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Production and optimization of L-citrulline by using watermelon peels

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Abstract
To develop an efficient method for the production of L-Citrulline, optimum conditions for the conversion of L-arginine to L-Citrulline by microbial L-arginine deiminases and for production of the enzyme were studied. A number of microorganisms were screened to test their ability to produce and accumulate L-Citrulline from L-arginine. Lacto bacillus was selected as the best organism for enzymatic method when compared to Bacillus subtilis and *E. coli*. The absorbance peak was obtained at 207nm. Optimum technique will be adopted based on the availability and activity of enzyme. Enzyme based involves the use of an enzyme produced by Lacto bacillus whereas the acid hydrolysis involves the chloroform extraction with the help of a strong acid. The anti-microbial and antifungal tests were done due to their potential to provide quality and safety benefits to many materials and methods.

Keywords: L-Citrulline, L-arginine deiminase, *Lacto bacillus*, *E. coli*, *Bacillus subtilis*, antimicrobial, antifungal

1. Introduction
Citrulline is formed from ornithine and carbamoyl phosphate in one of the central reactions within the urea cycle. In addition, it can be produced from arginine as a byproduct of the reaction catalyzed by the NOS family [10]. It is made of arginine by the enzyme trichohyalin at the inner root sheath and medulla of hair follicles. Arginine is first oxidised into N-hydroxyl-arginine, which is then further oxidized to citrulline concomitant with release of nitric oxide.
Several proteins contain Citrulline as a result of a post-translational modification. These citrulline residues are generated by a family of enzymes known as peptidylarginine deiminases (PADs), that convert arginine into citrulline in a method referred to as citrullination or deimination. Proteins that commonly contain citrulline residues include myelin basic protein (MBP), filaggrin, and several histone proteins, whereas other proteins, such as fibrin and vimentin are susceptible to citrullination during cell death and tissue inflammation.
Citrulline–arginine is a component of marine biomass originating from red algae, *Chondrus crispus*, which is found along the shores of the northern Atlantic Ocean. Being a stable dipeptide, it improves the bioavailability of the arginine by increasing skin energy levels, protecting the skin (in extreme conditions like cold and dry climates), encouraging cell growth and metabolism [24]. Therefore, it is used for prevention of skin aging. It also aids wound healing. The recommended dose is 100–1000 mg per day. In addition, it an important source for nitrogen and also it is essential for collagen synthesis [28].
On the other hand, L-Citrulline is not metabolized in the liver or intestine and does not induce tissue arginase, instead it inhibits its activity. L-Citrulline entering the vascular endothelium, kidney, and other tissues can be readily converted to L-arginine, thus raising tissue and plasma levels of L-arginine and increasing NO production. Supplemental L-Citrulline may be a therapeutic adjunct in disease states associated with "L-arginine deficiencies."

2. Uses of L-Citrulline
2.1 L-Citrulline Decreases Fatigue and Muscle Soreness
A study conducted on 18 men with fatigue reported that L-Citrulline reduced fatigue and increased ATP levels. All of them found a reduction in fatigue after exercise. L-Citrulline also increased the production of creatine phosphate, which is a form of energy reserves in the muscles. Another study (DB-RCT) of 41 men showed that citrulline malate reduced fatigue. Those receiving citrulline were able to do more reps per set of bench presses and experienced less soreness after exercise. A study (DB-RCT) on men found that L-Citrulline increased cycling speed and reduced muscle fatigue compared to that of placebo [16]. A randomized clinical trial performed on 30 heart failure patients showed that citrulline may enhance

exercise capacity. Those who were administered L-Citrulline were able to work out on a treadmill for a longer duration. As previously, mentioned healthy men who were given L-Citrulline also saw an increase in exercise capacity, either by being able to do more cycle faster or bench presses.

2.2 L-Citrulline Reduces Erectile Dysfunction

A single-blind trial of 24 men above the age of 55 showed that L-Citrulline increases the hardness of erections and improves erectile dysfunction (ED). Some men reported having more sex after treatment and being more satisfied. L-Citrulline supplements improved erectile dysfunction and reduced dysfunction caused by low blood pressure in rats [17]. This is likely due to an increase in nitric oxide levels. A study (DB-RCT) on 15 healthy men revealed that those who received L-Citrulline supplements had increased nitric oxide levels. A study of 10 children with a mitochondrial disorder found that L-Citrulline supplements effectively increased nitric oxide concentration due to an increase in arginine.

A combination of L-Citrulline and L-arginine supplements increased blood flow in the ears of rabbits. A study of 10 healthy young men found that nitric oxide plays an important role in regulating blood flow. Other studies, one being a Double Blind, Randomized, Controlled Trial, found that L-Citrulline efficaciously increases nitric oxide. This suggests that L-Citrulline may increase blood flow.

2.3 L-Citrulline Improves Heart Function

A study conducted on patients with heart failure found that L-Citrulline increased the volume of blood pumped by the heart's right ventricle and lowered blood pressure in the pulmonary artery [28]. A study of 35 patients showed that L-Citrulline improved the function of both the right and left ventricles of the heart as well as endothelial function. A study (DB-RCT) of 12 healthy young men showed that L-Citrulline decreased systolic blood pressure and increased nitric oxide levels. Nitric oxide causes blood vessels to dilate, which lowers blood pressure. Another study of 12 healthy young men found that L-Citrulline decreases both systolic and diastolic blood pressure.

2.4 L-Citrulline May Boost Immunity

A study (DB-RCT) on male cyclists demonstrated that L-Citrulline supplementation before exercise may help in boosting immunity. Neutrophils, a type of white blood cell, of the cyclists supplemented with L-Citrulline showed improved ability to fight infection by enhancing phagocytosis. A study suggests that use of L-Citrulline supplementation to treat sickle cell anemia exhibited that it may be a valuable treatment for the disease. The patients treated with L-Citrulline had lessened symptoms of the disease along with a reduction in white blood cells to normal counts [30].

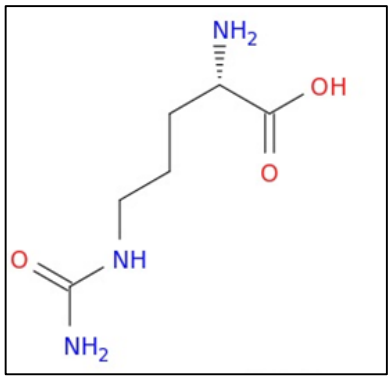


Fig 1: Structure of L-Citrulline (Molecular formula: $C_6H_{13}N_3O_3$, Molecular weight: 175.188g/mol, Physical description: chemical solid component, Melting point: 235.5C, pKa:2.43(at 25C), Solubility: 200g/L(at 20c)

3. Materials and Methods

3.1 Materials used

1. 70:30 Chloroform water mixture
2. MRS media (Beef extract 4gm, Yeast extract 5gm, dextrose 8gm, Ammonium citrate 0.8gm, Sodium acetate 2gm, Magnesium Sulphate 0.04gm, Manganese Sulphate 0.02gm, Polysorbate 804gm, Agar 6gm).
3. LB media
4. 2% solution of individual amino acids.
5. Solvent mixture of normal butanol, acetic acid and water in the ratio 12:3:5 by volume.
6. Ninhydrin reagent.

3.2 Sample collection

Watermelons were collected from K.R Market, Bangalore and were washed twice in running tap water followed by distilled water. Fruit flesh, rind and peel were separated. The rind was dried in Hot air oven at 100 ^{0}C.

Fig 2: Sample of Watermelon peel

3.3 Extraction

The extraction process was carried out in two methods:

1. Acid hydrolysis
2. Enzyme based

Enzyme based extraction involved the use of an enzyme produced by *Lacto bacillus* whereas the acid hydrolysis involved the chloroform extraction with the help of a strong acid.

For millennia enzymes have been utilized in the production of several food products. However, it was only a few decades ago that biocatalysis started to be considered as a valuable tool by the chemical industry. Examples of initial applications include the use of acylases, hydantoinases, and amino peptidases in the production of optically pure amino acids, and the use of nitrile hydratase in the enzymatic production of the bulk chemical acryl amide from acrylonitrile.

Since then, the industrial use of biocatalysis has expanded, with enzymes being utilized as catalysts in the industrial synthesis of bulk chemicals, food ingredients, pharmaceutical and agrochemical intermediates and active pharmaceuticals. Most these commercial enzymatic processes share several attributes: high product concentrations and productivities, no undesirable by-products and the use of enzymes that do not require expensive co-factors. Today, the industrial community sees biocatalysis as a highly promising area of research, especially for the development of sustainable technologies for the production of chemicals.

In addition, the development of a feasible biocatalysis process usually requires a major financial investment. Based on the type of biocatalyst to be utilised, specific reactor and hardware configurations are needed. Bio catalytic processes are typically highly heterogeneous and need specific designs of the catalyst–hardware interface to allow efficient immobilization and re-utilization. The lack of operational stability of certain enzymes when utilized in industrial scale processes is one of the constraints that have received more attention in recent years. Various techniques have been developed to better the stability so that enzymes can be used with organic solvents and at high temperatures and extreme pHs. Among these techniques are protein engineering, process modification and, as discussed in the next section, immobilization of the biocatalysts.

3.3.1 Method 1: Acid Hydrolysis

About 5gm of water melon rind was taken and 70:30 ratio of chloroform and water, was added to the sample. This solution was stirred with the help of a magnetic stirrer at 800rpm and 40^0C. The solution was filtered out and the filtrate was heated in a microwave oven for 3 minutes. The crude was extracted and centrifuged at 10000 rpm for 10 minutes. The process was repeated twice and the solution was taken in an eppendorf tube. Finally, the solution was analyzed using HPLC.

3.3.2 Method 2: Enzyme based

Lacto bacillus was grown in MRS medium (Beef extract 4gm, Yeast extract 5gm, dextrose 8gm, Ammonium citrate 0.8gm, Sodium acetate 2gm, Magnesium Sulphate 0.04gm, Manganese sulphate 0.02gm, Polysorbate 804gm, Agar 6gm). To the prepared media different concentrations of LB (0.5ml, 1ml, 1.5ml, 2ml) was added taking the sample at a constant concentration of 0.5 gram. To a different set of Petri plates the sample with concentrations of 0.2gm, 0.4gm, 0.6gm, 0.8gm was added keeping the LB concentration constant at 2 ml. After incubation the growth was noted down and HPLC analysis was performed.

3.4 Thin Layer Chromatography (TLC)

TLC is a type of planar chromatography routinely used by researchers in the field of phyto-chemicals, biochemistry, and so forth, to identify the components in a compound mixture, like alkaloids, phospholipids, and amino acids. Thin layer chromatography is also based on the principle of separation similar to other chromatographic techniques [12]. The separation relies upon on the relative affinity of compounds for the stationary and the mobile phase. The compounds under the effect of the mobile phase (driven by capillary action) move over the surface of the stationary phase. During this movement, the compounds with higher affinity for the stationary phase travel slowly while the others travel faster. Thus, separation of components in the mixture is obtained. Once separation takes place, the individual components are seen as spots at a respective level of travel on the plate. Their character or nature is identified by means of suitable detection techniques.

TLC plates of Silica Gel 60 F254 (Merck, Germany) were used. The plate served as a stationary phase in which at the bottom side, spots of the unknown samples were loaded using a capillary tube. For mobile phase, a solvent mixture of normal butanol, acetic acid and water in the ratio 12:3:5 was used. After the complete run by the mobile phase, the plate was dried at 105 °C for 5 mins. In order to observe the spots, a ninhydrin was sprayed onto the plate and was dried at 100 °C for 10mins. The Rf values were calculated.

Rf value can be calculated using the formula:

$$Rf = \frac{distance\ moved\ by\ the\ substance\ from\ origin}{distance\ moved\ by\ solvent\ from\ origin}$$

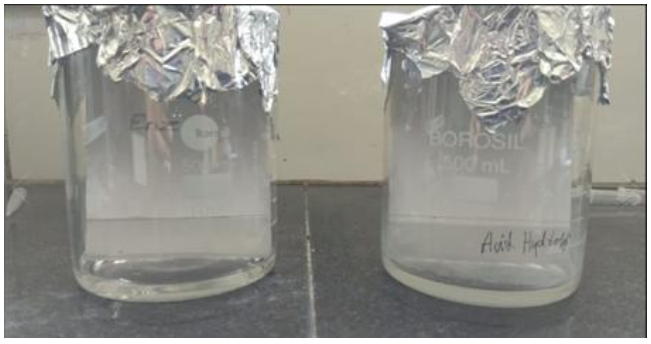


Fig 3: TLC method for enzyme and acid hydrolysis

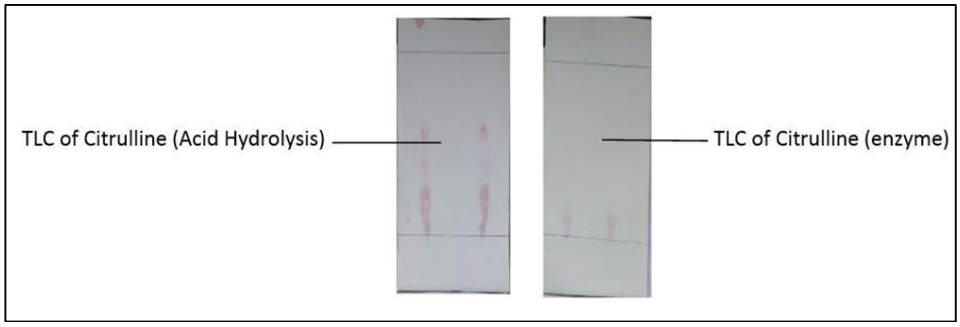


Fig 4: TLC plate after spraying Ninhydrin and drying

3.5 Antimicrobial activity

The antimicrobial activity was done taking three different microorganisms such as *Escherichia coli, Bacillus subtilis* and *Lacto bacillus*. Petri dishes with nutrient agar were inoculated with three different species of bacteria *Citrullus* sp. extracts were sterilized by passing each through a 0.22: m Millipore Round paper discs with a radius of 0.8 cm were dipped into extract and placed in the center on inoculated Petri dishes. Bacterial colonies were allowed to grow overnight at 37 °C, then the inhibition zone around the disc was measured. The zone of inhibition for three different species is as 8mm for lactobacillus, 4mm for bacillus subtilis and 5mm for Escherichia coli. Anti-microbial have gained interest from both academic research and industry due to their potential to provide quality and safety benefits to many materials. Anti-microbial packaging is the packaging system, which is able to kill or inhibit pathogenic microorganisms that are contaminating foods and thus prevent spoilage. Microbial contamination reduces the shelf life of foods and increases the risk of food-borne illness. The requirement for easily prepared, minimally processed and ready to eat fresh food products, poses major challenges for food quality and safety.

Application of anti-microbial treatment in food packaging is arousing interest of researchers due to its potential to provide quality and safety benefits and to extend the shelf life of the food. Anti-microbial food packaging improves safety by decreasing the rate of growth of specific microorganisms by direct contact of the package with the surface of foods. In the present study, we have selected Citrullus sp which is screened against multi drug resistant bacteria like *Bacillus subtilis, Lacto bacillus* (Carbohydrate Effects on the Inducement of the Arginine Deiminase Pathway Enzymes in Wine Lactic Acid Bacteria), Rhodococcus, and *Escherichia coli* and some fungal strains like, *Aspergillus niger, Aspergillus flavus and Trichoderma viridae.*

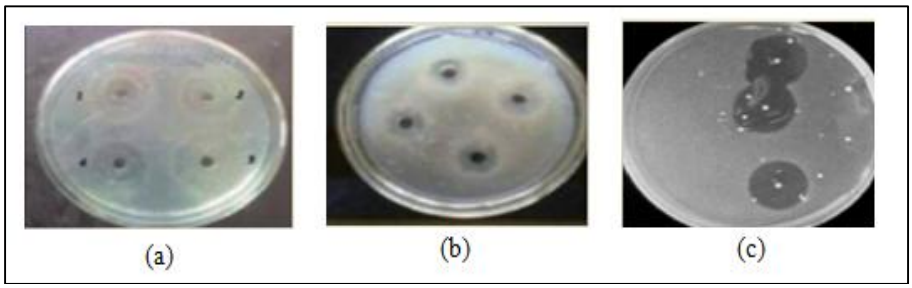


Fig 5: Zone of inhibition for various microbes – (a) *E. Coli* (b) *Bacillus Subtilis* (c) *Lacto Bacillus*

3.6 Anti-fungal activity

Antifungal susceptibility testing by disk diffusion method was carried out according to CLSI guidelines (CLSI document M44-A2) and manufacturer's instructions. The standard medium used for disk diffusion test was Mueller-Hinton agar supplemented with 2% dextrose and 0.5 µg/ml methylene blue. Addition of methylene blue in the medium has been found to promote the yeast growth and provide sharp zones of inhibition for the azole group of drugs. The antifungal disks tested was done using amphotericin B (100 units). Quality control test was done for two different microorganisms such as *Aspergillus niger* and *Trichoderma viridae* and the zone of inhibition was observed at 6mm and 7mm.

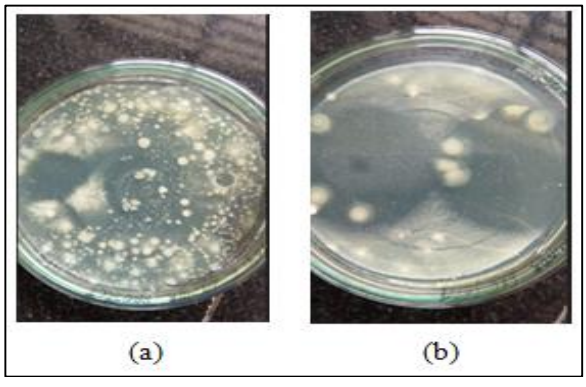
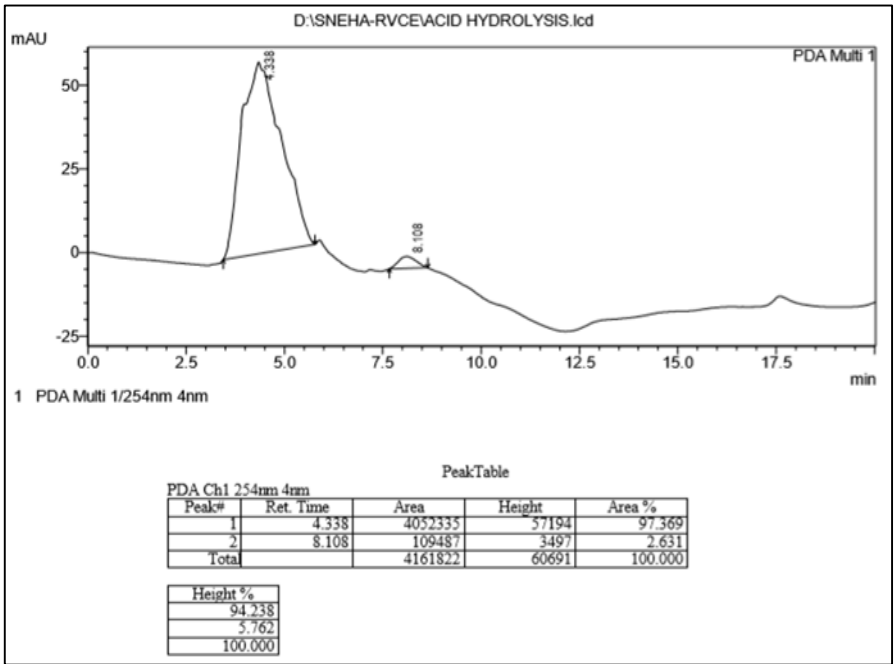


Fig 6: Antifungal activity of (a) *Aspergillus viger* (b) *Trichoderma viridae*

3.7 HPLC analysis

A rapid and versatile HPLC-UV method was used for the separation and quantification of L-Citrulline from watermelon. The rind of watermelon were extracted with acidic Chloroform and analyzed by HPLC using 0.03 mM phosphoric acid as a mobile phase. The separation was performed using Zorbax SB-Aq, Synergi Hydro-RP and Gemini C-18 columns and L-Citrulline was detected at 207 nm. L- Citrulline was well separated using Gemini C-18 column and the identity of peak was confirmed by mass spectral analysis. These results were further confirmed by derivatisation method. The developed method was used for the quantification of L-Citrulline. Thus, the method is easy and involves direct analysis of aqueous extracts. It can be used for rapid routine analysis of commercial samples. The role of watermelon extracts on smooth muscle relaxation markers was also studied. The sample extracts induced production of nitric oxide (NO) significantly at 25 ppm. This was also confirmed by measuring the change in the intracellular calcium, to induce relaxation of smooth muscle. It is suggested that watermelon extracts may have the potential to induce PDE-5A mediated smooth muscle relaxation.

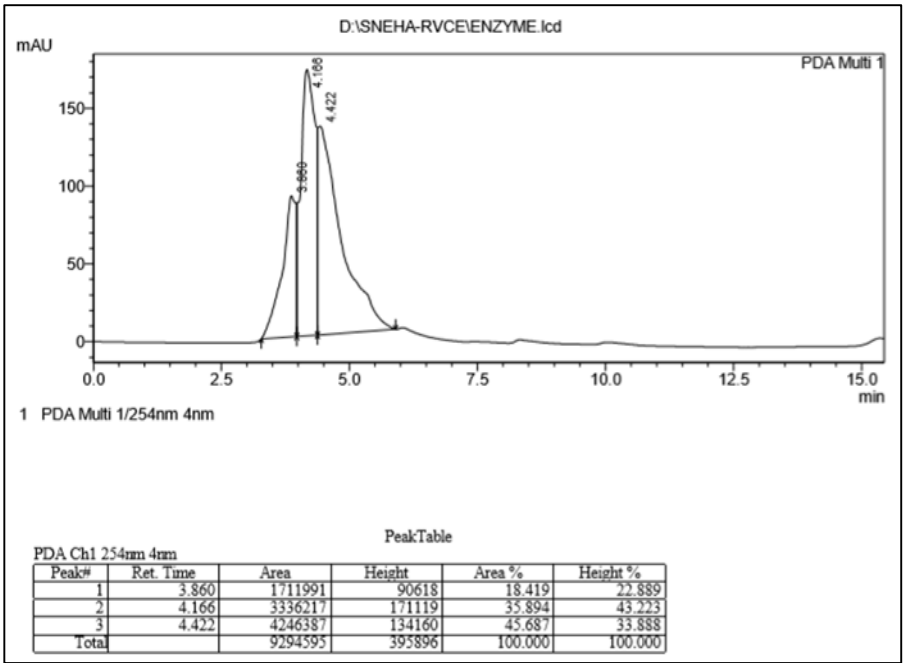


PeakTable

PDA Ch1 254nm 4nm

Peak#	Ret. Time	Area	Height	Area %	Height %
1	4.338	4052335	57194	97.369	94.238
2	8.108	109487	3497	2.631	5.762
Total		4161822	60691	100.000	100.000

Fig 8: HPLC of L-Citrulline extracted by Acid Hydrolysis



PeakTable

PDA Ch1 254nm 4nm

Peak#	Ret. Time	Area	Height	Area %	Height %
1	3.860	1711991	90618	18.419	22.889
2	4.166	3336217	171119	35.894	43.223
3	4.422	4246387	134160	45.687	33.888
Total		9294595	395896	100.000	100.000

Fig 9: HPLC of L-Cituline extracted by enzymatic method

4. Result and discussion

With the help of this study, we are trying to develop better ways for its extraction from waste resources such as watermelon rind. Purity of the extracted product was analyzed using HPLC for two methods such as acid hydrolysis and enzymatic method. The mobile phase used was water and acetonitrile 50:50 and the detector used was photodiode. Software such as LC solution software and the column used

was Gemini C18 column. For acid hydrolysis we got the peak at 4.3 min and for enzyme at 4.1 both showed the absorbance at 207nm. The extraction efficiency using thin layer chromatography technique was also done for both the methods of acid hydrolysis and enzymatic method is that the retention factor was obtained at 1.67 for acid method and 6.7 reading was obtained for enzyme hydrolysis. The antimicrobial and anti-fungal testes were done for which the zone of inhibition was observed 8mm for lacto bacillus and for fungi such as *Trichoderma viridae* some 10mm as observed.

5. Conclusion

From this analysis we can obtain an efficient method to determine the L-Citrulline using microorganism such as lacto bacillus the absorbance was observed at 207nm. The antimicrobial and antifungal tests were performed to determine the potential ability over the past few decades, these health benefits are under threat as many commonly used antibiotics have become less and less effective against certain illnesses not only because many of them produce toxic reactions, but also due to evolution of drug-resistant bacteria. Therefore, it is important to investigate newer drugs with lesser resistance. The antimicrobial activity is to determine the zone of inhibition of extracts on some bacterial and fungal strains. In the present study, the antimicrobial activity was determined in the extracts using agar disc diffusion method.

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7. Conflict of Interest

The authors listed in this paper have no conflict of interest known best from our side. There was also no problem related to funding. All authors have contributed equally with their valuable comments which made the manuscript to this form.

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