uniform distribution of the drug was shown after the determination of drug content **Table 3**.

TABLE 4: DRUG CONTENTS VALUES OF VARIOUS FORMULATIONS

Formulation code	Drug content
F1	81.25 ± 0.391
F2	80.10 ± 0.284
F3	82.32 ± 1.02
F4	83.11 ± 0.645
F5	86.49 ± 0.982

n = 3 ±SD

Viscosity Determination: The viscosity ranges of all formulations from 1230-1840 centipoises **Table 4**. Viscosity is directly proportional to polymer concentration; more the concentration of the polymer represents the higher viscosity in the formulation **Fig. 6**.

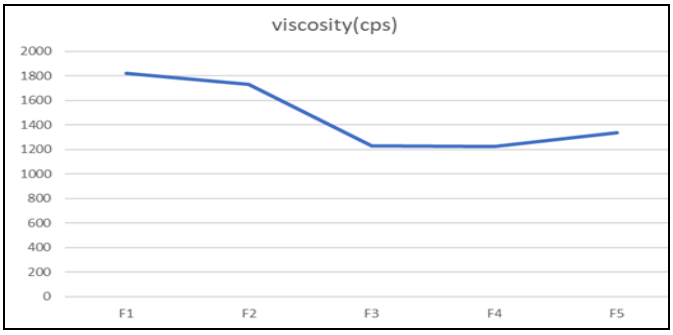

FIG. 6: GRAPHICAL REPRESENTATION OF VISCOSITY OF VARIOUS GEL FORMULATIONS

TABLE 5: VISCOSITY VALUES OF VARIOUS GEL FORMULATIONS

Formulation code	Dial reading (% torque)	Factor	Speed (rpm)	Viscosity
F1	1.82	1000	0.3	1820
F2	1.73	1000	0.3	1730
F3	1.23	1000	0.3	1230
F4	1.52	1000	0.3	1526
F5	1.34	1000	0.3	1340

***In-vitro* Drug Release Study:** The *in-vitro* release profile of all the prepared formulations was represented in **Fig. 7**. It was observed that the release of the drug from the formulation could be ranked in the following descending order: F4>F1>F5>F2>F3; the amount of drug release after 4 h was 90.64%, 83.49%, 80.86%, 80.24%, and 79.56% respectively **Table 5**. It was observed the factors which are affecting the drug release are polymer concentration and type of the polymer.

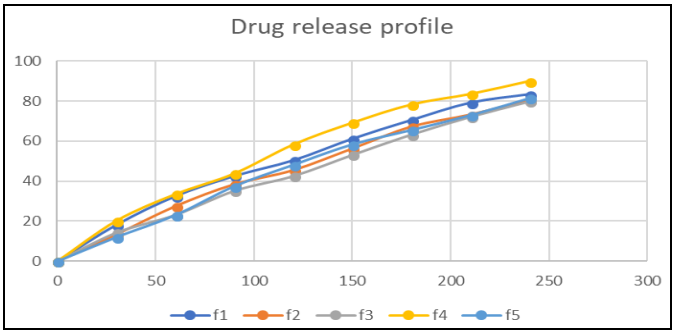

FIG. 7: DRUG RELEASE OF VARIOUS PREPARED GEL FORMULATIONS

Zone of Inhibition of Plant Extracts against *Candida albicans*: The effects of aqueous and methanolic extracts of concentration range between 10-60µL against *Candida albicans* were shown in **Table 8** and **9** respectively. The maximum activity against *Candida albicans* was observed in the case of *Azadirachta indica* and *Curcuma longa* at the concentration of 60 mg/ml and 40 mg/ml respectively **Table 7**.

TABLE 6: DRUG RELEASE STUDY OF FLUCONAZOLE GEL FORMULATION

Time (min)	% Cumulative drug release				
	F1	F2	F3	F4	F
0	0	0	0	0	0
30	18.24	13.42	14.35	20.21	12.02
60	32.51	27.52	23.24	33.54	23.18
90	42.64	38.47	35.28	43.50	37.65
120	50.45	45.62	42.67	58.22	48.25
150	61.21	56.38	53.27	69.37	58.45
180	70.30	67.20	63.87	78.35	65.87
210	78.21	73.33	72.04	83.64	73.15
240	83.49	80.24	79.56	90.64	80.86

TABLE 7: PHYSICAL PROPERTIES OF FLUCONAZOLE GEL FORMULATION

Formulation code	pH	Viscosity	Spreadability	Drug content (%)
F1	6.5	1820	4.5	81.25
F2	6.1	1730	4.3	80.10
F3	5.8	1230	5.1	82.32
F4	6.3	1526	4.6	83.11
F5	7.1	1340	4	79.49

TABLE 8: ZONE OF INHIBITION VALUES OF PLANT EXTRACTS

S. no.	Concentrations of extracts (mg/ml)	*Azadirachta Indica* (zone of inhibition in mm, diameter)	*Curcuma longa* (zone of inhibition in mm, diameter)
1	10	9	8
2	20	11	6
3	30	10	4
4	40	6	13
5	50	13	11
6	60	14	8

Zone of Inhibition of Plant Extracts in Combination with Fluconazole Gel against Candida Albicans: After incorporating the plant extracts in the gel formulations, it was observed that fluconazole shows a synergistic effect in very low proportion. A synergistic effect was observed in case of *Azadirachta indica*, and *Curcuma longa* **Table 8**.

TABLE 9: ZONE OF INHIBITION VALUES AFTER COMBINATION OF GEL FORMULATION WITH PLANT EXTRACT

S. no.	Concentrations (extracts + gel) (mg/ml)	The concentration of Fluconazole gel (%)	*Azadirachta indica* (zone of inhibition in mm, diameter)	*Curcuma longa* (zone of inhibition in mm, diameter)
1	10	1	11	13
2	20	1	7	14
3	30	1	8	9
4	40	1	9	15
5	50	1	12	8
6	60	1	16	15

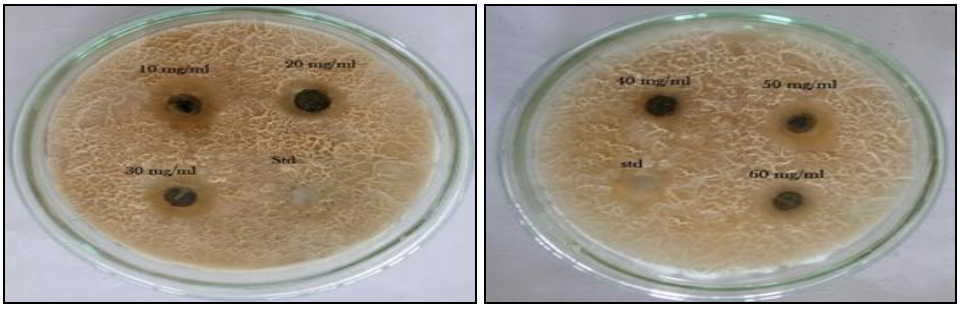

FIG. 8: EFFECT OF *AZADIRACHTA INDICA* EXTRACTS ON *CANDIDA ALBICANS*

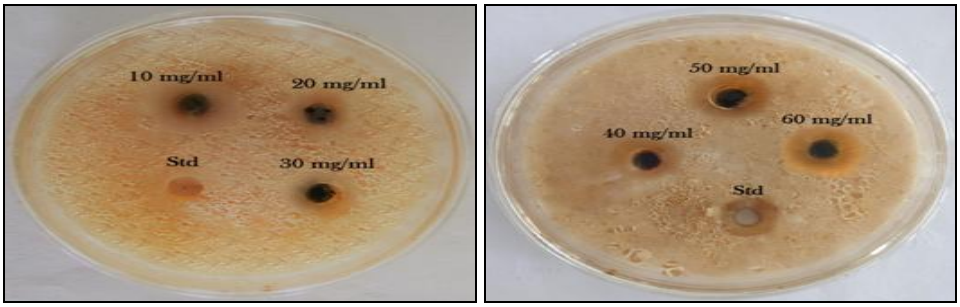

FIG. 9: EFFECT OF *A. INDICA EXTRACTS* IN COMBINATION WITH FLUCONAZOLE GEL ON *CANDIDA ALBICANS*

FIG. 10: EFFECT OF *CURCUMA LONGA* EXTRACTS ON *CANDIDA ALBICANS*

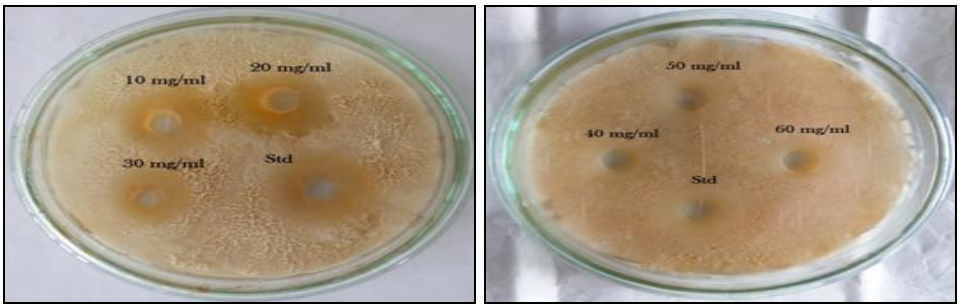

FIG. 11: EFFECT OF *C. LONGA EXTRACTS* IN COMBINATION WITH FLUCONAZOLE GEL ON *CANDIDA ALBICANS*

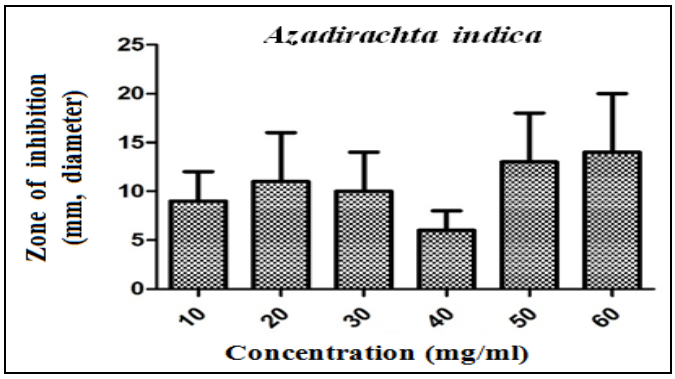

FIG. 12: ZONE OF INHIBITION VALUES OF *A. INDICA EXTRACTS* AGAINST *CANDIDA ALBICANS*

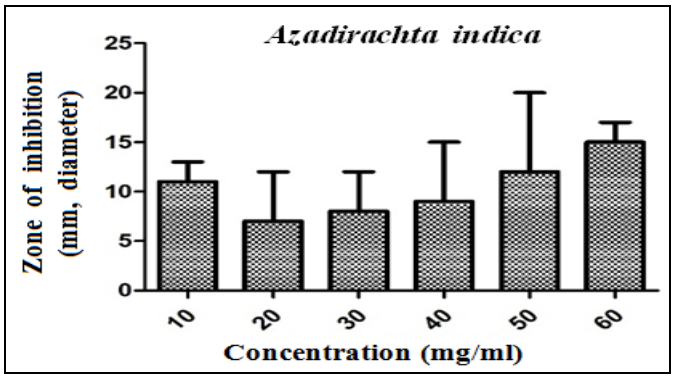

FIG. 13: ZONE OF INHIBITION VALUES OF *A. INDICA EXTRACTS* IN COMBINATION WITH FLUCONAZOLE GEL AGAINST *CANDIDA ALBICANS*

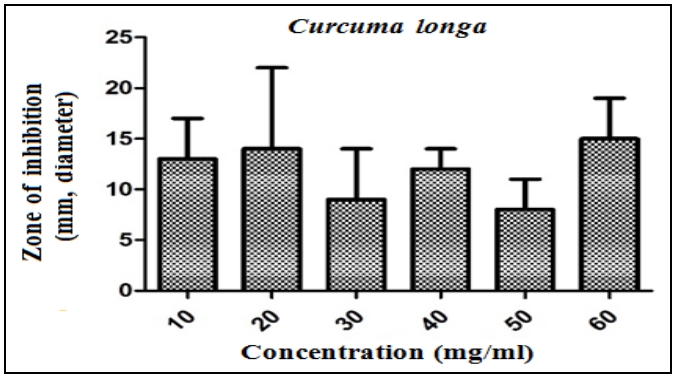

FIG. 14: ZONE OF INHIBITION VALUES OF *CURCUMA LONGA EXTRACTS* AGAINST *CANDIDA ALBICANS*

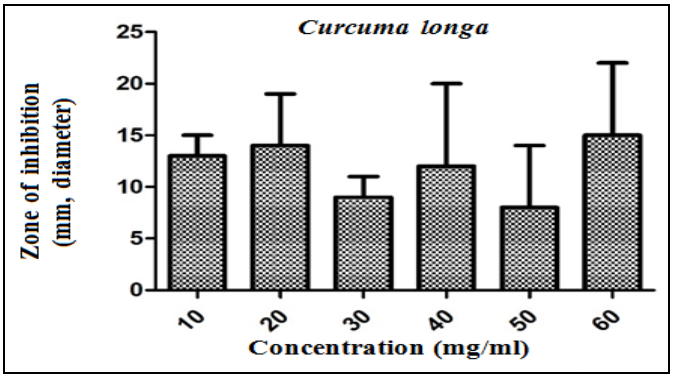

FIG. 15: ZONE OF INHIBITION VALUES OF *C. LONGA EXTRACTS* IN COMBINATION WITH FLUCONAZOLE GEL AGAINST *CANDIDA ALBICANS*

CONCLUSION: Current study indicates that the plant herbal extract and antimicrobial agents combination approach provides significant therapy for the development of potential treatment for microbial infections. The antifungal activity observed for the plant extracts used in the study indicates the further development of effective alternate antimicrobial remedies. Crude extracts of *Azadirachta indica* and *Curcuma longa* have been used previously against various pathogens and proved to be effective. Also they both have shown the synergistic effect with fluconazole against *Candida albicans*.

After performing all the physiochemical evaluations of the fluconazole gel, all the properties of gel were acceptable as an antifungal agent. The viscosity of the gel was optimum; pH value of all prepared formulations was under 5 to 7, which is acceptable for not causing any irritation and other side effects. All the plant extracts have shown antifungal activity along with synergistic action in combination with fluconazole.

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CONFLICT OF INTEREST: None declared.

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