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Research Article

Dermatophytes Isolation from Patients and In-vitro Application of Selected Natural Extracts and Oils for Anti-Dermatophytic Activity

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Abstract

Dermatophytosis, commonly known as ringworm, is caused by a group of fungi termed dermatophytes. It can invade the keratinized tissue of the skin, nails, and hair. Dermatophytosis is classified based on the site of infection, such as tinea pedis, tinea capitis, and tinea unguium. This study was performed at Bolan Medical College Hospital, Quetta, to isolate and identify the infectious agents of dermatophytes related infections in patients following all the codal formalities and ethical procedures. A total of 19 patients' samples were analyzed in the study, where ten samples were positive for infectious agents. The organisms were grown on specified media such as dermatophytes test medium agar, and potato dextrose agar, supplemented with antibiotics. The isolates were preliminary identified with the help of their growth on specific media and finally confirmed with the help of compound microscopic examination. Some common natural compounds, such as apple peel extract (APE), grapes peduncle extract (GPE), propolis oil (PO), and bitter apricot kernel oil (BAKO), were used against the dermatophytes isolates and were found positive for activity against a few isolates. The APE showed inhibitory activity of 31% against *Trichophyton tonsurans* and 80% against *Epidermophyton flocosum*, while the GPE showed 27% and 74%, PO showed 42% and 69%, and BAKO showed 78% and 70% activity against *Trichophyton tonsurans* and *Epidermophyton flocosum*, respectively. The bioactive compounds in these extracts were analyzed through Fourier Transformed Infrared Spectroscopy.

Keywords: Antifungal, antimicrobial, tinea, fungal infections, Quetta, Balochistan.

1. Introduction

Dermatophytes are pathogenic fungi that cause skin, hair, and nail infection in human beings (Nweze and Eke 2018). These fungi's most important and distinguishing feature is their keratinophilic and keratinolytic ability. These two properties help dermatophytes digest the skin's keratin protein for nitrogen sources (Sharma et al. 2015, Lakshmipathy and Kannabiran 2010). According to the world health organization (WHO) Report, fungal infection is a serious global health problem. The dermatophytic infection estimated by WHO accounts for 20% of all skin infections (Sinski and Kelley 1987). The fungal infection outbreak is higher in the tropics than in the less humid regions. Dermatophytic infection depends on factors such as the interaction with the host immune system, fungal metabolites,

site of infection, and pathogenesis of infecting species or strains of dermatophytes (Sharma et al. 2015).

Dermatophytes comprise three genera of fungi i.e. Trichophyton, Epidermophyton and Microsporum (RAHUL 2014). The macroconidia of Trichophyton are thinly walled with a smooth outer surface (Refai, Heidy, and Mahmoud 2013). Microsporum has micro and macroconidia, while the macroconidia are most prevalent. It has multi-septate hype with a rough outer layer of macroconidia (Lakshmipathy and Kannabiran 2010). Microsporum does not need any special nutritional requirements for its growth (Baghza, Al-Adhroey, and Ali 2016). The third genus of dermatophytes is Epidermophyton which form a bunch like macroconidia, while microconidia are completely absent throughout their life cycle. The only characteristic of Epidermophyton is the formation of a yellowish-brown colony on agar media (Lakshmipathy and Kannabiran 2010).

The chronic infection of dermatophytes is caused by anthropophilic species such as Trichophyton species, while the acute infection is caused by zoophilic species such as Microsporum (AL-Janabi 2014). The infection site distribution may help identify the pathogen (Coloe and Baird 2010). Among the infection of dermatophytes, tinea capitis is the most dominant in children. It is an infection of the head and can cause hair, nail, and skin infection in children. Poor hygiene and interaction with contaminated inanimate objects such as pillowcases, combs, nail cutters, and scissors enhance the transmission of dermatophytosis (RAHUL 2014). Other types of dermatophytic infections are tinea barbae, tinea cruris, (Behzadi, Behzadi, and Ranjbar 2014) and tinea corporis. These infections are commonly linked with different species of dermatophytes, Trichophyton, Microsporum, and Epidermophyton (Behzadi et al. 2013).

Due to the increasing infections associated with fungi, the pharmaceutical industry is focusing on developing good antifungal agents with low adverse effects, as the antifungal therapy needs to be used for an extended period of time. Extended fungal therapy may lead to drug resistance and other health complications. Unlike antibacterial susceptibility test *in vitro*, antifungal test takes much longer due to the slow rate of reproduction and their clinical appearance.

Some natural products have been used locally for the treatment of fungal infections. These local medicines are often prepared in the form of extracts or oils containing bioactive substances from medicinal plants (Savarirajan, Ramesh, and Muthaiyan 2021). These active biocomponents of natural products can be a good alternative to synthetic antibiotics, which will also be helpful for the control of antibiotic resistance (Webster et al. 2008). Traditional healers claim that certain medicinal plants are more effective compared to modern antibiotics in treating infectious diseases (Akbar et al. 2023). The potential use of folk medicine for treating infectious diseases produced by common pathogens must be evaluated scientifically (Sailaja 2014). Plant extract can be an ideal solution to the problem, which can be easily tested in vitro (Neela, Sonia, and Shamsi 2014). In this study, we have isolated the infectious agent responsible for dermatophytosis against which natural compounds were also tested successfully.

2. Material & Methods

2.1 Collection of Samples

The samples were collected from the Department of Dermatology in Bolan Medical College Hospital, and were brought to the

Department of Microbiology in University of Balochistan, for further analysis. The patients with skin and nails infection caused by fungal pathogens, confirmed by the dermatologist of the department, were included in the study (Figure 1), while the patients with bacterial or viral infections, confirmed by the specialists, were excluded. Out of the 90 patients examined for skin and nail infections, only 19 were recommended for dermatophyte sampling, collected from the patient's nails and hair. All the samples were taken from the lesions of the patients by following standard protocols. The site of infection was washed with 70% ethanol to avoid bacterial contamination (Tartor, El Damaty, and Mahmmod 2016).

The study was conducted after the ethical approval from the Institutional Bioethics Committee, whereas the samples were collected after the patients' informed consent.

2.2 Culture and Isolation

All the samples were aseptically collected and transported to the laboratory using standard protocols. The samples were inoculated into cycloheximide and chloramphenicol-supplemented Sabouraud Dextrose Agar (SDA) with slants for culturing (Matnani et al. 2012). All the samples were cultured at 37^0 C for 1 to 3 weeks of incubation (Shahitha, Saranya, and Poornima 2013).

2.3 Microscopic Identification

The culture slants, with mature growth of the fungal isolates, were subjected to microscopic examination using a compound microscope after proper staining with lactophenol cotton blue on clean, grease-free slides. A small inoculum was placed over the surface of the glass slide containing a small amount of lactophenol cotton blue stain with the help of a sterile wire loop and inoculating needles and teased for a few minutes to spread the mycelium uniformly, and then covered with a cover slip. Differences in the structure were recorded in all the species in their mycelium, microconidia, and macroconidia (Taha et al. 2017).

2.4 Culturing of the Isolates on Special Media

Some dermatophytes need a special selective media for their growth, which helps in their identification, such as dermatophytes test agar media. This media can be prepared by the addition of some antibiotics in SDA. These antibiotics are chloramphenicol 50 mg/L and cycloheximide 500 mg/L; phenol red was added as a pH and color indicator (Bedir et al. 2014). The growth of dermatophytes changes the color of media to brown from red (SIĞIRCI et al. 2019).

2.5 Essential Oil Extraction from Propolis and Bitter Apricot Kernels

Oil was extracted by hydro-distillation with the help of the all-glass Clevenger apparatus. An amount of 20 g of crushed propolis and bitter apricot kernels was distilled overnight using absolute ethanol as a solvent. Anhydrous sodium sulfate was added to the isolated essential oil to remove residual water for purification purposes.

2.6 Natural Extracts Preparation

APE and GPE were prepared by maceration method using 1:10 concertation in absolute ethanol in Food Microbiology and Bioprocess Technology Laboratory, Department of Microbiology in University of Balochistan, Quetta, and used against the specific dermatophytes isolated in the study following (Akbar et al. 2019).

2.7 Antifungal Activity

The antifungal activities of the natural agents (oils and extracts) were tested by adding the testing materials to pre-sterilized culture media just before its solidification, where the concentration of extract and oil was maintained at 50 µL/mL in culture media. The solidified media were then inoculated with the

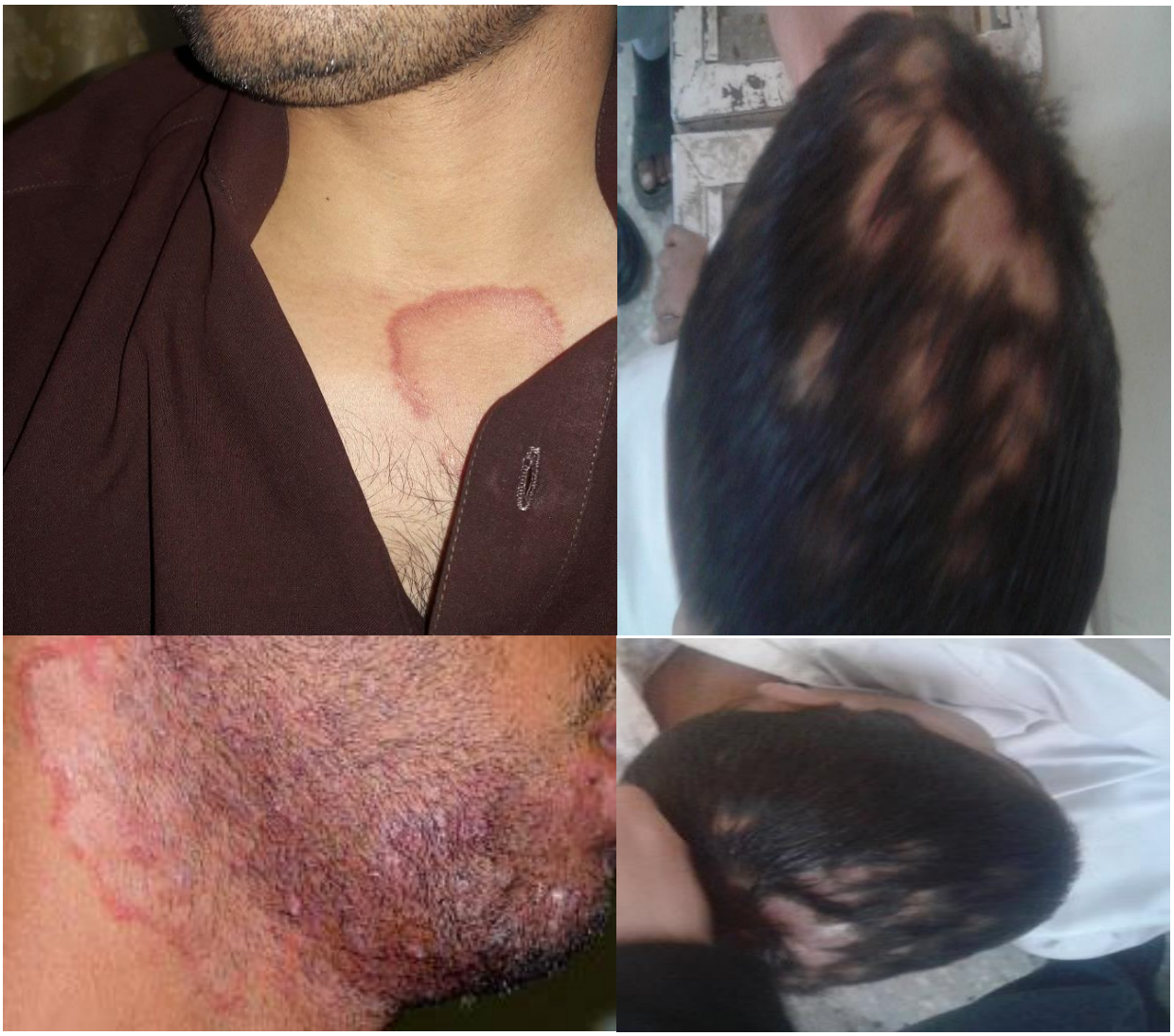

Figure 1. Patients with dermatophytic infections on skin and head.

target dermatophytes pathogens by placing 6 mm of inoculum at the center of agar plates. The media, free from anti-dermatophytic agents, was used as a negative control; on the other hand, antifungal drugs (Terbinafine) 50 µL/mL media was used as a positive control. The final growth was compared with the positive control following Abbott's formula for calculating the percentage of inhibition (Ahluwalia et al. 2012).

$$I\ (\%) = [(C-T)/C] \times 100$$

Where I = Inhibition, C =Positive control, T= Test

2.8 Fourier Transform Infrared (FTIR) Analysis of the Extracts and Oils

The analysis of major compounds through FTIR was done by adding around ten µL of sample on a diamond crystal of smart dura sample IR accessory. All the peak data were collected at room temperature. Spectral data were collected at room temperature and accumulated from 64 scans with a resolution of $1cm^{-1}$ in the range of $500\text{-}4000cm^{-1}$. A correction was applied by subtracting the background spectrum of air (Rodríguez et al. 2018).

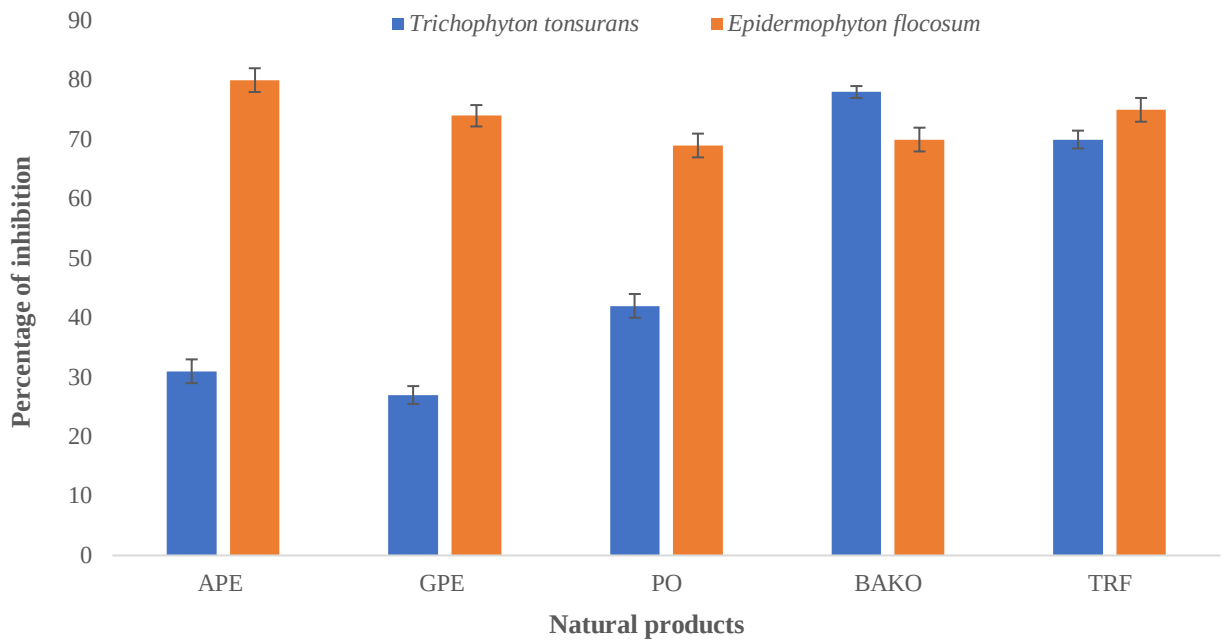

Figure 2. Anti-dermatophytic activity of the natural oils and extracts. APE= Apple peels extracts, GPE= Grape pedicel extracts, PO= Propolis oil, BAKO= Bitter apricot kernel oil, TRF= Terbinafine.

3. Results

From the nineteen samples collected, ten were confirmed positive for dermatophytes species, while the remaining belonged to *Aspergillus* species. Of these isolates, 30% were identified as *Epidermophyton flocosum*, while 70% of samples were identified as *Trichophyton* species. Within the *Trichophyton* species, 14% were *Trichophyton rubrum*, 71% were *Trichophyton tonsurans*, and 14% were *Trichophyton mentagrophytes* (Table 1).

Antifungal activity of APE, GPE, PO, and BAKO was performed by agar mix method. After three days of incubation, the results were calculated using the Abbot formula to find the zone of inhibition. The APE showed excellent activity (80% inhibition) against *Epidermophyton flocosum*, whereas its activity against *Trichophyton tonsurans* was not significant and could only inhibit 31% of its growth.

The GPE also showed significant activity against, *Epidermophyton flocosum*-74% and comparatively negligible activity against *Trichophyton tonsurans*-27%. The essential oil showed good potency against the target dermatophytes species. The activity of PO against *Epidermophyton flocosum* and *Trichophyton tonsurans* was 69% and 42%, respectively. BAKO showed significant activity against *Epidermophyton flocosum*-78% and *Trichophyton tonsurans*-71% (Figure 2).

The FTIR spectrum of BAKO revealed its three main compounds Eugenol acetate, carotenoid, and polyphenols, between the ranges 500 to 4000cm^{-1} of FTIR. The peaks range from 1665 to 4000. The peaks range from 1667 to 1695 cm^{-1}, most likely corresponding to carotenoids; the peak at 1750 cm^{-1} shows the possible presence of Eugenol acetate, while the peaks for polyphenol were found at 3583 cm^{-1}, 3393 cm^{-1}, 3396 cm^{-1}, 339 cm^{-1}. The FTIR analysis of APE showed the presence of three main compounds i.e. polysaccharide, galacturonic acid, and pyranose. The peaks 1606 and 1439 correspond to the presence of polysaccharides,

Table 1. Site of dermatophytic infections and the identified species

S#	Age (years)	Site of Infection	Sample Code	Result
1	10	Head/skull	01	*Trichophyton rubrum*
2	15	Foot	001	*Epidermophyton flocosum*
3	10	Head	02	*Trichophyton tonsurans*
4	13	Hand	002	*Trichophyton tonsurans*
5	06	Head	008	*Trichophyton mentagrophytes*
6	05	Head	007	*Trichophyton tonsurans*
7	07	Head	0009	*Trichophyton tonsurans*
8	14	Foot	004	*Epidermophyton flocosum*
9	08	Head	002	*Trichophyton tonsurans*
10	40	Toe's nail	S. nail	*Epidermophyton flocosum*

between 990-1120cm^{-1} reflecting the presence of galacturonic acid, while the highest peak obtained at 1013cm^{-1} indicates the presence of pyranose. FTIR analysis of GPE shows that there are three main bioactive compounds in it, namely pectin, vitamin C, and anthocyanin. The peaks for pectin were at 1639cm^{-1}, 1747cm$^{-1,}$ while peaks at 1600cm^{-1}, 1680cm^{-1}, 1750cm$^{-1,}$ and 2360cm^{-1} correspond to the presence of vitamin C. The anthocyanin peaks were found at 950-1200cm^{-1}. Two bioactive compounds are present in propolis, flavonoid, and phenolic resin. The corresponding peaks for flavonoid were received at 1619cm^{-1} (fisetin), 1652 cm^{-1} (hesperetin), and 1644 cm^{-1} (flavone), while the presence of phenolic resin was suspected at the range between 3650-3590cm^{-1}. During the FTIR peak data analysis, three different compounds, eugenol acetate, carotenoid, and polyphenols were assumed to be present in BAKO. We think these compounds are responsible for the antifungal activities of the oil. The presence of eugenol acetate ($C_{12}H_{14}O_3$) was confirmed in the oil through the gas chromatography and mass spectroscopy analysis during a detailed study of the oil.

4. Discussion

The dermatophytic species of fungi have been isolated from different sites of infection in the human body, and the use of plant extracts and essential oil of plants against these dermatophytes has been done in this study. During the six months duration of the study, we got 19 samples from the hospital, and 10 were identified as dermatophytes, while the remaining samples were of the *Aspergillus* species. In a cross-section study conducted in Kenya to investigate tinea capitis in school-going children (Moto, Maingi, and Nyamache

2015), the authors found 81.3% prevalence of dermatophytes, 61.3% Trichophyton spp., and 13.3% Microsporum spp. Similar results were recorded by (Zaki and El Emshaty 2014) while studying dermatophytes infection in diabetes patients, the authors concluded that the appearance of *Aspergillus* species in human infection is supported by two factors, (a) the host immune system, immunosuppressed people have a high chance of contracting *Aspergillus* related infectins, (b) trophic and humid region; regions with higher humid levels support the infection of said species. The majority of respondent (patients) 90% in this study were under 15 years old. Since children of this age group frequently play in the soil where they interact with geophilic groups of dermatophytes such as *Trichophyton rubrum*, and *Trichophyton tonsurans*, the most frequent pathogens of tinea capitis. Due to this reason, the *Trichophyton* species was the most predominant (70%) dermatophyte in our study (SIĞIRCI et al. 2019).

As mentioned previously, the APE showed inhibitory activity of 31% against *Trichophyton tonsurans* and 80% against *Epidermophyton flocosum*, the GPE showed 27% and 74%, PO showed 42% and 69%, and BAKO showed 78% and 70% activity against *Trichophyton tonsurans* and *Epidermophyton flocosum*, respectively. Similar results have been obtained previously. Apricot oil has shown comparatively high activity due to the presence of Eugenol acetate, which has high antimicrobial activity (Yulia 2005). Antifungal activity of oil compounds from Apricot, Propolis, and apple, grape extracts are reported here; however, its use against dermatophytes is rare (Akbar et al. 2022). We are reporting the antidermatophytic activities of these compounds for the first time.

FTIR analysis has shown that the apricot essential oil contains three main compounds, polyphenol, carotenoid, and Eugenol acetate. The presence of these compounds has been verified by comparing them with the peaks given in the literature (Zhou et al. 2016, Ivanauskas et al. 2008). The analysis of apple extract through FTIR found that polysaccharides, galacturonic acid, and pyranose are the main components. The peaks for polysaccharides were at 1606 and 1434, while the peaks which reflect the presence of galacturonic acid ranged from 1120cm^{-1} to 990cm^{-1}. The peaks for pyranose were obtained at 1013 cm^{-1}. Similar work has been reported previously(Dranca and Oroian 2019). The analysis of propolis through FTIR showed the presence of two bioactive compound-flavonoids and phenolic resin, which can affect the growth of fungal (KUZNIARZ 2014). The peaks for flavonoids were obtained at 1619cm^{-1} (fistin), 1652cm^{-1} (hesperetin), and 1644cm^{-1} (flavone), respectively. The same peaks were reported by (Heneczkowski et al. 2001). The peaks for phenolic resin were obtained in the range between 3650-3590cm^{-1}. Similar results were reported by (Edoga and Kovo 2006). Grape pedicle extract analysis through FTIR showed a diverse range of peaks. The peaks for pectin were at 1639cm^{-1} and 1747cm^{-1}, and peaks for vitamin C were obtained at 1600, 1680,1750,2360cm^{-1}. Peaks for anthocyanin were found in the range between 950-1200cm^{-1}. Similar results are reported by (Lekhuleni 2020, Zouambia et al. 2017, Zhao et al. 2015).

5. Conclusions

Dermatophytic infection is superficial and limited to the upper part of the skin, nails, and hair. Most of the patients in this study were under the age of 15 years, while the predominant infection recorded was tinea capitis which is caused by geophilic species of dermatophytes. Four different natural products (APE, GPE, BAKO, and PO) used in

the study were active against most of the target clinical isolates. BAKO and APE were found to be more effective against different species of dermatophytes. The bioactive compounds possibly responsible for the growth inhibition of the pathogenic fungal species present in the natural products were carotenoid, polyphenols, and eugenol acetate. In BAKO the eugenol acetate is proven to be antibacterial, antifungal, and anti-inflammatory. It was concluded that some natural extracts such as APE, GPE, BAKO, and PO could potentially limit the growth of dermatophytes, and their dermal applications for the control of dermatophytic infection are also in practice in indigenous societies.

Conflict of Interest

All contributing authors declare no conflicts of interest.

Funding

The study did not receive any external funding.

Ethics Approval

This study was approved by the University of Balochistan, Quetta, Pakistan.

Consent Forms

The written consent forms for patients were taken before inducting them into the study. These consent forms are available with the authors.

Data Availability

All the data related to this study is available with the authors.

Authors Contribution

AA conceptualized the study and wrote the final manuscript, GIK, NA, and ZG helped in the analysis and writing the first draft, did the experimental analysis, NA and SH contributed to manuscript writing and AA supervised the whole project and wrote the final manuscript.

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