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# *In Vitro* Antimicrobial Activity of The Filtrate Crude Extract Produced by *Aspergillus niger*

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## Abstract:-

**Introduction**: *Aspergillus niger* represents one of the fungi that can produce the secondary metabolites, including antimicrobial agents, industrial and biotechnological products. **Methodology:** *A. niger* was tested against *Candida albicans* on the same petri dish of potato dextrose agar at room temperature. *A. niger* was cultured in potato dextrose broth at 27 °C for 7 days. The fungal filtrate was separated from mycelia, and the filtrate was extracted for getting a crude blackish-brown extract. The GC-MS analysis used to identify the constituents of the extract. **Results:** The filtrate crude extract of *A. niger* exhibited the antimicrobial effects against *Staphylococcus aureus*, *Streptococcus mutans*, *Escherichia coli*, and *Candida albicans*.GC-MS analysis revealed that the extract contains [(5-methyl-2-phenylindolizine), (thiocarbamic acid, N,N-dimethyl, S-1,3-diphenyl-2-butenyl ester) and (22-beta.-acetoxy-3.beta.,16.alpha.-dihydroxy-13,28epoxyolean-2)].The extract had the toxic effect on a solution of the human red blood cells by using 300 µL of 10 mg/ml after 5 minutes while 100 µL and 200 µL of the same concentration did not appear the toxic effects during 1 hour of the testing time period. **Conclusions**: The extract of *A. niger* has the ability to produce the antimicrobial activity, so it very needs to separate its constituents into pure compounds for elucidating their chemical structure by using techniques of spectrometry. Then, the compounds can be separately testing against microbial pathogens, human normal cell to determine half lethal concentration ($LC_{50}$), and application of other biological tests such as test of $LD_{50}$. Finally, the identified and purified compounds will be tested by volunteers in order to identify the side effects and toxicity of these compounds to be implemented as drugs for treating diseases in the hospitals.

**Keywords:** *Aspergillus niger*, Antimicrobial Activity, GC-MS.

## Introduction:-

Although not all filamentous fungi have the ability to produce secondary metabolites, some fungi can give these metabolites. The secondary metabolites are compounds have no necessity in the normal growth and development. Mycotoxins and antimicrobial agents such as antibiotics considered as examples of metabolites. (Ellen and Barbara, 2008). *Aspergillus niger* represents one of the fungi that can produce the secondary metabolites such as miscellaneous indole groups, including tubingensin A, which inhibited Herpes simplex virus type 1 *in vitro* ( Cole and Schweikert, 2003). Also, *A. niger* is one of the living sources has an industrial and biotechnological importance, for instance producing citric acid and the enzymes. Additionally, the fungus is able to give foreign proteins (Atkinson and Sherwood,1994; Greval and Kalra, 1995; Magnuson and Lasure, 2004)).

*A.niger* can produce the ochratoxin A (OTA) in the contaminated food, examples, grapes and dried vine fruits besides wine ((Bayman *et al.*, 2002). This fungus isolated from the stored grains (rice) as well as some species of *Aspergillus*. The filtrate of *A. niger* did not give the identified mycotoxins compared with isolated fungi (Amadi and Adeniyi,2009).

The current study aimed to evaluate the filtrate crude extract of *A. niger* against some clinical isolates

of microbial pathogens. As well as for detecting the compounds that the filtrate crude extract contains.

## Materials and Methods:-

### Isolation and Morphological Identification:-

*Aspergillus niger* was isolated as a contaminated fungus by accidence in the Laboratory of Veterinary Medicine College, Thi-Qar University, Iraq in which it grew on the Himedia-potato dextrose agar (PDA) at the room temperature. The fungus was subcultured on the malt extract agar (MEA) and Czapex Dox Medium (CDM), both manufactured by Micromedia, and incubated at 27 °C for 6 days. The identification of fungus was carried out depending on the macroscopic and microscopic appearances where the lactophenol blue stain was used.

### Preliminary Antimicrobial Screening:-

A disc (7 mm in diameter) was taken from a colony of *A. niger* colony growing on the PDA Petri dish at a room temperature. The fungal disc left for 2 days until it grew. A tube of Himedia-potato dextrose broth (PDB) containing *Candida albicans* by which 0.1 ml of it inoculated around the disc to be incubated at the same conditions for 2 days until the inhibitory zone was observed around *C. albicans*.

### The process of Fermentation and Extraction:-

The two discs ( 7 mm diameter for each disc) were obtained from PDA dish containing 6-day old *A. niger* at 27 °C, and placed in 1000 ml flask containing 400 ml of a sterile PDB. The flask incubated at 27 °C for 7 days. The filtrate (400 ml) was separated from the mycelia by using filter paper that the filtrate pH was known (2.4) by pH meter. According to (Rao and Renn, 1963; Hanson, 2008), the filtrate was poured on the filter paper containing 2 grams of the activated charcoal like film on the paper. 100 ml of methanol-HCL (0.36 Normality) poured on the charcoal filter paper, and the filtrate left at 37 °C for 2 days until the evaporation completed, and the blackish-brown crude extract of fungal filtrate was obtained, and kept in a fridge. The activated charcoal used to separate proteins from the secondary metabolites.

### Secondary Antimicrobial Screening:-

An amount of the fungal filtrate was centrifuged at 13000 rpm for 5 minutes, aseptically the filter paper discs ( 6 mm in diameter) were impregnated in the filtrate, left until dried, and separately placed on the center of the nutrient agar (NA) dishes containing 0.1 ml of 1.5 x $10^9$ cell/ml of *Staphylococcus aureus, Streptococcus mutans,* and *Escherichia coli*. The dishes incubated at 37 °C for 2 days until the inhibitory zones were seen.

### Antimicrobial Activity of Fungal Filtrate Crude Extract:-

Aseptically, the NA dishes cultured with 0.1 ml of 1.5 x $10^9$ cell/ml of pathogenic *S. aureus*, and *E.coli* while PDA dishes used for pathogenic *C. albicans* at the same inoculum. The wells ( 7 mm diameter) were done in the center of all dishes and loaded by 100 µL of the fungal crude extract dissolved by dimethyl sulfoxide (DMSO). All dishes incubated at 37 °C for 2 days until the inhibitory zones were seen.

### Gas Chromatography-Mass Spectrometry(GC-MS) Analysis:-

The fungal crude extract was dissolved in DMSO and filtered by syringe Millipore ( µM 0.45) that the filtrate subjected to the GC-Mass spectrometry which carried out by gas chromatography-mass spectrometry, MSDCHEM\1\METHODS\MUAFAQ.M for the determination of negative ions(m/z) through using column characterized by HP-5MS, 5% Phenyl methyl Sillox(1629.5), 30m × 0.250 µm I.D. x 0.25 µm, SS., Inlet He, then application of the parameters (Table1).

Table 1: GC-MS parameters used to detect the compounds in the filtrate crude extract of *A. niger*.

| Analysis Parameters | |
|---|---|
| EMV mode | Gain Factor (1.00) |
| Resulting EM voltage | 1306 |
| Power capacity | 70 EV |
| Low Mass | 28.0 |
| High Mass | 441 |
| Threshold | 150 |
| Minimum quality for all narcotics | 90-97% |
| Flow rate | 1ml/min |
| Runtime | 24 min |
| Hold up time | 1.5288 min |
| Solvent delay | 3.00 min |
| Average velocity | 36.796 cm/sec |
| Temperature | Initial 70 °C to Maximum 375 °C |
| Pressure | 8.81 Psi |

### Statisticalal Analysis:-

The statistical analysis program was carried out by using GraphPad Prism 5. One- way ANOVA was used.

## Results:-

### Identification of *Aspergillus niger*:-

*Aspergillus niger* appeared as black to whitish black colonies possessing white edges on PDA. The

reversible colony was seen yellowish brown in color. No exudates, and topography was not flat as well as slightly aerial mycelium. On MEA, the colonies were grayish black had the greenish white edges. Reversible colony was brown in color with yellowish edges. No exudates, and topography was the leathery and slightly domed center of the colony, but the texture was little aerial mycelial. The growth on the CDM gave black colony with greenish-gray reversible. No exudates, and flat in topography while in a center of the colony was slightly domed, and the sporulation was less intense. The microscopic appearance revealed heads of the conidia were black while globose to subglobose conidia possessed a rough wall and dark blue in color. Additionally, the conidia were single or double besides chains. The conidiophores were septated, besides presenting vesicles on them (Fig.1).

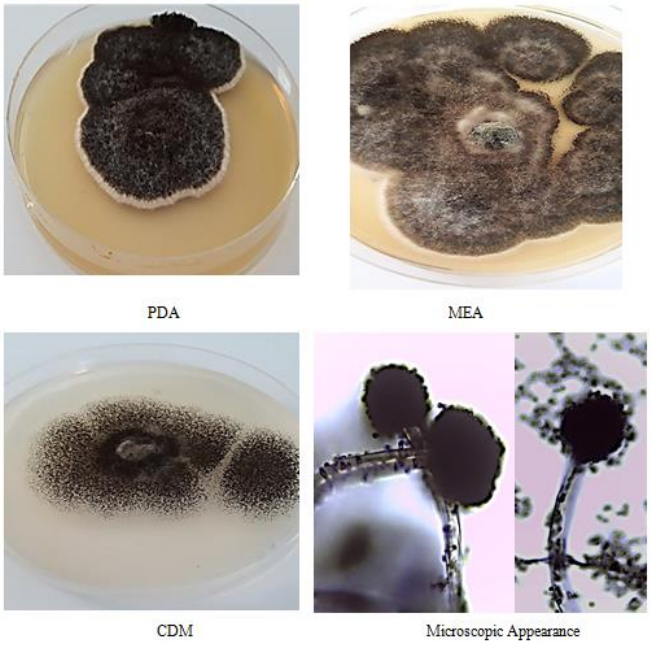


Fig. 1: Macroscopic appearances of 6 days old *A.niger* at 27 °C. PDA: Potato dextrose agar, MEA: Malt extract agar, and CDM: Czapex Dox Medium.

## Preliminary Screening of Antimicrobial Activity:-

*Aspergillus niger* grew on a Petri dish of PDA at the room temperature in which it produced the inhibition zone as a bioactive effect against *C.albicans* in the same conditions. (Fig. 2).

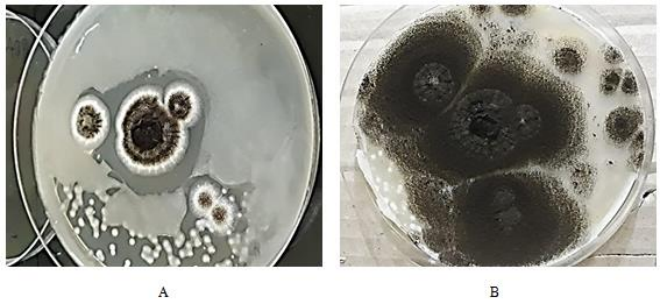


Fig.2: Preliminary antimicrobial screening of *A. niger* against *Candida albicans* at the room temperature. **A**: Inhibition zone (IZ) produced by 2 days old *A. niger* against *C.albicans*. **B**: The same dish represented continuous growth of *A. niger* (7 days in the age) for completely colonizing the dish for destroy the tested *C.albicans* was shown in the picture of A.

## Secondary Antimicrobial Screening of Fungal Filtrate:-

The filter paper discs which impregnated in the fungal filtrate revealed 11 mm in diameter as inhibition zones against *Streptococcus mutans* while no effect against *Escherichia coli* and *Staphylococcus aureus*. The filtrate of *A.niger* produced blackish-brown substance as a crude extract was weighted to be 300 mg/L.

## Antimicrobial Activity of Fungal Filtrae Crude Extract, and Its GC-MS Analysis:-

The crude extract of *A.niger* exhibited the highest inhibitory zone against *E.coli* and *C.albicans* followed by *S.aureus* while *S.mutans* had a less value of the inhibition (Table 2) and (Figures: 3 and 4). The GC-MS analysis detected that the filtrate crude extract of *A.niger* contains three compounds besides DMSO as the sample solvent (Table 3) and ( Fig.5).

Table 2: Antimicrobial activity of the filtrate crude extract (10 mg /ml) produced by *A.niger* against microbial pathogens.

| Inhibitory Zones (mm) | | | | |
|---|---|---|---|---|
| *S.aureus* | | *S.mutans* | *E. coli* | *C.albicans* |
| 18 | | 10 | 20 | 20 |
| Tetr. | 26 | 29 | 8 | ---- |

Tetr.: disc contains 30 µg pure tetracycline was used as control.

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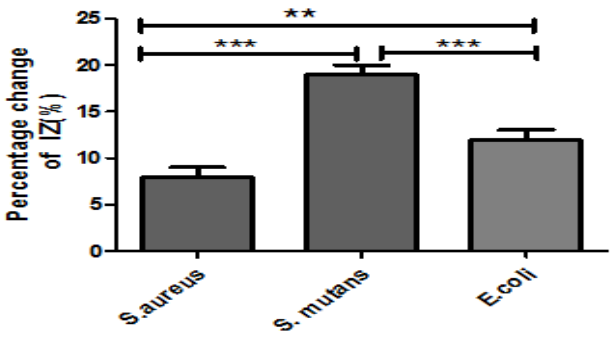



Fig.3: There were significant results among tested microorganisms which were inhibited by filtrate crude extract of *A.niger*. One- way ANOVA was used, and the statistical analysis was carried out by using GraphPad Prism 5. One or more stars indicates to present the significant results.

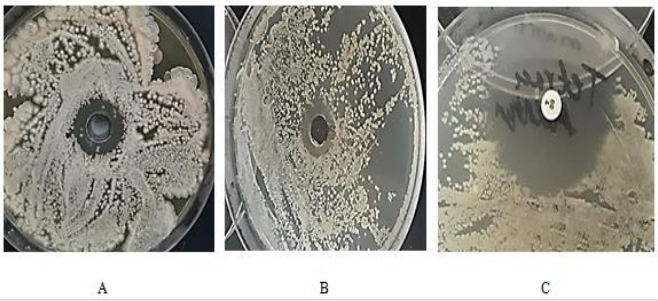
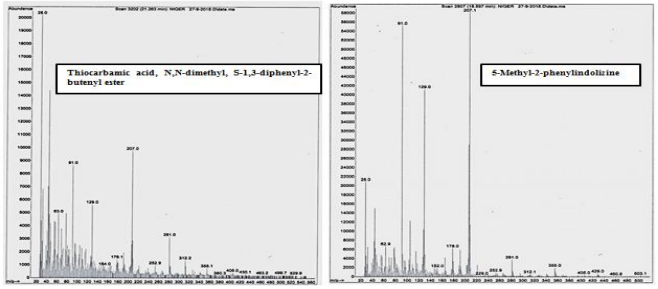



Fig. 4: Antimicrobial activity of the filtrate crude extract produced by *A.niger* against two microbial pathogens. **A**: Inhibition of *C.albicans* by fungal filtrate crude extract. **B**: Inhibition of *S.aureus* by fungal filtrate crude extract. **C:** Inhibition of *S.aureus* by tetracycline disc contains 30 µg.

Table 3: GC-MS analysis of the filtrate crude extract produced by *A. niger*.

| Compounds | Retention Time (min.) |
|---|---|
| DMSO ( sample solvent ) | 3.111 |
| 5-Methyl-2-phenylindolizine | 18.897 |
| Thiocarbamic acid, N,N-dimethyl, S-1,3-diphenyl-2-butenyl ester | 21.263 |
| 22-Beta.-acetoxy-3.beta.,16.alpha.-dihydroxy-13,28 epoxyolean-2 | 21.891 |



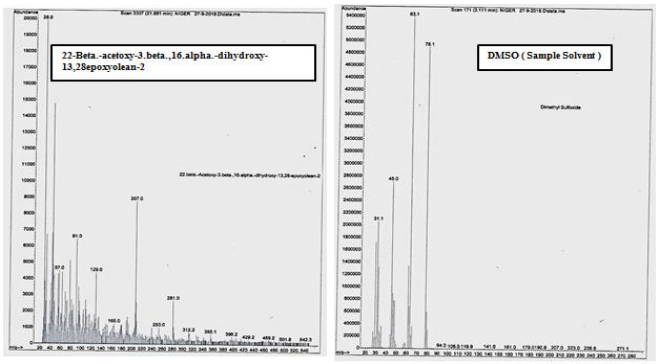




Fig. 5: (5-Methyl-2-phenylindolizine), (thiocarbamic acid, N,N-dimethyl, S-1,3-diphenyl-2-butenyl ester) and (22-Beta.-acetoxy-3.beta.,16.alpha.-dihydroxy-13,28epoxyolean-2) of *A.niger* extract besides DMSO, sample solvent, detected by GC-MS.

## Preliminary Toxicity Test:-

The test of toxicity resulted in a hemolysis of the red blood cells by using 300 µL of 10 mg/ml obtained from the filtrate crude extract of *A.niger* after 5 minutes of the test while 400 µL and 500 µL of the same concentration led to the hemolysis after 3 and 1 minutes of the test respectively ( Table 4 ) and (Fig.6).

Table 4: Hemolysis of human red blood cells (RBCs) due to *A.niger* filtrate crude extract at room temperature.

| Volumes | Results | Time period (1 hr) of The Test |
|---|---|---|
| 100 µL of 10 mg/ml | No hemolysis | During 1 hr of the test |
| 200 µL of 10 mg/ml | No hemolysis | During 1 hr of the test |
| 300 µL of 10 mg/ml | Hemolysis | After 5 min. of the test |
| 400 µL of 10 mg/ml | Hemolysis | After 3 min. of the test |
| 500 µL of 10 mg/ml | Hemolysis | After 1 min. of the test |

Fig.6: Hemolysis of human red blood cells (RBCs) due to *A.niger* filtrate crude extract at room temperature. From left to right: First: control tube containing blood, normal saline and 100 µL of D.W, second, third and fourth: blood hemolysis of 300 µL, 400 µL, and 500 µL respectively.

*Aspergillus* species are cosmopolitan and more common fungi in the warm climates. There are several species isolated from the thermal environment (Pitt, 1994; Pitt *et al*., 2000; Klich, 2002a; Klich ,2002b; Domsch *et al*., 2007). Our results showed *A. niger* isolated a contaminating fungus from The Laboratory of Veterinary Medicine College, Thi-Qar University, Iraq, in July which was characterized by high temperature ( 46- 50 °C). Therefore, it may be considered as thermotolerant species, despite that, its growth was well at 27°C. *A. niger* has ability to produce the secondary metabolites, such as tubingensin A which represented an antiviral agent against Herpes simplex virus type 1 *in vitro* (Cole and Schweikert,2003). The secondary metabolites, including antimicrobial compounds, are given by a fungus reaching maintenance phase (idiophase ) by which its living and died cells are equal in their number. The idiophase comes after lag phase that a fungus adapts in its fermenting medium and tropophase which gives a rapid and increasing growth of the fermented fungus (Hanson, 2008). The current study used potato dextrose broth as a fermenting medium to perform the fermentation process that gave the filtrate of *A.niger* which was extracted and led to obtaining the filtrate crude extract. Researchers showed that filtrate extract of fermented *A. niger* inhibited some microorganisms, including *S.aureus*, *E.coli*, and *C.albicans* (Fawzy *et al*., 2011; Kalyani and Hemalatha, 2017; Thoratiand and Mishra, 2017). Our study agreed with the mentioned researchers in which the extract of *A. niger* exhibited the inhibition against some microbial pathogens (Tabel 2) and (Fig.4).

GC-MS analysis of *Dieffenbachia picta* (Araceae) showed its leaf and stem essential oil contains 5-methyl-2-phenylindolizine. The essential oil exhibited inhibition values against some microbial pathogens, examples, *S.aureus*, *E.coli*, and *C.albicans* (Oloyede *et al*., 2011). Also, 5-methyl-2-phenylindolizine detected by GC-MS technique from the essential oil of six different parts of *Blighia unijugata* (Moronkola *et al*., 2017). Ethanolic crude extract of *Salacia oblonga* contains 5-methyl-2-phenylindolizine which inhibited *S.aureus*, *S. epidermidis*, and *E. faecalis* (Musini *et al*., 2013). Thiocarbamic acid, N, N-dimethyl, S-1,3-diphenyl-2-butenyl ester detected in the extract of *Irpex Lacteus*, wild fleshy fungus, had no activity against microbial pathogens (Chaudhary and Tripathy, 2016). The GC-MS of present study detected 5-methyl-2-phenylindolizine, and thiocarbamic acid, N, N-dimethyl, S-1,3-diphenyl-2-butenyl ester within the filtrate crude extract of *A.niger* (Table 3) and (Fig.5). It may be indicated to attribute the antimicrobial activity of the fungal extract was due to present 5-methyl-2-phenylindolizine.

Also, 22-beta. -acetoxy-3. beta.,16. alpha.-dihydroxy-13,28 epoxyolean-2 might result the antimicrobial activity. Additionally, it may be said that the same gene is in the genome of *D. picta*, *B. unijugata*, *S. oblonga* and *A.niger* led to producing the antimicrobial compound.

Biotransformation of the substrate occurs depending on the affinity between the substrate and substance mixed with it, such as substrate affinity with the specific enzymes. Usually, one component of the mixture has a high affinity while other components possess low affinity. All components lead to get biotransformation. The toxicity of the mixture may occur not because of the major compounds within a substance but it may be due to toxic effects of the impurities mixed with the compounds (Dekant and Vamvakas, 2005). The current study showed the filtrate crude extract of *A.niger* resulted in the hemolysis of RBCs through using 300 µL of 10 ml/ml ( Table 4) and (Fig. 6) Although, hemolysis of RBCs caused by the fungal extract, it may be attributed to the toxicity of impurities mixed with the extract, or due to used 300 µL considered a large dose in comparison with 100 and 200 µL of the same concentration which did not give the hemolysis (Table 4). Therefore, the separation, purification and separately testing of the compounds in the fungal filtrate crude extract need to be studied as future work.

## Conclusions:-

The filtrate crude extract of *A.niger* had the antimicrobial effect on some microbial pathogens. Therefore, it very needs to separate and purify these compounds to be separately tested against more pathogens. Additionally, they need to be tested to reveal their toxic effects on the human normal cell lines, and laboratory animals in order to determine half lethal concentration ( LC50) and half lethal dose ( $LD_{50}$). Also, these purified compounds can be subjected to the techniques of spectrometry for elucidation of their chemical structure. Finally, they can be tested through volunteers, then implemented as antibiotics for treating the diseases. The study can be performed in the

molecular aspect for detecting the gene that codes to produce 5-methyl-2-phenylindolizine either in the genome of fungi or plants.

## References:-

Amadi, J. E., and Adeniyi, D. O. (2009). Mycotoxin Production by Fungi Isolated from Stored Grains. *Afri. J.Biotech.* 8 (7):1219-1221.

Atkinson, T. and Sherwood, R.F.(1994). Biotechnology Handbook, Springer Science and Business Media New York. Pp:15-74.

Bayman, P.; Baker, J.L.; Doster, M.A.; Michailides, T.J.; Mahoney, N.E. (2002). Ochratoxin Production by the Species *Aspergillus ochraceus* Group and *Aspergillus alliaceus*. *Appl. and Enviro. Microbiol*. 68: 2326–2329.

Chaudhary, R. and Tripathy, A. (2016). Isolation and Identification of Bioactive Compounds from *Irpex Lacteus* Wild Fleshy Fungi. *J. Pharm. Sci. and Res.* 7 (7): 424-434**.**

Cole, R.J. and Schweikert, M. A**.** (2003). Hand Book of Secondary Fungal Metabolites, Vol.1. Academic Press, An imprint of Elsevier Science, USA. P:513.

Dekant, W. and Vamvakas, S**.** (2005). Ullmann's Industrial Toxicology. WILEY-VCHVerlag Gmb and Co. KGaA, Weinheim. P: 23.

Domsch, K. H.; Gams, W. and Anderson, T.H. (2007). Compendium of Soil Fungi. 2nd Ed. Eching, IHW-Verlag, The Netherlands. P: 860.

Ellen, M.F. and Barbara, J H. (2008). Secondary Metabolism: Regulation and Role in Fungal Biology. *Current Opinion in Microbiol*., Elsevier. 11: 481-487.

Fawzy, G.A.; Al-Taweel, A.M. and Melake, N.A. (2011). *In Vitro* Antimicrobial and Anti-Tumor Activities of Intracellular and Extracellular Extracts of *Aspergillus niger* and *Aspergillus flavus* var. *columinaris*. *J. Pharm. Sci. Res.* 3 (1): 980-987.

Hanson, J.R.(2008). Chemistry of Fungi. The Royal Society of Chemistry, UK. Pp: V- 23.

Greval, H.S. and Kalra, K. L. (1995). Fungal Production of Citric Acid. *Biotechnol. Advance*. 13: 209–234.

Kalyani, P, Hemalatha, K.P.J. (2017). In Vitro Antimicrobial Potential of *Aspergillus niger* (MTCC-961). *Intern. J. Chem.Tech. Res.* 10 (.4):430-435.

Klich, M.A. (2002a) Identification of Common *Aspergillus* species. Central Beureau voor Schimmel Cultures, Utrecht, The Netherlands. P:121.

Klich, M. A. (2002b) Biogeography of *Aspergillus* species in Soil and Litter. *Mycol. Lawrence* 94: 21-27.

Magnuson, J.K. and Lasure, L.L. (2004). Organic Acid Production by Filamentous Fungi. In: Tkacz JS, Lange L (eds.). Advances in Fungal Biotechnology for Industry, Agriculture, and Medicine. Kluwer Academic, New York. Pp: 307–340.

Moronkola, D.O.; Faruq, U.Z.; Adigun, O.A. and Ajiboye, C.O. (2017). Essential Oil Compositions of Leaf, Stem-Bark, Stem, Root, Flower, and Fruit with Seed of *Blighia unijugata* Baker (Sapindaceae). *Afri. J. Pharmac. and Pharmacol.* 11 (7): 108-119.

Musini, A.; Rao, M. J. P., and Giri, A. (2013). Phytochemical Investigations and Antibacterial Activity of *Salacia oblonga* Wall Ethanolic Extract. *Annals of Phytomed*. 2(1): 102-107.

Oloyede, G.K.; Onocha, P.A. and Abimbade, S.F.(2011). Chemical Composition, Toxicity, Antimicrobial and Antioxidant Activities of Leaf and Stem Essential Oils of *Dieffenbachia picta* (Araceae). *Europ. J. Sci. Res.* 49(4): 567-580.

Pitt, J.I. (1994). The Current role of *Aspergillus* and *Penicillium* in Human and Animal Health. *J. Med. Vet. Mycolo*. 32: 17-32.

Pitt, J. L.; Samson, R .A. and Frisvad, J .C. (2000). List of Accepted Species and Their Synonyms in the Family Trichocomaceae. In: Integration of Modern Taxonomic Methods for *Penicillium* and *Aspergillus* Classification. Samson RA, Pitt JL. Ed. Harwood Academic Publishers, Australia. Pp: 9-49.

Rao, K.V. and Renn, D. W. (1963). Antimicrobial Agents: Chemotherapy.P:77 Cited by Alfadhul, S. A.M. **(**2004). Production of Nucleoside Antibiotic from Locally Isolated *Streptomyces rimosus.* M.Sc. Thesis, College of Science, University of Basrah. P:39.

Thorati, M. and Mishra, J.K.(2017). Antibacterial Activity of Crude Extract from *Aspergillus niger* Isolated from The Stilt Roots of *Rhizophora apiculata* along South Andaman Coast, India. *J .Pharmacog.and Phytochemi*.

6(5): 1635-1638.