Annals of R.S.C.B., ISSN:1583-6258, Vol. 25, Issue 4, 2021, Pages. 1498 - 1507
Received 05 March 2021; Accepted 01 April 2021.

Investigation of Toxic Secondary Metabolites Produced by Some Dermatophytes

[1]-Abeer Mohammed Ali Al-garawyi, - [2]Adnan Hamad Aubaid, - [3]Adnan Waheed AL- Bederi

Biology Dept., Education for Pure Science College/Al-Muthanna University.
Microbiology Dept., College of Medicine,University of Al-Qadisiyah.
Anatomy Dept., College of Medicine, University of Al-Qadisiyah

Abstract:

The study included 71 samples of hair fragments, nail clippings, and skin scrapings which were collected from patients whom admitted to Department of Dermatology at Al-Diwaniyah Teaching Hospital during the period from March 2018 to August 2018, all specimens were examined by direct KOH 10% mount smear and cultured in sabourauds dextrose agar to determine the dermatophyte species.The laboratory examination results showed that out of 71 samples processed,49 (69%) were positive in both KOH mount and culture . The main causative agents of human dermatophytes were as follow:*Trichophytonrubrum* 31(63.3%), *Trichophytonmentagrophytes* 11(22.4%), *Microsporumcanis* 5(10.2%) and *Epidermophytonfloccosum* 2(4.1%) . *Trichophytonrubrum*was selected for its ability of toxic secondary metabolites production based on the efficiency of production among tested dermatophytes.Thin layer chromatography results of chloroform extracts revealed three spots with different colors named as follows; TRA compound (dark blue), TRB compound (dark brown), and TRC compound (light blue) under UV light have different with rate of flow values equal to 12.1, 48.5, and 84.8 respectively .
==========

Keywords: *T. rubrum* , Mycotoxins, TRA, TRC .

Correspondence author E.mail:**aliabeer297@gmail.com**

Introduction:

Dermatophytosis is a disease of global significance caused by pathogenic keratinolytic fungi called dermatophytes in both animals and humans, earlier all pathogenic dermatophytes were classified into three genera, namely *Microsporum, Trichophyton* and *Epidermophyton(1)*.Globally, approximately 20 – 25% of the population is affected by superficial mycosis with a pre-ponderance of dermatophytosis however, over the past two decades, there has been a dramatic increase in the incidence of dermatophytosis in humans as a result of socioeconomic problems, large- scale international travels, immigration from tropical countries, and contact with animals, particularly pets. The increasing age and the use of immunosuppressive drugs are the predisposing factors for the rise of dermatophytosis induced morbidity in humans(2) Dermatophytes are grouped according to their natural habitat as anthropophiles (human associated), zoophiles (animal associated) and geophiles (soil dwelling). Dermatophytes produce a variety of virulence enzymes such as keratinase, protease, phospholipase, lipase and elastase upon different substrate specificities, which are involved in the pathogenicity of host tissues(3).Among the non-enzymatic virulence factors, *T. rubrum* produces a mycotoxin called xanthomegnin, which is known to be produced by food-borne *Penicillium* and *Aspergillusin vitro* and *in vivo* causing nephropathy and death in animals, the xanthomegnin is the major substance that gives red pigmentation on reverse of the *T. rubrum* culture and it was observed in infected skin and nail specimens. The

Annals of R.S.C.B., ISSN:1583-6258, Vol. 25, Issue 4, 2021, Pages. 1498 - 1507
Received 05 March 2021; Accepted 01 April 2021.

xanthomegnin level is varied among the clinical samples(4). Fewdermatophyte species produce melanin or melanin like compounds *in vitro* and *in vivo* and play similar role in the pathogenesis of dermatophytic disease in infection of *Microsporiumgypseum* , *Epidermophytonfloccosum* , *T.mentagrophytes* and *T.rubrum* based on the known role of melanins in other pathogenic fungi , one of the striking features of these fungi in pure culture is its intense pigmentation , usually blood red and yellow occasionally (5). This study aimed to evaluation and detection the toxic secondary metabolites produced by dermatophytes and determination of skin histological changes after subcutaneous injection with different extracted compounds .

Materials and Methods :

-Collection of specimens: The present study included collection of 71 samples including hair fragments, nail clippings, and skin scrapings were collected from patients of both sexes and different ages whom admitted to Department of Dermatology at Al-Diwaniya Teaching Hospital during period from February 2018 to January 2019.The specimens were divided into two portions; first portion was examined immediately under light microscope for direct examination and second portion was cultured in Sabouraud dextrose agar(6,7)

-Extraction of toxic secondary metabolites from liquid media :

Sabouraud dextrose broth (SDB) was used to detection of toxic secondary metabolites produced by dermatophytic fungi , the maximum production of toxic secondary metabolites were obtained at the 18th days of incubation period in this media. After that, 25 flask filled 250 ml of SDB after sterilization and cooling, the culture media was inoculated with agar blocks of 5 mm of pure isolates grown onSDA for 7 days at a rate of one disk per flask, except one flask left without inoculation as a control for comparison, and incubated at 28 °C for three weeks. After incubation, the entire contents were filtered with a sterile, clean gauze and then chloroform was added to the broth (1:1) in a separation funnel. The mixture was shaken for a few minutes then filtered an upper layer containing spores and mycelia, and a lower layer containing chloroform and toxic secondary metabolites. The bottom layer filtered through a Whatman No. 1 filter paper then concentrated by using rotary evaporator to approximately 1 ml in dark bottles(8,9).

-Detection of toxic secondary metabolites by using Thin Layer Chromatography technique (TLC) :

This experiment was carried out in a toxicology laboratory at the College of Applied Medical Sciences / Karbala University , where used Thin Layer Chromatography plates (TLC) with dimensions of 20 * 20 cm, after activated in the electric oven at a temperature of 105 0C for an hour before use(10,11).

-Determination of UV absorbance:

Investigation of toxic secondary metabolites manufactured bydermatophytes species, the UV absorption spectrum used to identifysecondary metabolites bandsin sample in a slica gel plate.

Study of toxic effects *(In vivo):*

The total number of animals in these groups was 20 rats (5 rats in each group), as well as in this study three compounds were examined by subcutaneous injection according to Tovar *et al.(12),* after dissolved in dimethyl sulfoxidethe following steps was done :

1. Group 1: Rats were injected subcutaneously with a single dose 16 µg \kg from weight body of toxic secondary metabolites A compound .

Annals of R.S.C.B., ISSN:1583-6258, Vol. 25, Issue 4, 2021, Pages. 1498 - 1507
Received 05 March 2021; Accepted 01 April 2021.

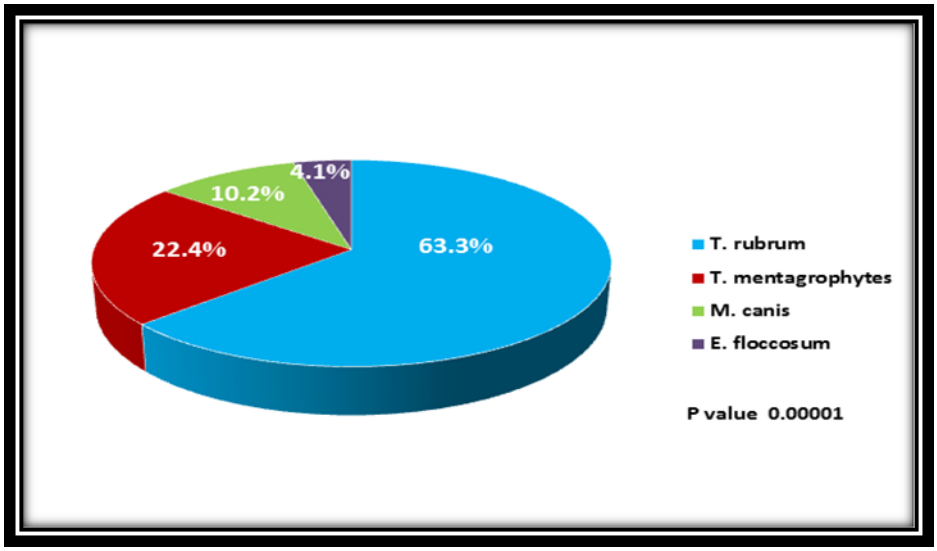
2. Group 2: Rats were injected subcutaneously with a single dose 16 µg \kg from weight body of toxic secondary metabolites B compound.
3. Group 3: Rats were injected subcutaneously with a single dose 16 µg \kg from weight body of toxic secondary metabolites C compound.
4. Group 4: Rats were injected subcutaneously with same dose of normal saline only as a control .

Preparation of histological sections :

The rats were anesthetized after three weeks from injection ,the skin samples taken with 1cm³ after sacrificed of experimental animals and taken from injected area with infection symptoms .The processing and staining techniques were done according to Suvarna*et al.(13)*

Results and Discussion :

Seventy one specimens were accumulated from patients having symptoms of dermatophytoses , the results showed 49 (69%) were positive in both KOH mount and culture , *T. rubrum* was the most frequent etiological agent 31(63.3%) followed by *T. mentagrophytes*11(22.4%). While *M. canis* and *E. floccosum* isolates were found in 5(10.2%) and 2(4.1%) respectively (Fig. 1).



Figure(1):Distribution of fungal species among positive cultures.

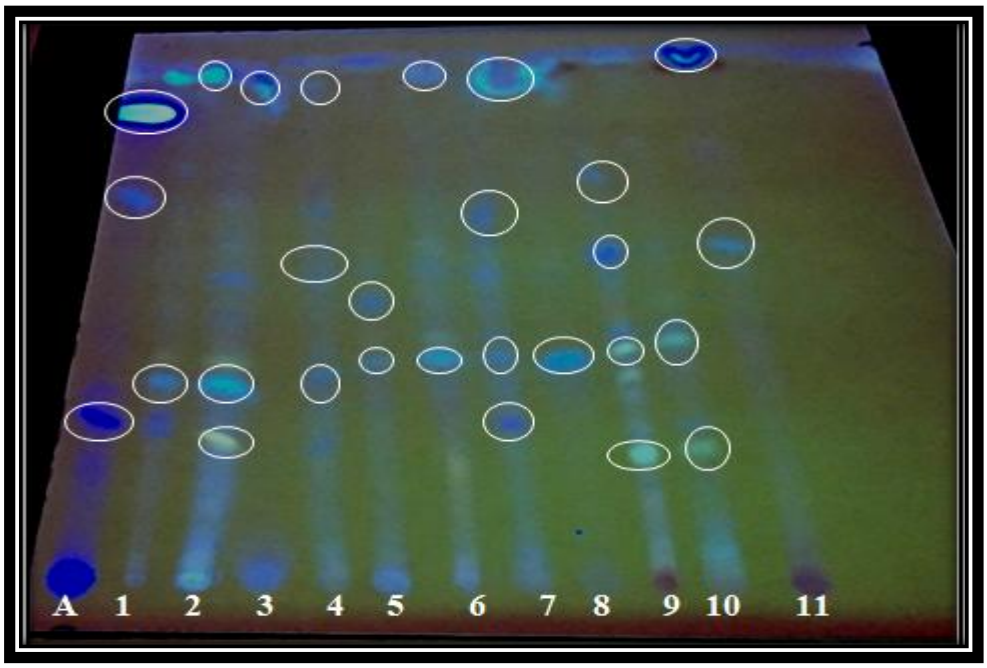
The statistical analysis showed that there was a highly significant difference among dermatophytes species at P≤0.05. This result closely similar to result of (14) who presented *T. rubrum* was the most frequent etiological agent 50% followed by *T. mentagrophytes* 32. 5%, *M. canis*10% , *T. sudanese* 2.5%, *T. schoenleinii* 2.5% and *E. floccosum*2.5%. While, the present study disagreed with(15) who exposed the main causative of dermatophytes infections was *M. canis*with 40.91 % cases followed by *T. tonsurans*32.73 % cases and*T.verrucosum*17(15.45 %) cases.

Detection of some dermatophytes ability to produce toxic secondary metabolities :

This study showed that 49(100%) culture positive isolates possess the ability to produce toxic secondary metabolitiesinSabouraud dextrose broth (SDB)when chemically analyzed by using TLC technique, in addition to, the results of current study showed that there are 23 blue spots of toxic secondary metabolitiesinTLC plate

Annals of R.S.C.B., ISSN:1583-6258, Vol. 25, Issue 4, 2021, Pages. 1498 - 1507
Received 05 March 2021; Accepted 01 April 2021.

under UV light similar to aflatoxins (AFB1) in color and Rf. as compared with standard AFB1, as well as there are other 3 spots that do not resemble aflatoxins, with different bright colors and Rf. (Fig.2).



Figure(2): Detection of toxic secondary metabolities by using TLC technique; A: Standard AFB1, 1: T. rubrum,2: T. mentagrophytes, 3: M. canis and 4: E. floccosum extracts grown in the NB; 5: T. rubrum, 6: T. mentagrophytes, 7: M. canis and 8: E. floccosum extracts grown in the CEB; 9: T. rubrum, 10: T. mentagrophytes and 11: M. canis extracts grown in the SDB after18 days of incubation at28 ◦ C .

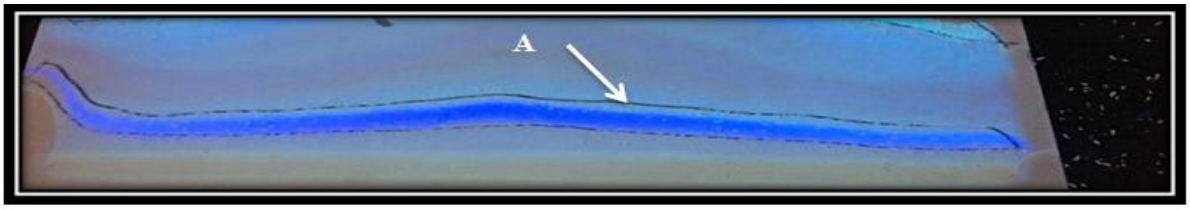
Due to the large number of secreted compounds from isolated fungi, *T. rubrum* selected for production of toxic secondary metabolities. Thin layer chromatography (TLC) results of chloroform extracts isolated from *T. rubrum* after cultivated in SDB, three spots were separated with rate of flow (Rf.) values equal to 12.1, 84.8 and 48.5, (table 1) and (Fig.3,4 and 5)

Table (1): Thin layer chromatography results of extracted compounds isolated from *T. rubrum.*

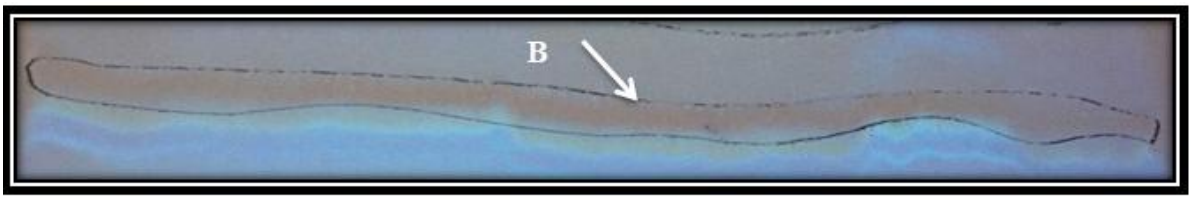
Compounds	Test	Color	Rf.
TRA	UV radiation at a wavelength of 365 nm	Dark blue	12.1
TRB	UV radiation at a wavelength of 365 nm	Dark brown	48.5
TRC	UV radiation at a wavelength of 365 nm	Light blue	84.8

TRA= Trichophytonrubrum A compound ; TRB= Trichophytonrubrum B compound; TRC= Trichophytonrubrum C compound; Rf= Rate of flow ; UV= Ultraviolet light .

Annals of R.S.C.B., ISSN:1583-6258, Vol. 25, Issue 4, 2021, Pages. 1498 - 1507
Received 05 March 2021; Accepted 01 April 2021.



Figure(3): Thin layer chromatography (TLC) separation of chloroform extracts isolated from Trichophytonrubrum. (A): TRA

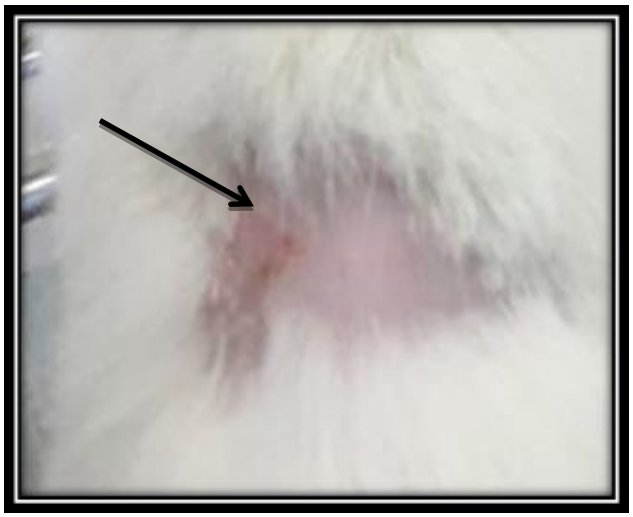


Figure(4): Thin layer chromatography (TLC) separation of chloroform extracts isolated from Trichophytonrubrum . (B): TRB

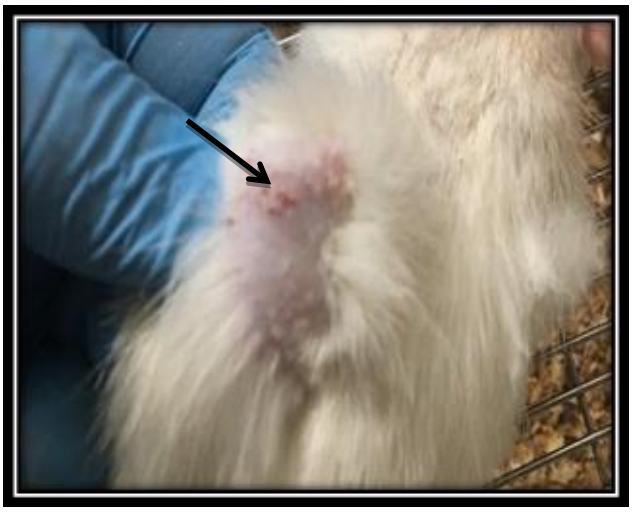
Figure(5): Thin layer chromatography (TLC) separation of chloroform extracts isolated from Trichophytonrubrum. (C): TRC

Detection of cytotoxic activity of secondary metabolities produced by *T.rubrum* (*In vivo*) :

The macroscopic examination for the skin tissue of the rats treated with secondary metabolites TRA, TRB and TRC compounds separately produced by *T. rubrum* showed clear changes after three weeks of S.C.injection such as; skin lesions appeared as erythematic and scaly ovoid with crusty edges in compared with control group(Figs. 6, 7,8 and 9) .

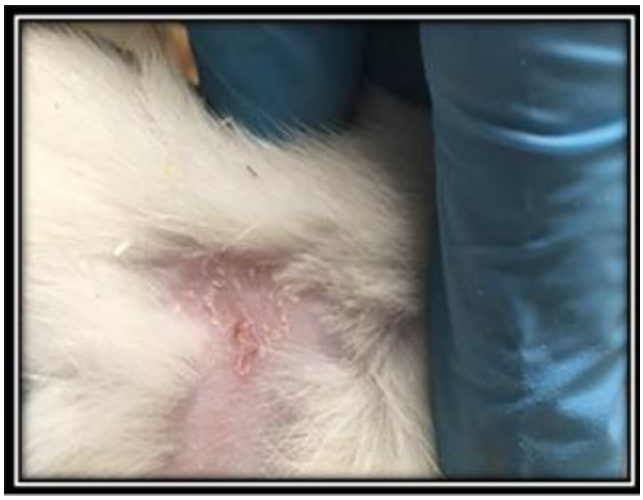


Figure(6): Shows mild erythema with crusty edges after 3 weeks from subcutaneous injection with TRA compound.



Figure(7): Shows erythema and crusting of area after 3 weeks from subcutaneous injection with TRB compound .

Annals of R.S.C.B., ISSN:1583-6258, Vol. 25, Issue 4, 2021, Pages. 1498 - 1507
Received 05 March 2021; Accepted 01 April 2021.



Figure(8): Shows scaly ovoid with crusty edges and erythema after 3 weeks from subcutaneous injection with TRC compound .

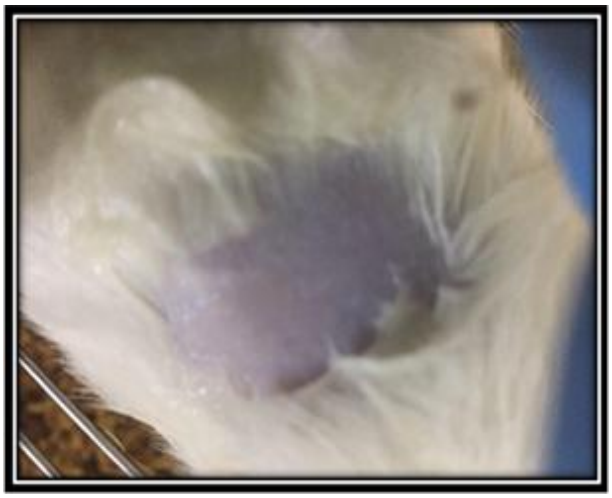


Figure (9): Shows the control group of rat skin tissue

The microscopic examination for the skin tissue sections of the rats treated with secondary metabolites TRA, TRB and TRC compounds separately, produced by *T. rubrum* also showed clear changes in these tissues after three weeks of S.C. injection such as; heavy chronic inflammatory cell in upper dermis, oedema, destruction of hair follicles, spongiosis of epidermis cells , and stratified epithelium hyperplasia as a compared with control group (Figs.10, 11, 12, 13 and 14).

Annals of R.S.C.B., ISSN:1583-6258, Vol. 25, Issue 4, 2021, Pages. 1498 - 1507
Received 05 March 2021; Accepted 01 April 2021.

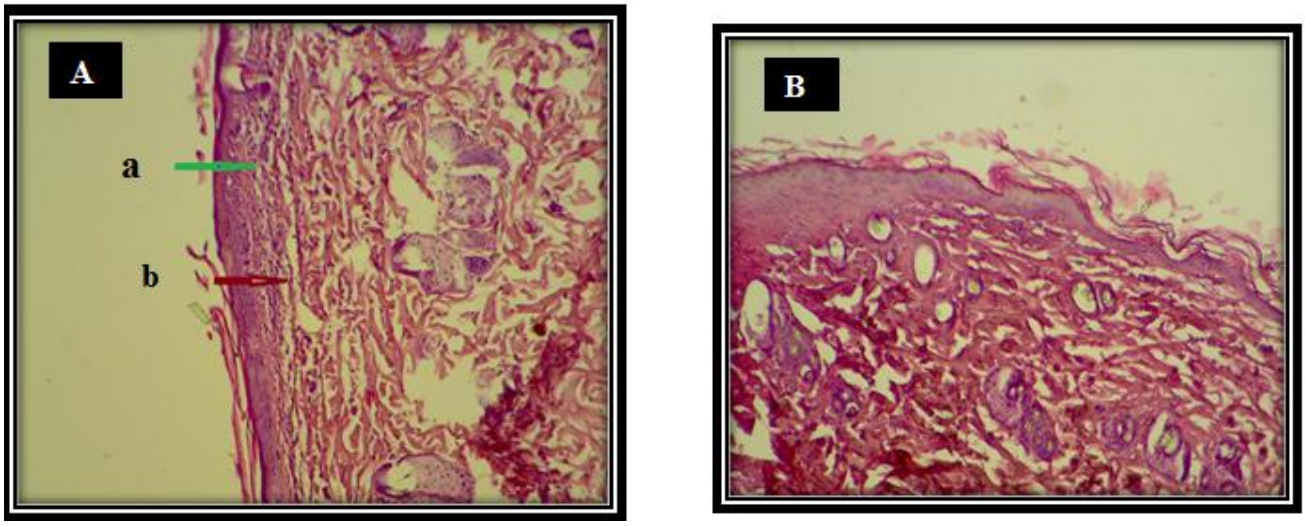


Figure (10): (A): Cross section of rat skin after injection with TRA compound shows heavy chronic inflammatory cells in upper dermis (a) and oedema (b) (H&E, X40). (B): Cross section of rat skin for the control group shows epidermal stratified epithelium and dermis layer (H&E, X40).

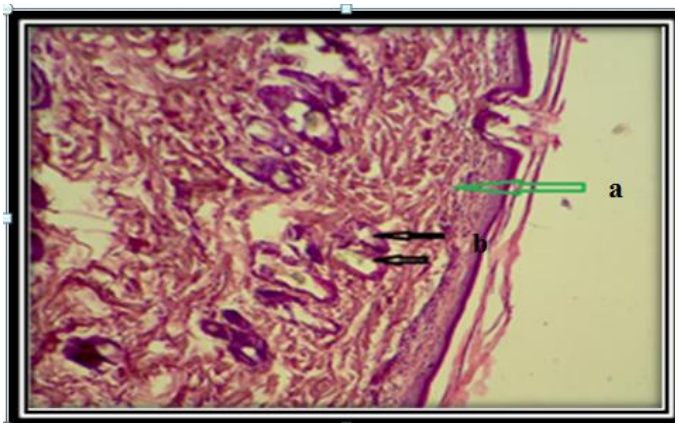


Figure (11): Cross section of rat skin after injection with TRB compound shows heavy chronic inflammatory cells infiltration (a) and destruction of hairs follicles (b) (H&E, X40).

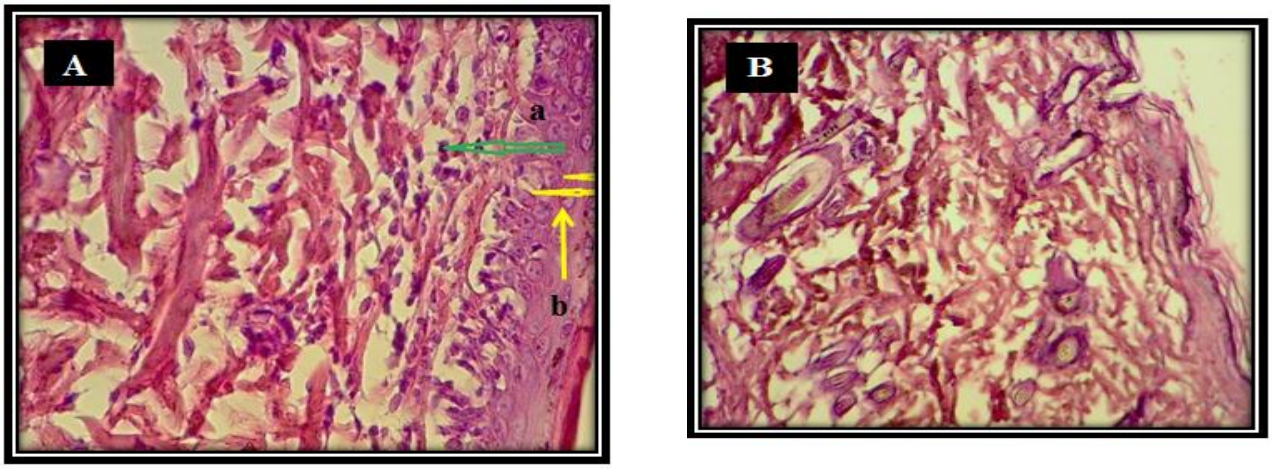


Figure (12): (A): Cross section of rat skin after injection with TRB compound shows heavy chronic inflammatory cells infiltration (a) and spongiosis of epidermis cells (b) (H&E, X40). (B): Cross section of rat skin for the control group shows epidermal stratified epithelium and dermis layer (H&E, X40).

Annals of R.S.C.B., ISSN:1583-6258, Vol. 25, Issue 4, 2021, Pages. 1498 - 1507
Received 05 March 2021; Accepted 01 April 2021.

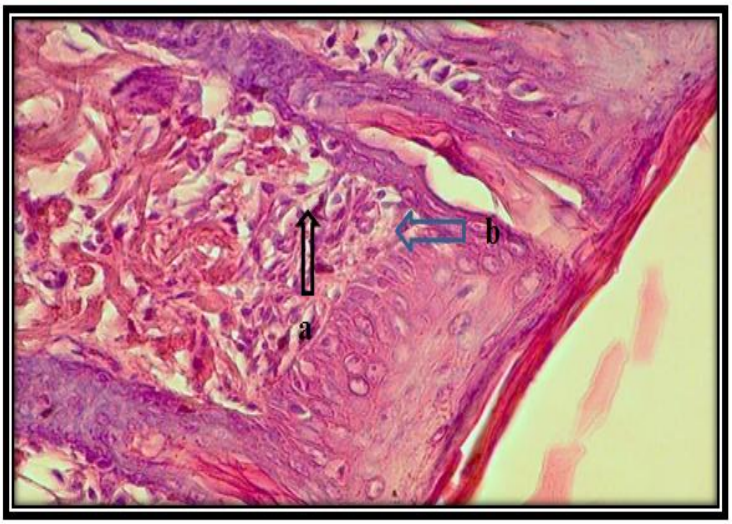


Figure (14): Cross section of rat skin after injection with TRC compound shows destruction of hairs follicles (a) stratified epithelium hyperplasia(b) (H&E, X40).

The cause of these changes in skin tissues is that the mycotoxin compounds induced dermal toxicity by oxidative stress, which induces a ribotoxic stress response and subsequent activation of mitogen activated protein kinases (MAPKs) pathways. Then, this stimulates expression of c-fos and c-jun, resulting in the induction of keratinocyte apoptosis. In addition, the keratinocytes affected primarily by ribotoxic stress release TNF-α and IL-1β, and these cytokines are also thought to be involved in the mechanisms of mycotoxin induced keratinocyte apoptosis(16). From above the results, the secondary metabolites have toxic effects (mycotoxins) in biological system of laboratory rats. There are no previous studies on the extraction of toxic metabolic compounds from dermatophytic fungi for the purpose of comparison, but it will discuss close studies to this topic, where Yarom*et al.(*17)suggested that T-2 toxin-induced epidermal degeneration might be secondary to ischemia brought about by micro vessel degeneration in the dermis . In1999, Albarenque*et al.*(18), started a series of studies to clarify the mechanisms of T-2 toxin-induced dermal toxicity using Wistar-derived hypotrichotic WBN/ILA-Ht rats focusing on the expression of apoptosis related oncogenes and cytokines, this is the first report of mycotoxin induced apoptosis in the skin(19,20) showed in theirs study that involvement of reactive oxygen species (ROS) in Citrinin mediated toxicity characterized by apoptosis in certain in vitro models(16) revealed the correlation of elevated ornithine decarboxylase activity with apoptotic cell death in normal keratinocytes via the induced generation of reactive aldehydes and H2O2, followed by subsequent activation of the ataxia-telangiectasia mutated DNA damage response pathway. Aflatoxin B1 acts as a skin tumor initiator through reactive metabolite formation, lipid peroxides (LPO) mediated oxidative stress, and glutathione-S-transferase (GST)-mediated AFB1-DNA adduct formation. Like in the case of Patulin (PAT), AFB1 may also have skin tumorigenic potential as a promoter and/or a complete carcinogen in mouse skin after long-term and higher dose application(21,22).

Annals of R.S.C.B., ISSN:1583-6258, Vol. 25, Issue 4, 2021, Pages. 1498 - 1507
Received 05 March 2021; Accepted 01 April 2021.

Conclusions:

This study proved that the ability of all dermatophytes isolates produce mycotoxins that are not similar to aflatoxins in chemical structures and properties in spite of the similarity in Rf. and colures under UV light as compared with standard AFB1. As well as, this study demonstrated for the first time at the international and local level the ability of the *T. rubrum* to produce three toxins named TRA, TRB and TRC, which have toxic effects in the physiological, biochemical and skin histological parameters for white rats males with very low concentrations.

References:

1-**Begum**, J.; Mir, N. A.; Lingaraju, M. C.; Buyamayum, B. and Dev, K. (2020). Recent advances in the diagnosis of dermatophytosis. *Journal of Basic Microbiology*, 60(4):293–303.

2-Gordon, A. K.; McIver, C.; Kim, M.; Murrell, D. F. and Taylor, P. (2016). Clinical application of a molecular assay for the detection of dermatophytosis and a novel non-invasive sampling technique. *Pathology*, *48*(7): 720-726.

3-Zhan, P. and Liu, W. (2017). The changing face of dermatophytic infections worldwide. *Mycopathologia*, *182*(1-2): 77-86.

4-Elavarashi, E.; Kindo, A. J. and Rangarajan, S. (2017). Enzymatic and non-enzymatic virulence activities of dermatophytes on solid media. *Journal of clinical and diagnostic research*, 11(2): 23-25.

5-Cavallini, C.; Vitiello, G.; Adinolfi, B.; Silvestri, B.; Armanetti, P.; Manini, P. and Menichetti, L. (2020). Melanin and Melanin-Like Hybrid Materials in Regenerative Medicine. *Nanomaterials*, 10(8): 1518.

6-Kaur, R.; Kashyap, B. and Bhalla, P. (2008). Onychomycosis-epidemiology, diagnosis and management. *Indian Journal of Medical Microbiology*, 26(2):108-116.

7-Hasson,S.O.;Adnan.Hamad Aubaid Al-Hamadani and Al-Azawi,I.H.(2018). Occurrence of Biofilm Formation in Serratia fonticola and Pantoea sp. Isolates among Urinary Catheterized Patients.Nano Medicine and Engineering ,10(3):295-304.

8-Al-Jumaili, S. A. (2014) . Mycotoxins. Book house, first edition, Karbala, Iraq, PP.75-94.

9-Al-Shawi, H.; Aubaid,A.H. and Al-Dujaili,N.H.(2018).A novel antibiotic-like substance isolation from a dermatophyte , Trichophyton rubrum. Reviews in Medical Microbiology, 29(2):126

10-Hasson,S.O.; Al-Awady ,M. and Adnan.Hamad Aubaid Al-Hamadani. (2019).Boosting Antimicrobial Activity of Impenem in combination with Silver Nanoparticles towards S.fonticola and Pantoea sp.Nano Biomedicine and Engineering , 11(2):200-214.

11-Al-Jumaili, S. A. and Al-Mousawi(2011). Isolation and diagnosis of fungi associated with imported apple and study of toxicological effects of *A. terreus* in male white Rat. *Al-Kufa University Journal for Biology*, 3(2): 66-72 .

12-Tovar, E. A.; Essenburg, C. J. and Graveel, C. (2017). In vivo Efficacy Studies in Cell Line and Patient-derived Xenograft Mouse Models. *Bio-protocol*, 7(1) : 1-15.

13-Suvarna, K. S.; Layton, C. and Bancroft, J. D. (2019). Pigments and minerals. Bancroft's Theory and Practice of Histological Techniques, Eighth edn: Elsivier. PP.73-113 .

14-Mohammed, N.M.(2015). Molecular characterization of some dermatophytic fungi in Al-Diwania province and the effect of silver nano particles on their growth. M. Sc. Thesis., College of science, Al-Qadisiyah Univ., Iraq.

15-Najem, M. H.; Al-Salhi, M. H. and Hamim, S. S. (2016). Study of dermatophytosis prevalence in Al-Nassiriyah city− Iraq. *World Journal of Pharmacy and Pharmaceutical sciences*, 4(4): 166-172.

16-Doi, K. and Uetsuka, K. (2014). Mechanisms of mycotoxin-induced dermal toxicity and tumorigenesis through oxidative stress-related pathways. *Journal of toxicologic pathology*, 27(1): 1-10.

17-Yarom, R.; Bergmann, F. and Yagen, B. (1987). Cutaneous injury by topical T-2 toxin: involvement of microvessels and mast cells. *Toxicon, 25*(2): 167-174.

18-Albarenque, S. M.; Shinozuka, J.; Iwamoto, S.; Nakayama, H. and Doi, K. (1999). T-2 toxin-induced acute skin lesions in Wistar- derived hypotrichotic WBNIILA-Ht rats. *Histology and histopathology*, *14*(2): 337-342.

19-Liu, B. H.; Yu, F. Y.; Wu, T. S.; Li, S. Y.; Su, M. C.; Wang, M. C. and Shih, S. M. (2003). Evaluation of genotoxic risk and oxidative DNA damage in mammalian cells exposed to mycotoxins, patulin and citrinin. *Toxicology and applied pharmacology*, 191(3): 255-263.

20-Chen, C. C. and Chan, W. H. (2009). Inhibition of citrinin-induced apoptotic biochemical signaling in human hepatoma G2 cells by resveratrol. *International journal of molecular sciences*, 10(8): 3338-3357.

21-Wei, G.; DeFeo, K.; Hayes, C. S.; Woster, P. M.; Mandik-Nayak, L. and Gilmour, S. K. (2008). Elevated Ornithine Decarboxylase Levels Activate Ataxia Telangiectasia Mutated–DNA Damage Signaling in Normal Keratinocytes. *Cancer research*, *68*(7): 2214-2222.

22-Al-Azawi,I.H.; .;Adnan.Hamad Aubaid Al-Hamadani.and Hasson,S.O.(2018).Association between biofilm formation and susceptibility to antibiotics in Staphylococcus lentus isolated from urinary catheterized patients. International Journal of research in pharmaceutical sciences,9(SPL1):1309