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RESEARCH PAPER

INVESTIGATING SYNERGISTIC ACTIVITY OF METHANOLIC EXTRACT OF *CURCUMA LONGA* AND *MORINGA OLIFERA* FOR IN VITRO ANTIOXIDANT AND ANTIBACTERIAL ACTIVITIES

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The serious challenges posed by the antibiotic resistance to the drug discovery were answered by the natural products as starting points in the antibiotic drug discovery. Natural products are safe, effective and therapeutic agents which are being currently used for treatment of various diseases due to their chemical and biological diversity. *Moringa olifera* fruits and *Curcuma longa* rhizomes have potential uses like anti-cancer, anti-inflammatory, antimicrobial, hepatoprotective etc. individually. Hence, an attempt was made to prove the synergistic nature of these plants by screening for *in vitro* antioxidants and antibacterial activities. The qualitative screening proved that the methanolic extracts were found to be rich in phytoconstituents like alkaloids, flavonoids, glycosides phenols etc. Hence, methanolic extracts were selected for the *in vitro* antioxidant screening using DPPH and H_2O_2 free radical scavenging methods. The IC_{50} was calculated which proved that the combined mixture of plants has higher activity than the *Curcuma longa* and *Moringa olifera* but was less than the standard, ascorbic acid. Further, *in vitro* antibacterial activity was studied using agar well diffusion method against gram-positive *Streptococcus aureus*, *Bacillus subtulis* and gram-negative *Escherichia coli* and *Proteus vulgaris*. Based on the measured zone of inhibition, the combined mixture was shown to have the higher antibacterial activity than the individual extracts. Hence, the synergistic effect of methanolic extracts was proved. Further, *in vivo* study may provide a strong correlation for pharmacological activity.

Key words: *Moringa olifera*, *Curcuma longa*, Antioxidant, Antibacterial, Synergistic activity.

INTRODUCTION

The natural herbs have been potential lead candidates in various disease ailments even in the dreadful disease like cancer, HIV etc [1]. The natural herbs are popular now days as cure for ailments due to their non-toxic, cost-effective and eco-friendly benefits. There are lot of concerns and growing demand for the development of antibiotics from the natural sources especially plants as the literature supports lot of chemical diversity in the phytomolecules, capacity as broad spectrum antibiotics [2]. The growing antibiotic resistance has posed to the potential challenges to the human kind in search of new antimicrobials with potent combat for antimicrobial resistance. Few to several plants were identified and used as successful antibiotics in herbal cure [3]. The growing demand has identified synergistic activity of herbs as cure and novel approach against the infections [4]. The natural product

chemistry is evolving revolutionary division under pharmaceutical sciences with advantages of roots in combinatorial chemistry, biodiversity and also potent pharmacological evidence. The natural products are found to be chemically diverse, potent, easily available, low cost aids with synergistic activity but suffer solubility issues which can be addressed by converting them into novel drug delivery system. Hence, the novel approach of targeting an infection with combination of two or more herbs having a positive effect is an effective strategy. The word "synergism" in pharmaceutical terminology denotes an additive effect of two or more drugs with potentiating effect on the target in comparison to the entities [5-7]. Thus, the concept can be exploited by synthesizing a lead compound with combination of two or more extracts of plants [8-13].

The plant *Curcuma longa* (Zingiberaceae) is active pharmacologically with its phyto-constituents like gingerols, shagoals etc and known widely as anti-inflammatory, anticancer, antidiabetic, antiviral and antimicrobial [14-16]. The plant *Moringa olifera* (Moringaceae) is popular plant known for its antidiabetic, neuropharmacological action, along with broad spectrum antimicrobial activity [17-19]. The positive effects of these plants enhanced their usage in the regular life contributing to the physiological health. The literature findings suggest a strong correlation of plant extracts with synergistic activity. There are no reports on the combined extract. Thus, the two plants were taken to derive a single plant extract with potent synergistic nature.

MATERIALS AND METHODS

All the materials used for the study were of laboratory grade from Merck Chemicals. The equipments used for the study were calibrated and properly maintained before and after the study.

Procedure of Soxhlet extraction for Moringa olifera fruits

The soxhlet apparatus was initially loaded with small piece of cotton to prevent the entry of plant material into siphon tube. 800 g of dried fruit powder was loaded into the main glass chamber of the Soxhlet extractor. The Soxhlet extractor was then placed onto a 2 litre round bottom flask containing the solvent *i.e.* methanol (nearly 1000-1500 ml) after defatting with hexane followed by chloroform. The Soxhlet is then equipped with a condenser [20-22]. The solvent was heated to reflux using a heating mantle. The solvent vapour was allowed to travel up to the distillation arm and into the chamber containing the plant material. The condenser ensures that any solvent vapour cools and drips back down into the chamber housing the flower material. The chamber containing the plant material slowly fills with warm solvent. Some of the desired compound will then dissolve in the warm solvent. The Soxhlet chamber was almost full and the chamber automatically emptied by the siphon side arm, with the solvent running back down to the distillation flask. This cycle was allowed to repeat many times, over a time period of 12 h. During each cycle, a portion of the non-volatile compound dissolves in the solvent. The solvent from the distillation flask was collected, filtered and then concentrated using rotary evaporator. It is labelled as hexane fraction and weighed as 20 g. The procedure is repeated with chloroform and methanol for 48 h till the plant material is exhausted of all polar components (**Figure 1**).

Procedure of Soxhlet extraction for Curcuma longa rhizomes

The soxhlet apparatus was initially loaded with small piece of cotton to prevent the entry of plant material into siphon tube. 800 g of dried rhizome powder was loaded into the main glass chamber of the Soxhlet extractor. The Soxhlet extractor was then placed onto a 2 litre round bottom flask containing the solvent *i.e.* methanol (nearly 1000-1500 ml) after defatting with hexane followed by chloroform. The Soxhlet is then equipped with a condenser [14, 24-26]. The solvent was heated to reflux using a heating mantle. The solvent vapour was allowed to travel up to the distillation arm and into the chamber containing the plant material. The condenser ensures that any solvent vapour cools and drips back down into the chamber housing the fruit material. The chamber containing the plant material slowly fills with warm solvent. Some of the desired compound will then dissolve in the warm solvent. When the Soxhlet chamber was almost full, the chamber was automatically emptied by the siphon side arm, with the solvent running back down to the distillation flask. This cycle was allowed to repeat many times, over a time period of 12 h. During each cycle, a portion of the non-volatile compound dissolves in the solvent. The solvent from the distillation flask was collected, filtered and then, concentrated

***Moringa olifera* fruits**

Fruits were collected at local market (January 2020), dried and powdered

Powdered 800 g

The powdered flowers was extracted by the soxhlet, simultaneously with *n*-hexane, chloroform and methanol

- **Hexane Extract** ML-HE
- **Chloroform Extract** ML-CH
- **Methanol Extract** ML-ME

Fig. 1. Extraction of *Moringa olifera*

***Curcuma longa* rhizomes**

Rhizomes were collected at local market (January 2020), dried and powdered

Powdered 800 g

The powdered flowers was extracted by the Soxhlet, simultaneously with *n*-hexane, chloroform and methanol

- **Hexane Extract** CL-HE
- **Chloroform Extract** CL-CH
- **Methanol Extract** CL-ME

Fig. 2. Extraction of *Curcuma longa*

using rotary evaporator. It is labelled as hexane fraction and weighed as 22 g. The procedure is repeated with chloroform and methanol for 48 h till the plant material is exhausted of all polar components (**Figure 2**). Combined extraction procedure of individual plant parts is shown in the **Figure 3**.

Individual plant part (100 g)

Fruits were collected at local market (January 2020), dried and powdered

Powdered 800 g

The powdered parts were extracted by the Soxhlet, simultaneously with *n*-hexane, chloroform and methanol

- **Hexane Extract** MC-HE
- **Chloroform Extract** MC-CL
- **Methanol Extract** MC-ML

Fig. 3. Combined extraction procedure of individual plant parts (to prove synergism)

Antioxidant activity

The antioxidant activity was evaluated by following methods:

DPPH free radical scavenging assay

Different volumes (10, 20, 30, 40, 50, 60, 70, 80, 90, and 100 µl) of methanol extracts were mixed with DPPH radical in methanol (2.2 mg/l, 200 µl) in a 96-well microplate. The final volume of each well was made up to 300 µl by adding the appropriate amount of methanol. The mixture was shaken gently on a microplate reader. The control (containing all reagents except the test compound) and standards were subject to the same procedure [25-28]. The free radical scavenging activity was expressed as the percentage inhibition of free radical generation by the sample, and calculated using the following formula:

$$I\ (\%)\ \text{of DPPH radical scavenging effect} = [(A_{control} - A_{sample})/A_{control}] \times 100 \quad (1)$$

where $A_{control}$ is the absorbance of the control, and A_{sample} is the absorbance of the sample at 515 nm. The samples were analyzed in triplicate.

Hydrogen peroxide scavenging activity
The scavenging activity of extract towards hydrogen peroxide radicals was determined for selected extracts. Solution of hydrogen peroxide (40 mM) was prepared in phosphate buffer pH 7.4 and its concentration was determined by measuring the absorbance at 560 nm using UV spectrophotometer [25-28]. 0.1 mg/ml of the extract was added to hydrogen peroxide solution and absorbance measured at 560 nm using UV spectrophotometer against a blank solution containing phosphate buffer without hydrogen peroxide. The percentage of hydrogen peroxide scavenging by the extract and standard compound was calculated using the given formula:

Percentage scavenged [H_2O_2] =
$1 - \text{Abs (standard)}/\text{Abs (control)} \times 100$ (2)

where, Abs control was the absorbance of the control (without extract) at 560 nm; Abs sample was the absorbance in the presence of the extract at 560 nm. The experiment was repeated in triplicate.

Antibacterial activity
Culture and maintenance of microorganisms
Pure cultures of all experimental bacteria and fungi were obtained from the Microbial Type Culture Collection and Gene Bank (MTCC), Institute of Microbial Technology (IMTECH), Chandigarh. The pure bacterial cultures were maintained on nutrient agar medium and fungal culture on potato dextrose agar (PDA) medium. Each bacterial culture was further maintained by sub culturing regularly on the same medium and stored at 4°C before use in experiment [27-30].

Microbiological screening
Media preparation and its sterilization:
For agar well diffusion method, susceptibility was tested on solid (Agar-agar) media in petri plates. For bacterial assay nutrient agar (NA) (40 g/l) and for fungus PDA (39 g/l) was used for developing surface colony growth [28]. The suspension culture, for bacterial cells growth was done by preparing 2% Lauria Broth (*w/v*), and for fungus cells growth, 2.4% (*w/v*) PDB (Potato dextrose broth) was taken for evaluation. All the media prepared was sterilized by autoclaving the media at (121°C) for 20 min.

Agar well diffusion method:
Agar well-diffusion method was followed to determine the antimicrobial activity. Nutrient agar (NA) and Potato dextrose agar (PDA) plates were swabbed (sterile cotton swabs) with 8 h old - broth culture of respective bacteria and fungi. The wells (10 mm diameter and about 2 cm a part) were made in each of these plates using sterile cork borer [29]. Stock solution of each plant extract was prepared at a concentration of 1 mg/ml in different plant extracts *viz.* methanol extracts of each plant and polyherbal extract. About 100 µl of different concentrations of plant solvent extracts were added using sterile syringe into the wells and allowed to diffuse at room temperature for 2 h. Control experiments comprising inoculums without plant extract were set up. The plates were incubated at 37°C for 18-24 h for bacterial pathogens and 28°C for 48 h fungal pathogens. The diameter of the inhibition zone (mm) was measured and the activity index was also calculated. Triplicates were maintained and the experiment was repeated thrice, for each replicates, the readings were taken in three different fixed directions and the average values were recorded [30].

Test for antibacterial activity:
The antibacterial assay was carried out by micro dilution method in order to determine the antibacterial activity of compounds tested against the pathogenic bacteria. The bacterial suspensions were adjusted with sterile saline to a concentration of 1.0 × 107 CFU/ml. The inoculum were prepared and stored at 4°C. Dilutions of the inoculums were cultured on solid medium to verify the absence of contamination and to check the validity of the inoculums. All experiments were performed in duplicate and repeated three times.

RESULTS AND DISCUSSION
Physical nature of the extracts
The percentage yield of the extract was found to be 20.5% for mixture of extracts and the extract was found be semi solid reddish brown in color. The methanolic extract of *M. olifera* and *C. longa* were brown in color and found to have 18.7% and 17.6% yields respectively (**Table 1**).

Preliminary phytochemical screening
The qualitative analysis of the methanol, chloroform and hexane extracts of *M. olefera* is shown in the **Table 2**. Based on the preliminary phytochemical tests of the extracts, more constituents were observed. The methanolic

extract was found to contain alkaloids, flavonoids, terpenoids, carbohydrates etc. which may be responsible for more therapeutic activity in the chloroform and hexane extracts. The qualitative analysis of the all three extracts of *C. longa* is shown in the **Table 3**.

Table 1. Physical status of methanolic extracts

S. No.	Extract	Mixture of methanolic extracts	*M. olifera* methanolic extract	*C. longa* methanolic extract
1.	Yield	35.5%	20.5%	26.5%
2.	Colour	Reddish brown	Dark brown	Yellowish brown
3.	Nature	Semisolid	Semisolid	Semisolid

Table 2. Results for preliminary phytochemical screening (*M. olefera*)

Constituents	Methanol extract	Chloroform extract	Hexane extract
Alkaloids	+	–	–
Glycosides	+	+	–
Trace metals	+	+	–
Saponins	+	+	+
Tannins	–	–	–
Phenols	+	+	+
Flavonoids	+	+	+
Amino acids and proteins	+	–	–
Carbohydrates	+	+	+

– Not present, + Present

Table 3. Results for phytochemical preliminary tests (*C. longa*)

Constituents	Methanol extract	Chloroform extract	Hexane extract
Alkaloids	+	–	–
Glycosides	+	+	–
Trace metals	+	+	–
Saponins	+	+	+
Tannins	–	–	–
Phenols	+	+	+
Flavonoids	+	+	+
Amino acids and proteins	+	–	–
Carbohydrates	+	+	+

– Not present, + Present

Based on the preliminary phytochemical tests of the extracts, more constituents were observed in the methanolic extract. It was found to contain alkaloids, flavonoids, terpenoids, carbohydrates etc which may be responsible for more therapeutic activity in the chloroform and hexane extracts. The qualitative analysis of the methanol, chloroform and hexane extracts of mixture of *M. olifera* and *C. longa* is shown in the **Table 4**. Based on the preliminary phytochemical tests of the extracts, more constituents were observed in the methanolic extract. It was found to contain alkaloids, flavonoids, terpenoids, carbohydrates etc which may be responsible for more therapeutic activity in the chloroform and hexane extracts.

***In vitro* Antioxidant activity**

DPPH radical scavenging activity

DPPH is a relatively stable free radical and the assay determines the ability of methanolic extract to reduce DPPH radical to the corresponding hydrazine by converting the unpaired electrons to pair once. **Figure 4** illustrates the effect of methanolic extract on DPPH radicals compared to standard. IC_{50} values

of ascorbic acid, mixture and the methanol extracts was found to be 14.18, 19.78, 21.78, 22.68 µg/ml respectively (**Figure 5**). Percentage scavenging of DPPH radical examined at different concentrations of extract was depicted in **Table 5**.

Table 4. Results for phytochemical preliminary tests (*M. olifera* and *C. longa*)

Constituents	Methanol extract	Chloroform extract	Hexane extract
Alkaloids	+	–	–
Glycosides	+	+	–
Trace metals	+	+	–
Saponins	+	+	+
Tannins	–	–	–
Phenols	+	+	+
Flavonoids	+	+	+
Amino acids and proteins	+	–	–
Carbohydrates	+	+	+

– Not present, + Present

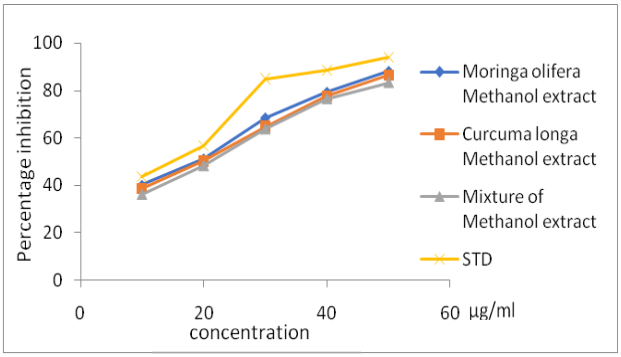


Fig. 4. Graph showing percentage inhibition of extracts by DPPH method

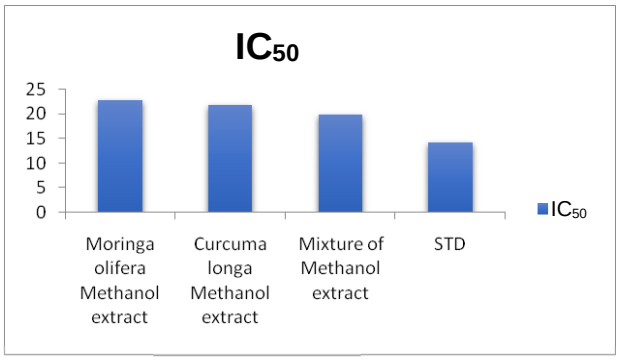


Fig. 5. IC_{50} values of different extracts on the DPPH radicals

Table 5. Effect of extracts on DPPH radicals

Concentration (µg/ml)	Log concentration	*Moringa olifera* methanolic extract	*Curcuma longa* methanolic extract	Mixture of methanolic extract	STD
10	1	40.2±0.2	38.7±0.1	36.2±0.2	43.7±0.3
20	1.30103	51.3±0.4	50.2±0.1	48.3±0.3	56.7±0.2
30	1.4771213	68.6±0.1	64.7±0.2	63.7±0.1	84.9±0.1
40	1.60206	79.4±0.3	77.4±0.4	76.4±0.4	88.4±0.3
50	1.69897	88.2±0.21	86.2±0.3	83.2±0.2	93.9±0.2
IC_{50}	–	22.88±0.3	21.78±0.3	19.78±0.3	14.18±0.2

The percentage Inhibition was found to be high in methanol extract of mixture when compared to the standard. It was having lower antioxidant capacity.

When compared to individual extracts, the mixture of extracts was found to have high antioxidant capacity and the IC_{50} values were near to the standard which proves its antioxidant activity.

Hydrogen peroxide scavenging activity

Hydrogen peroxide is a weak oxidizing agent and can inactivate a few enzymes by oxidation of essential thiol (-SH) groups. The scavenging activity of hydrogen peroxide by ascorbic acid and the methanol extract is studied. IC_{50} values of ascorbic acid, methanol extract were found to be 0.025, 1.26 µg/ml respectively (**Figure 6, 7**). Results are depicted in **Table 6**.

Table 6. Effect of extract on H_2O_2 radicals

Concentration (µg/ml)	Log concentration	*M. olifera* methanolic extract	*C. longa* methanolic extract	Mixture of methanolic extract	STD
10	1	79±0.2	78±0.2	78±0.2	88.3±0.1
20	1.30103	89±0.3	95±0.3	87±0.3	89.3±0.3
30	1.47712	92±0.4	98±0.4	92±0.4	91.9±0.1
40	1.60206	96±0.1	99±0.1	92±0.1	94±0.3
50	1.69897	98±0.2	99±0.2	98±0.2	98.5±0.2
IC_{50}	–	1.35±0.3	1.39±0.3	1.30±0.3	0.127±0.2

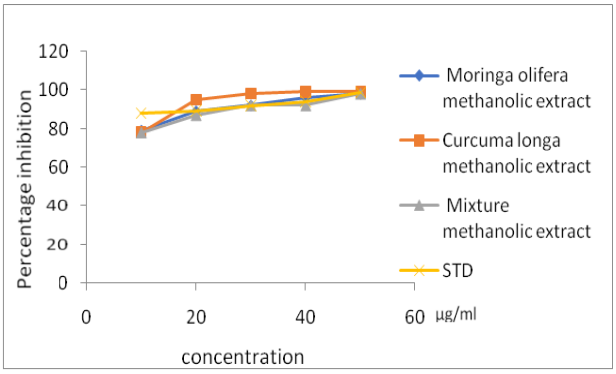


Fig. 6. Graph showing % scavenging activity of extract by H_2O_2 method

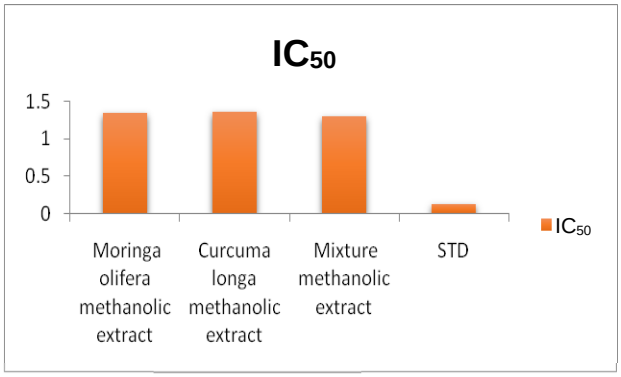
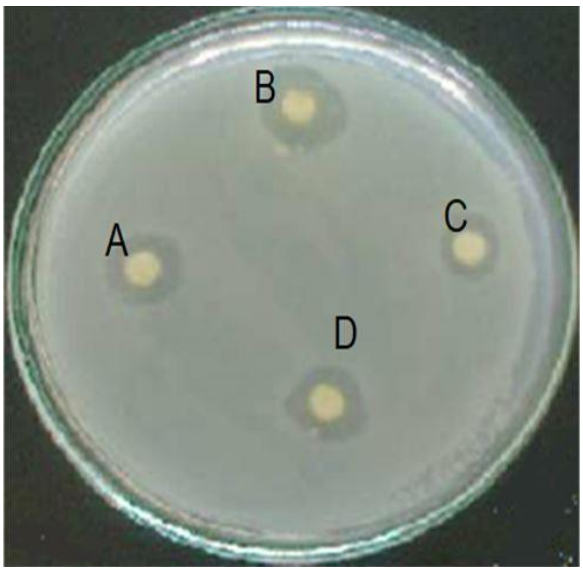


Fig. 7. IC_{50} values of mixture, individual extracts and standard

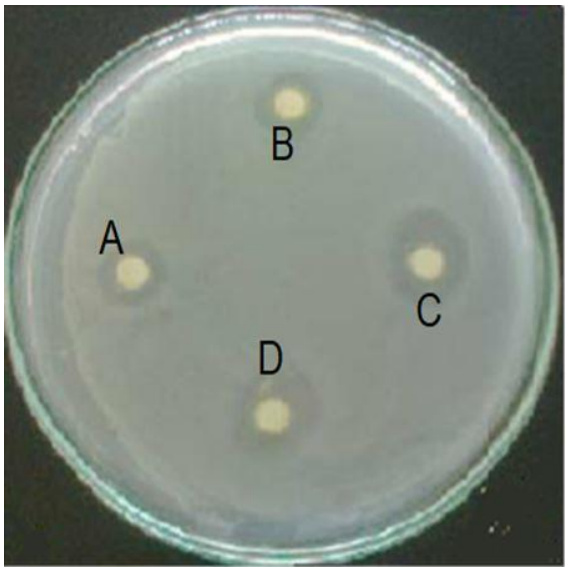
The H_2O_2 free radical scavenging activity was found to be high in methanol the IC_{50} values were near to the standard which proves its antioxidant activity.

In vitro antibacterial activity

The antimicrobial activity was studied using agar well diffusion method and the zone of inhibition were calculated in mm. The antimicrobial activity was tested against gram-negative (*E. coli* and *P. vulgaris*) and gram-positive (*S. aureus* and *B. subtilis*) using agar well diffusion method using the standard Streptomycin. The zone of inhibition was calculated in mm for all the methanolic extracts and the standard. It was observed the standard has highest activity and the mixture was found to have higher activity the individual extracts (**Figure 8**). Order of activity: streptomycin > mixture > *C. longa* > *M. olifera*.



(i)



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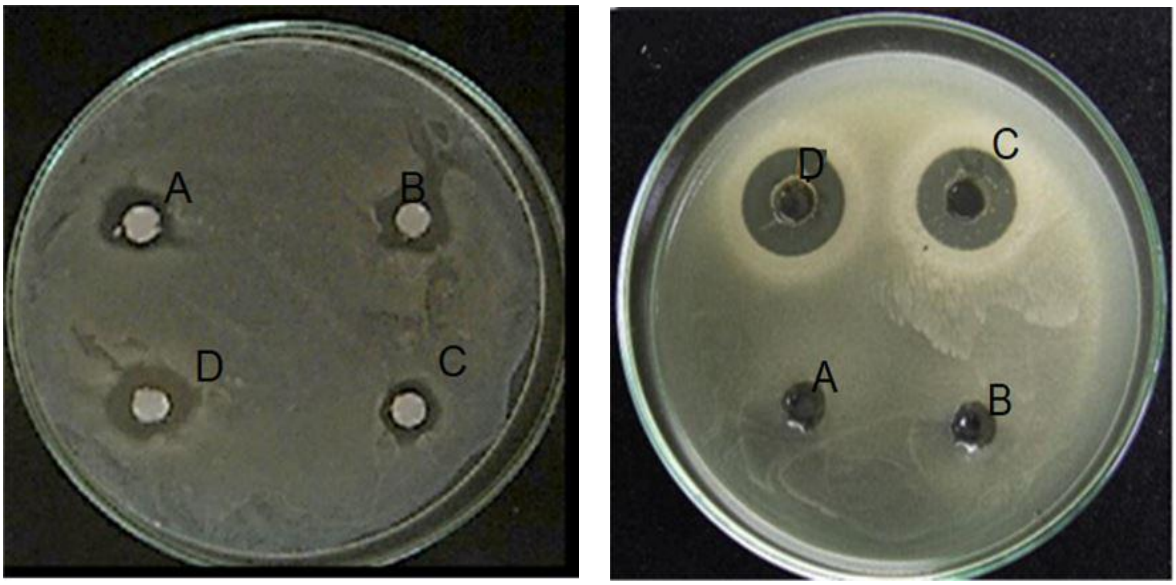

(iii) **(iv)**

Fig. 8. Antibacterial activity of extracts against (i) *S. aureus*, (ii) *E. coli*, (iii) *B. subtilis*, (iv) *P. vulgaris*
(A: Methanolic extract of *M. olifera* fruits; B. Methanolic extract of *C. longa* rhizomes;
C: Methanolic extract of combined extracts; D: Standard – Streptomycin)

The synergism nature of the plants had increased activity than the individual extracts and it was found to be close to the standard (**Table 9**).

Table 9. Comparison of zone of inhibition of extracts with standard streptomycin

Zone of inhibition (diameter in mm) of inhibition in mm				
Methanolic extracts of plants				
Bacterial species	**Streptomycin (10 µg)**	***Moringa olifera***	***Curcuma longa***	**Mixture of *Moringa olifera* and *Curcuma longa***
Escherichia coli	17	14	15	16
Proteus vulgaris	25	18	20	22
Staphylococcus aureus	24	16	18	22
Bacillus subtilis	20	15	16	18

CONCLUSION

The present study describes synergistic effect of *M. olifera* fruit and *C. longa* rhizome methanolic extracts for antioxidant and antibacterial activities. Based on the preliminary phytochemical tests of the extracts, more constituents were observed in the methanolic extract that was found to contain alkaloids, flavonoids terpenoids, carbohydrates etc. which may be responsible for therapeutic activity in all the extracts of each of plant material. The extract was found to have antioxidant properties with mixture having more potential than the individual extracts and the IC_{50} values were near to standard, ascorbic acid. Hence, it was found that the synergistic nature of *M. olifera* and *C. longa* are advantageous than the individual extracts. The extract was further screened for antibacterial activity using agar well diffusion method where the zone of inhibition as a sign of measurement of activity was studied. The zone of inhibition was found to be high for mixture when compared to *C. longa* methanolic extract and least was found for *M. olifera* extracts. The synergistic nature of *M. olifera* and *C. longa* was advantageous than the individual extracts. Further, the preclinical study can prove the strong correlation of the pharmacological activity.

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