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ANTI-DANDRUFF POTENTIAL OF ETHANOLIC EXTRACT OF CARICA PAPAYA LEAVES AND ITS SYNERGISTIC EFFECTS WITH KETOCONAZOLE

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ABSTRACT

Dandruff, a major worldwide problem, characterized by massive desquamation of scaly flakes of stratum corneum. The role of fungus, *Malessezia furfur* as a causative agent in pathogenesis of dandruff has been suggested. Natural plant extracts with bioactive phytoconstituents serve as safer alternative over synthetic antidandruff shampoos. Although, role of *Carica papaya* leaves as an antifungal agent has been demonstrated, its imperative role against *Malassezia furfur* has remained elusive. In the present study, dried powdered *Carica papaya* leaves were extracted in ethanol using maceration method. The preliminary qualitative phytochemical screening of ethanolic extract of papaya leaves was conducted. Moreover, *in vitro* antifungal activity of papaya leaves extract against *M. furfur* was assessed using broth dilution and agar cup plate methods. The synergistic activity of ethanolic extract of papaya leaves in combination with ketoconazole was performed using agar cup plate assay. The phytochemical screening revealed the presence of alkaloids, flavonoids, saponins, steroids, carbohydrates, tannins, phenolic compounds and proteins. The ethanolic extract of papaya leaves exhibited antifungal activity at (2 mg/ml) and (2.2 mg/ml) concentration with zone of inhibition of 8±0.35 mm and 10±0.15 mm respectively in agar cup plate assay and minimum inhibitory concentration (MIC) at (1.8 mg/ml). Moreover, in combination study, papaya leaves extract with ketoconazole (2 mg/ml+1 mg/ml) showed synergistic effect with zone of inhibition of 15±0.50 mm as compared to ketoconazole control (12±0.27 mm). We suggest that ethanolic extract of papaya leaves serve as potential antifungal agent for dandruff management.

KEYWORDS: Carica papaya leaves, Antifungal, Antidandruff, Minimum inhibitory concentration, Zone of inhibition, Phytochemical screening.

INTRODUCTION

Dandruff is the most common chronic scalp abnormal skin condition characterized by excessive scaling and shedding of epidermal cells from the scalp. It is manifested in the form of itching, flaking, irritancy and redness of scalp with mild inflammation.[1,2,3] and is prevalent in almost half of the population at the pre-pubertal age of any gender. The etiology of dandruff mainly involves hyperkeratinization of scalp epidermal cells, over-production of sebum, individual susceptibility and microfloral metabolism. The colonization of unipolar lipophilic yeast, *Malassezia furfur* (*Pytirosporum ovale*) found on human skin flora (75% to 98%) played vital role in pathogenesis of dandruff. Cutaneous *Malassezia* fungus requires external source of lipids (saturated free fatty acids) for its survival and therefore converts the sebum into fatty acids via oxidation of triglycerides by stimulating lipase enzyme, which accelerates hyperproliferation of keratinocytes.[4,5] The accumulation of excess unsaturated fatty acids, such as oleic acid, on the skin causes skin irritation.[6] Owing to its remissions, relapses and repeated necessity of treatment, dandruff management became a challenge to clinicians and cosmeceutical companies. Although several synthetic commercial anti-dandruff shampoos like, zinc pyrithione, ketoconazole, climbazole (imidazole derivatives), salicylic acid, selenium sulphide and coal tar are available in the market. However, widespread use of these synthetic antifungal drugs revealed numerous side effects (pruritus, burning, edema, erythema, dry skin, headache and difficulty in breathing, tightness in the chest) and developed resistance against fungus upon its chronic use.[7,8,9] The potency and efficacy of synthetic drugs can be improved by combination therapy with novel herbal formulations possessing anti-*Malassezia* activity that could overcome the limitations with the current antidandruff treatment.

Herbal extracts are enriched with several phytochemicals (secondary metabolites) like, flavonoids, tannins, alkaloids, terpenoids, sterols and carbohydrates that contributes to its antimicrobial/anti-fungal properties.[10,11] Papaya, botanical name *Carica papaya* L., belonging to the family *Caricaceae*, is a small tree or an evergreen shrub medicinal plant with powerhouse of nutrients. The whole plant parts (fruit, roots, bark, peel, seeds and pulp) is known to possess medicinal properties. Interestingly, papaya leaves have shown to contain many active constituents including, alkaloids (carpinine, carpaine, carpasemine,

pseudocarpaine, dehydrocarpaine I & II), flavonoids (myricetin, kaemferol, quercetin), proteolytic enzymes (papain, chymopapain), sulfurous compounds (benzyl iso-thiocyanate), caretinoids, triterpenes, amino acids, vitamin C and E.[12,13,14] *Carica papaya* leaves exhibits wide spectrum biological activities such as anti-inflammatory, anti-malarial, hepatoprotective, cardioprotective, antioxidant, antispaspmodic, anti-cancer, immunomodulatory, anti-aging, antiviral, antibacterial and antifungal.[14,15,16,17,18] Substantial evidences demonstrated antifungal potential of *Carica papaya* leaves against, *candida albicans, Rhizopus stolonifer, Fusarium spp., Colletotrichum gloeosporioides and Colletotrichum gloeosporioides*.[9,18,19,20] However, putative role of *Carica papaya* leaves against dandruff causing yeast, *Malassezia furfur* has remained yet unexplored. Hence, the present investigation was undertaken to unveil *in-vitro* antifungal potential of ethanolic extract of *Carica papaya* leaves against *Malassezia furfur* for dandruff management employing broth dilution and agar cup plate assays. Moreover, the synergistic effect of ethanolic extract of *Caricia papaya* leaves with ketoconazole was also assessed using agar cup plate method.

MATERIALS AND METHODS

Materials

The active Papaya leaves were procured from local suppliers and authenticated at Department of Botany, R.T.M., Nagpur University.

Figure 1: The authenticated *Carica papaya* leaves.

Preparation of plant extract

The samples of papaya leaves were sorted, washed and shade dried at room temperature for 15 days. The plant materials were crushed well into fine powder in an electronic grinder and kept into air tight polythene bags for further use and stored at room temperature. Approximately 40 gm of papaya leaves powder were soaked and macerated in 400 ml of 96% ethanol for 3 days with occasional stirring. After extraction, the extract was decanted and filtered through whatman filter paper. Ethanolic crude extract was obtained by evaporating the solvent using rotary evaporator and water bath at 60°C. The ethanolic extract was weighed (3 gm) and then stored in the refrigerator at 4°C until use.

Phytochemical screening

Carica papya leaves extract was subjected to phytochemical screening for presence of phytoconstituents such as alkaloids, flavonoids, saponins, steroids, carbohydrates, tannins, phenolic compounds and proteins with the following standard procedures.[21,22,23]

1) Detection of flavonoids

A. Alkaline reagent test: To one ml solution of the extract, 1 N NaOH solution was added to give yellow colour. This colour vanishes after addition of few drops of dilute acid indicating the presence of flavonoids.

B. Shinoda test: To the test solution, few magnesium turnings and Concentrated HCl was added drop wise. If the pink scarlet, crimson red or occasionally green to blue colour obtained after few minutes, shows the presence of flavonoids.

2) Detection of alkaloids

The extract (50 mg) was stirred with few ml of dilute hydrochloric acid and filtered. The filtrate was tested carefully with various alkaloid reagents as follows.

A. Mayer's Test: To a few ml of filtrate, a drop or two of Mayer's reagent were added by the side of the test tube. A white or creamy precipitate indicated the positive test.

B. Dragendorff's test: To a few ml of filtrate, 1-2 ml of Dragendorff's reagent was added. A prominent yellow precipitate indicated the test as positive.

3) Detection of carbohydrates

The extract (100 mg) was dissolved in 5 ml of water and filtered. The filtrate was subjected to the following tests.

A. Molisch's test

To 2 ml of filtrate, two drops of alcoholic solution of α-naphthol were added, the mixture was shaken well and 1 ml of concentrated sulphuric acid was added slowly along the sides of the test tube and allowed to stand. A violet ring indicated the presence of carbohydrates.

B. Benedict's test

To 0.5 ml of filtrate, 0.5 ml of Benedict's reagent was added. The mixture was heated on a boiling water bath for 2 min. A characteristic coloured precipitate indicated the presence of sugar.

4) Detection of glycosides

For detection of glycosides, 50 mg of extract was hydrolysed with concentrated hydrochloric acid for 2 hrs on water both, filtered and the hydrolysate was subjected to the following test.

A. Keller killiani test: The test solution with few drops of glacial acetic acid in 2 ml of 5% $FeCl_3$ and concentration sulphuric acid from side of the test tube-Lower layer reddish brown and upper layer (bluish green) indicates the presence of glycosides.

5) Detection of saponins by foam test

2 ml of papaya extract was diluted with 2 ml of distilled water. The suspension was shaken continuously in a graduated cylinder for 15 min. A stable two cm layer of foam indicated the presence of saponins.

6) Test for steroids

0.5 g of ethanolic papaya leaf extract was dissolved in 2ml of sulphuric acid and the color change from violet to blue or green, confirmed the presence of steroids.

7) Detection of Proteins and Amino acids

The extract (100 mg) was dissolved in 10 ml of distilled water and filtered through whatman No.1 filter paper and the filtrate was subjected to tests for proteins and amino acids.

A. Biuret test: An aliquot of 2 ml of filtrate was treated with one drop of 2 % copper sulphate solution. To this, 1 ml of ethanol (95%) was added, followed by excess of potassium hydroxide pellets, pink colour in the ethanolic layer indicated the presence of proteins.

8) Detection of tannins

A. Ferric chloride test: An extract (50 mg) was dissolved in 5 ml of distilled water. To this, few drops of neutral 5% ferric chloride solution were added. Appearance of brownish green or blue black colour indicated the presence of tannins.

9) Test for phenols

Few drops of Ferric chloride solution were added in 1ml of papaya leaf extract. Appearance of intense colouration, suggested the indication of phenol.

Test microorganism

In the present study, the standard fungal culture of *Malassezia furfur* (*M. furfur*) (MTCC:1374) was procured from Institute of Microbial Type Culture Collection and Gene Bank, Chandigarh (MTCC), India.

Preparation of the media

The culture of *Malassezia furfur* was prepared by using Sabouraud dextrose agar (SDA). The Loop-full of fungal culture was inoculated in the SDA medium and incubated for 72 hours at room temperature.

***In vitro* antifungal assays of an extract**

Preparation of stock solution of papaya leaves extract

The stock solution was prepared by weighing 200 mg of ethanolic papaya leaves extract and dissolving it in 100 ml of water to obtain concentration of 20 mg/ml.

Agar cup plate method

The antifungal activity against *M. furfur* was investigated by agar cup plate method according to the procedure described by Shaikh et al. (2010) and Ruth and Miller, 2015.[24,25] with some modifications. Sabouraud dextrose agar medium was poured into the petriplate. After the medium got solidified, the 0.5 ml of diluted fungal suspension was swabbed on respective nutrient agar plates. Then, a hole with a 1 cm diameter was punched aseptically with a sterile cork borer. The 100 µL (0.1 ml) of ethanolic papaya extracts with 1.8 mg/ml, 2 mg /ml and 2.2 mg/ml concentrations were added into each wells respectively. Ketoconazole (1 mg/ml) solution was used as a reference standard control. The plates were incubated at 28 °C for 48 hrs. To elucidate synergistic antifungal activity of *Carica papaya* leaves extract with ketoconazole (1.8 mg/ml+1 mg/ml and 2 mg/ml+1 mg/ml), two combination studies were

performed (Figure 4). The antifungal activity was determined by measuring the diameter of zone of inhibition (mm) around the well by vernier caliper. The data were recorded in terms of mean±standard deviation.

Broth dilution method

The antifungal activity of ethanolic extract of *Carica papaya* leaves were evaluated using broth dilution method according to the procedure described by Shaikh et al. (2010)[24] with some modifications. The broth/tube dilution test is the standard method for determining levels of microbial resistance to an antimicrobial agent. Serial dilutions of the test agent was made in a liquid microbial growth medium which is inoculated with a standardized number of organisms and incubated for a prescribed time. At the end of the incubation period (generally 24-48 hours), the tubes are visually examined for the presence or absence of turbidity.

Determination of minimum inhibitory concentration (MIC)

Minimum inhibitory concentration (MIC) is the lowest concentration of extract that inhibits the growth of test pathogens by inhibiting the visual appearance of turbidity. In the present study, the microbial work was carried out in an aseptic area and the MIC was evaluated by preparing the inoculum of microorganisms from nutrient broth cultures. Initially, the nutrient broth was prepared and sterilized by autoclave using 15 Ib pressure at 121 °C for 15 min. The medium was poured into the test tubes. The constant volume of 0.1 ml of standard *M. furfur* inoculum was added in the test tubes. The extracts were serially diluted and exact amount of extract was added using sterile pipettes as indicated in the Table 2 to obtain a final volume of 10 ml. Ketoconazole (1 mg/ml) was used as a reference standard. The tubes were incubated at temperature 30° C for 48 hours. The test tubes were evaluated for growth of fungus by observing the presence or absence of turbidity. The test procedure was repeated in triplicates to measure reproducibility of the results. The lowest concentration which showed the absence of turbidity was recorded as the MIC value.

RESULTS AND DISCUSSION

Preliminary phytochemcial screening

Phytochemicals are constituents synthesized naturally by plants as the primary or secondary metabolites, possessing enormous pharmacological activities. Therefore, to evaluate the presence of active phytoconstituents in the ethanolic extract of *Carica papaya* leaves, a preliminary phytochemical screening assay was performed. In the present investigation, the extract revealed the presence of alkaloids, flavonoids, saponins, steroids, carbohydrates,

tannins, phenolic compounds and proteins, as depicted in Table 1. The results corroborate previous findings suggesting the existence of different bioactive secondary metabolites in herbal plants and *Carica papaya* leaves (flavonoids, alkaloids, phenols and tannins) that might be responsible for their antimicrobial attributes.[9,18,23,26,27]

In vitro Antifungal/Antidandruff activity of *Carica papaya* leaves extract

In the present study, the ethanolic extract of *Carica papaya* leaves exhibited significant antifungal activity in both broth dilution and agar cup plate methods. The broth dilution assay showed dose-dependent inhibition of *M. furfur* growth with the increasing concentrations of the ethanolic extract of *Carica papaya* leaves. Table 2. demonstrates the antifungal activity of ethanolic extract of *Carica papaya* leaves as revealed by absence of turbidity at 1.8 mg/ml, 2 mg/ml and 2.2 mg/ml relative to control group. The lowest concentration, MIC of the extract that inhibited the growth of *M. furfur* was found to be at 1.8 mg/ml relative to control group as evident in Figure 2. Moreover, in agar cup plate method, the ethanolic *Carica papaya* leaves extract showed significant antifungal activity at 2 mg/ml and 2.2 mg/ml concentration with zone of inhibition of 8±0.35 mm and 10±0.15 mm (Figure 3.) respectively as compared with ketoconazole control (11mm±0.62 mm). Table 3. summarizes the dose-dependent zone of inhibitions of ethanolic *Carica papaya* leaves extract at different concentrations against *M. furfur*. The present findings confirmed an antidandruff potential of ethanolic extract of *Carica papaya* leaves as revealed by inhibitory activity against fungus, *M. furfur*.

Moreover, combination of ethanolic extract of papaya leaves with ketoconazole at 1.8 mg/ml+1 mg/ml and 2 mg/ml+1 mg/ml concentrations exhibited synergistic effect with the zone of inhibitions of 10±0.34 mm and 15±0.50 mm respectively as compared with *Carica papaya* leaves extract alone (7±0.14 mm or 8±0.35 mm respectively) and ketoconazole control (12±0.27 mm; Figure 4 and Table 4). Figure 5. summarizes the comparative study of combination of ethanolic extract of *Carica papaya* leaves alone and ethanolic extract of *Carica papaya* leaves+ketoconazole. The results of present study are in accordance with the previous findings. Recently, ethanolic extract of *Carica papaya* leaves demonstrated significant antifungal activity against *Candida albicans* at 1 mg/ml concentration, and the effect was potentiated with flucoconazole.[9] Additionally, *Carica papaya* leaves exhibited antifungal activity against several fungal species including, against, *Candida albicans, Rhizopus stolonifer, Fusarium spp. and Colletotrichum gloeosporioides and Colletotrichum gloeosporioides.*[9,18,19,20,28] It has been postulated that papaya leaves contain several

phytoconstituents (alkaloids, flavanoids, tannins) which attributes to its antimicrobial and antifungal effects.[18] These bioactive components found in extracts have potential to inhibit the cell wall synthesis and promotes the leakage of cytoplasmic organelles.[29] Carpaine is a major alkaloid found in papaya leaves which possess antimicrobial property.[30] and also provide protection against skin associated problems like pimples, freckles and acne.[31] Tannins inhibit the cell wall synthesis by forming irreversible complexes with proteins.[32] In the light of above findings, we suggest that ethanolic extract of *Carica papaya* leaves serve as novel herbal antifungal agent for dandruff management as monotherapy or as combination therapy with synthetic agents.

CONCLUSION

The present study for the first time unveils the novel potential of ethanolic extract of *Carica papaya* leaves as an antifungal agent against dandruff causing yeast, *M. furfur*. Moreover, the extract has significantly exhibited synergistic activity with ketoconazole (standard antidandruff agent). Taken together, we suggest that *Carica papaya* leaves extract might be safer, potent and effective herbal bioactive antidandruff agent that could be used alone or in combination with commercially available synthetic drugs. This combination approach might not only improve the efficacy and potency but also aid in lowering the dose and side effects associated with synthetic antidandruff formulations.

Conflict of interest

Authors declare no conflict of interest.

Acknowledgment

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Table 1: Evaluation of phytochemical screening of *Carica papaya* leaves.

Secondary Metabolites	Name of the Tests	*Carica papaya* leaves Ethanolic extract
Flavonoids	Alkaline reagent Test	+
	Shinoda test	
Alkaloids	Mayer's Test	+
	Dragendorff's Test	
Carbohydrates	Molish's Test	+
	Benedict's Test	
Glycosides	Keller Killiani Test	+
Saponins	Foam Test	+

Proteins and Amino Acids	Biuret Test	+
Phenols and Tannins	Ferric Chloride Test	+
Steriods	Salkowski test	+

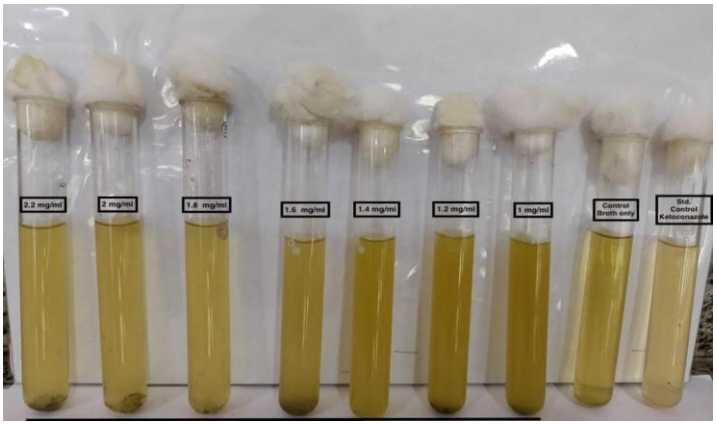

Figure 2: The minimum inhibitory concentration (MIC) of ethanolic extract of *Carica papaya l*eaves using broth dilution method.

Table 2: Evaluation of minimum inhibitory concentration (MIC) against *Malessezia furfur* using broth dilution method.

Sr. no	Amount of Extract (ml) (Stock: 20 mg/ml)	Amount of medium (ml) Broth+ inoculm	Total volume of solution (ml)	Concentration of extract in final solution (mg/ml)	Turbidity
1)	0.5	9.5 ml	10 ml	1 mg/ml	+++
2)	0.6	9.4 ml	10 ml	1.2 mg/ml	+++
3)	0.7	9.3 ml	10 ml	1.4 mg/ml	++
4)	0.8	9.2 ml	10 ml	1.6 mg/ml	+
5)	0.9	9.1ml	10 ml	1.8 mg/ml	-
6)	1	9 ml	10 ml	2 mg/ml	-
7)	1.1	7.9 ml	10 ml	2.2 mg/ml	-
8)	-	10 ml (Broth only)	10 ml	0	-
9) Ketoconazole (Standard control)	1 ml (Stock: 10 mg/ml)	9 ml	10 ml	1 mg/ml	-

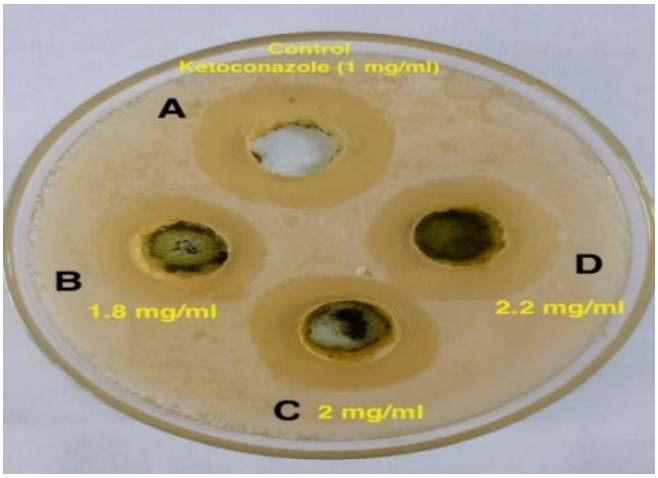

Figure 3: The zone of inhibitions of ethanolic extract of Carica papaya leaves against Malessezia furfur at different concentrations using agar cup plate method.

A - Standard control Ketoconazole (1 mg/ml)

B - Ethanolic *Carica papaya* leaves extract (1.8 mg/ml)

C - Ethanolic *Carica papaya* leaves extract (2 mg/ml)

D - Ethanolic *Carica papaya* leaves extract (2.2 mg/ml)

Table 3: Dose-dependent antifungal activity of *Carica papaya* leaves (Test) against *Malessezia furfur* using agar cup plate method.

Ethanolic papaya leaves extract (Test)	**Concentration**			**Control (Ketoconazole)**
	1.8 mg/ml	**2 mg/ml**	**2.2 mg/ml**	**1mg/ml**
Zone of inhibition (Mean±SD)	7±0.14 mm	8±0.35 mm	10±0.15 mm	11mm±0.62 mm

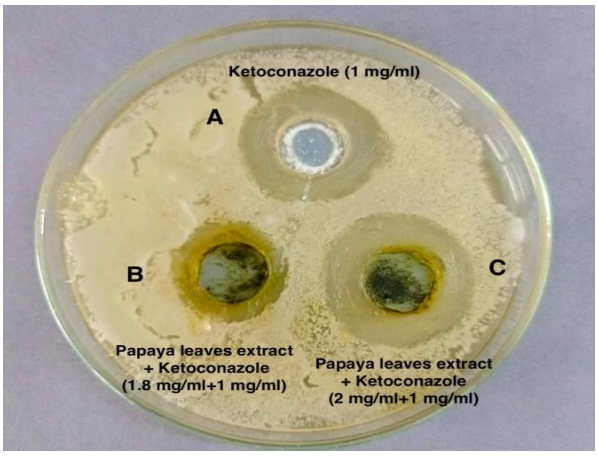

Figure 4: The zone of inhibitions of combination of papaya leaves extract with ketoconazole and against *Malessezia furfur* at different concentrations using agar cup plate method.

A – Ketoconazole (1 mg/ml)

B - Ketoconazole (1mg/ml) + *Carica papaya* leaves extract (1.8 mg/ml)

C – Ketoconazole (1 mg/ml) + *Carica papaya* leaves extract (2 mg/ml)

Table 4: The zone of inhibitions of ethanolic extract of *Carica papaya* leaves (Test) in combination with control against *Malessezia furfur* using agar cup plate method.

Concentration	Ethanolic papaya leaves extract alone (Test)		Ethanolic papaya leaves extract (Test) + Control		Control (Ketoconazole)
	1.8 mg/ml	2 mg/ml	1.8 mg/ml +1 mg/ml	2 mg/ml +1 mg/ml	1 mg/ml
Zone of inhibitions (Mean±SD)	7±0.14 mm	8±0.35 mm	10±0.34 mm	15±0.50 mm	12±0.27 mm

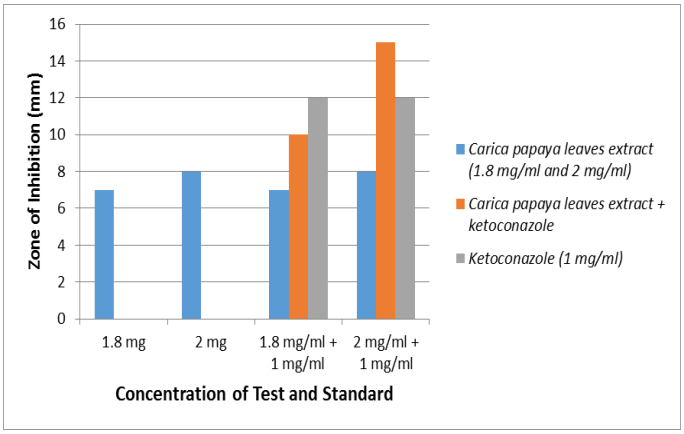

Figure 5: The comparative study of combination of ethanolic extract of *Carica papaya* leaves alone and ethanolic extract of *Carica papaya* leaves + Ketoconazole.

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