**Title page**

**SAFETY STUDY OF A PHARMACOPOEIA-BASED FORMULATION USED IN DEPRESSION AND ANXIETY**

**Type of article:** Researcharticle

**Authors: Nikhat Fatimaa\*** Sumbul Rehmanb, Abdur Rauf c, NazishSiddiquid

PG Scholara\*, Assistant Professorb, Associate Professorc,d

**Affiliation:**

a\*,b,c, dDepartment of Ilmul-Advia (Unani Pharmacology), Faculty of Unani Medicine, AMU, Aligarh

Dear Editor,

We want to publish our research paper entitled “Safety study of a Pharmacopoeia-based formulation used in Depression and Anxiety” in your esteemed journal of repute. The paper reflects the experimental study as per WHO guidelines on the safety aspect of herbal drugs. The study has been done on formulation including *Terminalia chebula*Retz., *Emblica officinalis,Rosa damascena* Linn., *Lavendulastoechas,Polypodium vulagre* Linn., *Cuscutareflexa*Roxb., which are claimed to be effective in various ailments with a specific recommendation for psychological disorders. The study is a hands-on proof for its safe use in clinics for the same. Therefore, we would like to request you to publish the abovesaid research paper in your esteemed journal of repute.

We would like to mention that this manuscript has not been submitted to any other journal and published elsewhere. We will be highly obliged for your kind consideration.

Hope to have your positive response.

Best regards,

Dr. Nikhat Fatima (Corresponding Author)

Department of Ilmul-Advia (Unani Pharmacology)

Faculty of Unani Medicine

Aligarh Muslim University (AMU)

Aligarh (202001)

Uttar Pradesh, India.

E-mail: [nicky.alig1993@gmail.com](mailto:nicky.alig1993@gmail.com)

Contact no. +91 8267876071

**Abstract**

**Introduction**: Safety study is one of the essentialparameters of standardisation of natural origin drugs and is now obligatoryunder World Health Organization recommendations to ensure the safety of a drug before any preclinical or clinical studies.Between 70 and 80 percent of people worldwide receive their primary healthcare through non-conventional medicine, primarily from herbal sources, as per a report published by the World Health Organization.

**Methods**: In this present study, a pharmacopoeia-based formulation(PBF) claimed to be used for psychological diseases in Unani classical text was prepared using *Terminalia chebula*Retz.(fruit),*Emblica officinalis*(fruit)*,Cuscutareflexa*Roxb. (whole plant),*Rosa damascena* Linn.(flower), *Lavendulastoechas*(flower)*,Polypodium vulagre* Linn. (rhizome). These single drugswere collectedand authenticated from CSIR- NIScPR, New Delhi, and then the prepared powdered formulation was subjected tothe safety study screeningformicrobial load, heavy metals, aflatoxin and pesticide residue using LC-MS/MS, GC-MS/MS and ICP-MS.

**Results:** The results indicated that microbial load, specific pathogens, aflatoxinsB1, G1, B2, G2and pesticides residue were absent, while heavy metals like As, Pb, Hg and Cdwere found below the limit of quantification.

**Conclusion**: Excess amounts of heavy metals or the presence of aflatoxins in medicinal plants may lead to serious side effects like hepatoxicity, nephrotoxicityand carcinogenicity etc. WHO has now set the limits of these toxic contaminantsfor better efficacy in managing diseases. From this study, it is concluded that the formulation was safe and free from toxic contaminants and microbes.Hence,it can be used effectively in preclinical and clinical trials.

**Keywords:** Safety study, Pharmacopoeia-based formulation (PBF), World Health Organization (WHO).

**Introduction**

The need to include herbal medications in pharmacovigilance systems is rising as the usage of herbal products and medications increasing globally. For instance, in 2000, more than 158 million Americans spent almost US$ 17 billion in the United States. More recently, research found that more than 70% of Germans claimed to use "natural remedies," and for the majority of them, herbal medications were their first option for treating minor illnesses or disorders [1]. Therefore, to provide standard quality products, safety study of these herbs or herbals preparation comes first as safety is an essential component of quality control and a key element in the provision of herbal products for therapeutic use.

There is a prevalent misperception among people that "natural" inevitably equals "safe" and that medicines of natural origin are innocuous and risk-free. Though, instances of contamination of raw medicinal herbs and their preparations have become increasingly common.Some medicinal plantsare toxic by nature, while others are the result of past or ongoing exposure to contaminants like industry emissions, lingering chemical residues, radionuclides and metalsthat occur naturally in the ground or the atmosphere.The conventional systems used for the harvesting, manufacturing, transportation, and storage of herbal medicines result in increased contamination and microbial growthpropagation that may be due to unhygienic equipment, places and environmental factors (temperature, light, moisture and insects/ moulds) during collection, transportation and storageas a result of this quality of the drug suffers [2].Contamination of herbal medicinal drugs may also arise because of adulterationwith other substancesand pharmaceuticals; for instance, NSAIDs, corticosteroids and benzodiazepineslead to adverse effects. Adverse events can also be caused by the inappropriate administration,inaccurate and incorrect usage of the false species of plants of medicinal plants by consumers and healthcare professionals, intake of products contaminated with harmful substances like pathogenic microorganisms, toxic metals, and pesticide and fertilizer residues. Consequently, the safety of medicinal herbs has become a significant concern for the welfare of humankind[1,3].

Globally, the prevalence of psychological diseases is increasing daily, among which depression and anxietyhavebecome the leading causes of disability worldwide,affecting more than 264 million people. Close to 800 000 people die due to suicide every year[4]. By 2030, it is expected that depression will be one of the third disorders contributing to the universal disease burden [5]. To cope with the current scenario,herbal approaches from Unani classical literature were explored, and apharmacopoeia-basedformulation(PBF)was prepared using single herbal drugs,namely *Terminalia chebula*Retz. (*Halelakabuli*),*Emblica officinalis*(*Amla*)*,Cuscutareflexa*Roxb. (*Aftimoon*), *Rosa damascena* Linn.*(Gul-e-surkh)*, *Lavendulastoechas*(*Ustukhudoos*)*, Polypodium vulagre* Linn.*(Bisfaij*), which was subjected to safety study screening as per the WHO guidelines before its usage in preclinical research in animal model of depression and anxiety, becausethe quality assurance of plant drugs is considered a pre-requisite for their further evaluation for biological activity. These single drugs areexhilarants (*mufarreh-wa-muqawwi-e-qalb*), brain tonic (*munaqqi-e-dimagh*), concoctive and purgative ofphlegm and blackbile (*munzijwamushil-e-balgham-wa-sauda*)on the basis of Unani classical literature [6-10]. These six drugs are among the 63 single natural drugs specifiedby *IbnSina* in *RisalaAdviaQalbia,* useful in cardiac disorders as well as psychiatric ailments. Because the brain and heart are linked in terms of emotional disorders, all exhilarant drugs, whether single or compound formulations are considered beneficial in treating psychiatric illnesses like depression and anxiety[11]. “*RisalaAdviaQalbi*a” itself is a tremendous, systematized, original, authentic research work of that era by *IbnSina*.

**Materials and Methods**

**Collection and authentication of ingredients of PBF**

All single crude drugs of the PBF were procured from DawakhanaTibbiyaCollege A.M.U Aligarh except *Emblica officinalis (Amla)*, which was directly plucked from the tree (S.S south hostel, AMU Aligarh). The drugswere identified on the basis of ethno-botanical literature and were further subjected to microscopical analysis for confirmation,followed by authentication by Dr. Sumbul Rehman, in the Pharmacognosy section, Department of IlmulAdvia (Unani Pharmacology), Faculty of Unani medicine, A.M.U. Aligarh.Every single drug used for the preparation of PBF was also authenticated by CSIR- National Institute of Science Communication and Policy Research, New Delhi, with the authentication number provided in Table 1.The specimen of all the single drugs used in PBFhas been submitted to *Mawalid-e-Salasa* Museum of Department of IlmulAdvia (Unani Pharmacology), Faculty of Unani Medicine, A.M.U. Aligarh and their voucher number are provided in Table 1for record and future reference (Fig. 1).

**Preparation of Sample**

Fresh *Emblicaofficinalis (Amla)* was washed, sliced and dried in sunlight, all other single drugs were also cleaned from undesirable substances like earthy and foreign material such as pieces of wood, twigs, etc., and dried in sunlight and then powdered in an electrical grinder as per Unani pharmacopoeia at Pharmacy lab of Department of IlmulAdvia (Unani Pharmacology), A.M.U. Aligarh. The powdered sample was then sifted through sieve number 80 to ensure that the particle size was uniformand kept separated in air-tight containers. The powders of different drugs were then mixed in definite proportions, as given in Table 1, to form PBF [12, 13].

The powder was then examined to determine the microbial load, heavy metals, aflatoxins and pesticides residueat Delhi Test House (DTH), Azadpur, Delhi-110033 [QR-0302 Report No 2346220905IM69015,Sample Dated05/09/2022, Reported on 22/09/2022].

**Tests for Microbiological determination**

**Total viable aerobic count (TVC)**

The total viable aerobic count (TVC) of the test drug was performed as described in the test procedure, employing plate count findings to detect the antibacterial activity of the test sample.

**Pre-treatment of the test sample**

Depending upon the type of herbal drug sample tested, it was dissolved using an appropriate technique, and any antibacterial properties were removed by dilution or neutralization. The test sample was diluted using MM1275-500G from Hi-media Labs in Mumbai, India, which is a buffered sodium chloride-peptide solution with a pH of 7.0.

**Plate count for bacteria**

In a petri dish with a diameter of 90 mm, 1 ml of the pre-treated test sample was mixed with approximately 15 ml of the liquefied casein-soybean digest agar at a temperature not more than 45 °C.Alternatively, the test sample was spreadon the surface of the solidified medium. Two plates were made using the same dilution, inverted, and incubated at 30-35 °C for 48-72 hours unless a more accurate count was achieved in a shorter time. The number of colonies that consequently formed was counted, and the results were calculated using the plates having the greater number of colonies, up to a maximum of 300.

**Plate count for fungi**

In a petri dish with a diameter of 90 mm, 1 ml of the pre-treated test sample was mixed withabout 15 ml of liquefiedSabouraud glucose agar with antibioticsat a temperature not more than 45 °C. Alternatively, the test sample was spreadon the surface of the solidified medium. Two plates were made using the same dilution, inverted, and incubated at 20-25˚C for 5 daysunless a more accurate count was achieved in a shorter time. The number of colonies that consequently formed was counted, and the results were calculated using the plates with fewer than 100 colonies [14].

**Determination of Heavy metals**

Heavy elements such as Lead, Mercury, Arsenicand Cadmiumwere identified using ICP-MS in the test sample.

**Determination of Aflatoxins**

**Sample preparation:** The presence of aflatoxins B1, G1, B2and G2in the test sample of the given herbal drug powder was detected with LC-MS/MS.The test drug sample (2gm) and 60% acetonitrile/water (20ml)weremixedfor 2 minutesat high speed.The combined sample was centrifuged at 1600 rpm for ten minutes. The supernatant was collected and diluted with 2ml filtrate in 48 ml of Phosphate Buffered Saline (PBS, pH 7.4) to produce a solvent concentration of 2.5% or less; methanol/water was prepared with 2 ml of test sample diluted with 14 ml of Phosphate Buffered Saline (PBS, pH 7.4)to get a solvent concentration of 10% or less. At a flow rate of 5 ml/min, the sample diluent was run over the immunoaffinity column. The column was then rapidly blown with air to dry after being rinsed with 20 ml of distilled water at a flow rate of around 5 ml/min.The sample elute was mixed with 1.5 ml of distilled water. A sample volume of 500μlwas injected into the LCMS-MS.By comparing sample peak heights or areas to the total aflatoxin standard, the concentration of aflatoxin in the sample was determined[14,15].

**Determinationof pesticide residue**

GC-MS/MS was used to conduct the test to evaluate certain pesticide residues such as organophosphorus, organochloride and pyrethroid compounds [16].

**Result**

Microbial load determinationshowninTable 2 and 3 demonstrated that the total bacterial count was found to be 1270 cfu/gm, while the total yeast and mould count was found to be 80 cfu/gm and the specific pathogens such as *Escherichia coli, Staphylococcus aureus,Salmonella*and*Pseudomonas aeruginosa*werefound to be absent.The heavymetal analysis in Table 4 revealed that metals like Arsenic, Lead, Mercury and Cadmium were found below the limits of quantification (BLQ),whileaflatoxinsB1, G1, B2and G2,depicted in Table 5, and pesticide residue, as shown in Table 6found to be absent in the test sample.

**Discussion**

The aim of safety profiling of medicinal plants is to produce good quality products and prevent defects from occurring in herbal preparation and increase the global acceptance of the traditional system of medicine, which is based on herbs. This can be achieved by quality control as quality is important in every product however, it is vital in medicine because it involves life, and there is no secondary quality in drugs. Most of the time, the plant origin drugs are contaminated with microbes; for instance, resistant bacterial strains sometimes accompanied with the drugs produces health risk on consumption. A study revealed the incidence of bacteria that are resistant to antibiotics in 29 herbal supplements that were acquired from local retailers in the USA. The resistant strains of *Bacillusspp*.,*Staphylococcusspp*, *Enterobactercloacae*, *Erwiniaspp*., *Ewingellaamericana* and *Stenotrophomonasmaltophilia*were isolated[17]. Similarly, mycotoxins produced as secondary metabolites by moulds can be toxic to human health. Moulds of the *Aspergillus* and *Penicillium* genera are the common contaminants of grain and peanuts, producing aflatoxins which can be strong carcinogens [18]. Contamination of heavy metals like Arsenic, Cadmium, Mercury and Lead in medicinal plants can be due to environmental factors, as metals are freely distributed in the soil and water. They may be transferred from contaminated soil, air, and water to plants, which may later be consumed by humans and accumulate in the body,resulting in various health hazardous disorders like lung cancer, respiratory diseases, allergic dermatitis, neurological disorders, liver and kidney damage [19,20]. Therefore, they should be absent, or if they are present in any herbal sample, it should be within permissible limits. A permitted limit for their concentration in the plants has been established by WHO; thus, maximum health benefits can be obtained from herbal sources.

**Conclusion**

The formulation was found to be safe as microbial load, heavy metals, aflatoxins and pesticideswere found to be within permissible limits. Hence the formulation can be considered as harmless and can be used safely in preclinical and clinical studies. It is a hands-on proof and will provide future assistance for its usage in psychological disorders.

**Acknowledgments**

The authors are thankful to the Department of IlmulAdvia (Unani Pharmacology), Aligarh Muslim University, Aligarh (AMU), for providing Lab space and Delhi Test House (DTH), New Delhi, for specific experimental analysis.

**References**

1. World Health Organization (WHO). Guidelines on safety monitoring of herbal medicine in pharmacovigilance system.Geneva: WHO; 2004.
2. World Health Organization (WHO). Guidelines for assessing quality of herbal medicine with reference to contaminants and residues.Geneva: WHO;2007.
3. World Health Organization (WHO). National policy ontraditional medicine and regulation of herbal medicines reportof a WHO global survey. Geneva: WHO; 2005.
4. World Health Organization (WHO). Depression. Factsheet. Geneva*,* Switzerland: WHO; 2020
5. Mathers CD, Loncar D. Projections of global mortality and burden of disease from 2002 to 2030. PLoS medicine. 2006 Nov 28;3(11):e442.
6. Ibn Baitar ZB. Al JameulMufradat al AdviawalAghziya (Urdu translation). Vol I.New Delhi: CCRUM; 1986.
7. Central Council for Research in Unani Medicine. Standardisation of Single Drugs of Unani Medicine. New Delhi: CCRUM; 1992.
8. Ibn Baitar ZB. Al JameulMufradat al AdviawalAghziya (Urdu translation). Vol IV.New Delhi: CCRUM; 2003.
9. Ghani N. KhazianulAdvia (Musawwar Edition). Darya Ganj, New Delhi:Idara Kitab-ul-Shifa; 2011.
10. Ibn Sina. Al Qanoon fil Tib (Urdu translation by Kantoori G. H.). Daryaganj, New Delhi: Eijaz Publishing House; 2010.
11. Ibn Sina. RisalaAdviaQalbia (Farsi Tarjuma). Aligarh Muslim University, Aligarh: Publication Division; 1996.
12. Central Council for Research in Unani Medicine. The Unani Pharmacopoeia of India. Part I.New Delhi: CCRUM; 2007.
13. Central Council for Research in Unani Medicine. The Unani Pharmacopoeia of India.VolVI(I).New Delhi: CCRUM; 2009.
14. Ramkrishanan G, Gayathri V, Sathia S, Parameswari RP, Saravana CB. Physicochemical and phytochemical standardization of Thraatchathichooranam- A polyherbal formulation. Journal of pharmaceutical Science & Research. 2015; 7(6):305-313.
15. Ventura M, Gómez A, Anaya I, Díaz J, Broto F, Agut M, Comellas L. Determination of aflatoxins B1, G1, B2 and G2 in medicinal herbs by liquid chromatography–tandem mass spectrometry. Journal of Chromatography A. 2004 Sep 3;1048(1):25-9.
16. Maobe GAM, Gatebe E, Gitu L, Rotich H, Profile of Heavy Metals in Selected Medicinal Plants used for the treatment of Diabetes, Malaria and Pneumoniain Kisii region, Southwest Kenya. Global Journal of Pharmacology. 2012; 6(3):245-251.
17. Brown JC, Jiang X. Prevalence of antibiotic-resistant bacteria in herbal products. Journal of food protection. 2008 Jul;71(7):1486-90.
18. Fuat Abd Razak M, Aidoo KE, Candlish AGG. Mixed herbal drugs: inhibitory effect on growth of the endogenous, mycoflora and aflatoxin production. Mycopathologia 2009;167: 273-86.
19. Caldas ED, Machado LL. Cadmium, mercury and lead in medicinal herbs in Brazil. Food and chemical toxicology. 2004 Apr 1;42(4):599-603.
20. Kosalec I, Cvek J, Tomic S. Contaminants of medicinal herbs and herbal products. Arhiv za higijenuradaitoksikologiju. 2009 Oct 1;60(4):485.

**Table1: Details of ingredients used in PBF** [12,13]

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| **Scientific name& Family** | **Unani name** | **Parts used** | **Quantity**  **(In gm)** | **Authentication No.** | **Voucher No.** |
| *Terminalia chebula* Retz  (Combretaceae) | *Halelakabuli* | Fruit rind | 40 | (NIScPR /RHMD/ Consult/ 2023/ 4319-20-2) | SC-0311/22 |
| *Emblica officinalis*(Euphorbiaceae) | *Amla* | Fruit | 40 | (NIScPR /RHMD/ Consult/ 2023/ 4319-20-1) | SC-0309/22 |
| *Cuscutareflexa*Roxb.  (Convolvulaceae) | *Aftimoon* | Whole plant | 40 | (NIScPR /RHMD/ Consult/ 2023/ 4319-20-6) | SC-0307/22 |
| *Rosa damascena* Linn.  (Rosaceae) | *Gul-e-surkh* | Flower | 60 | (NIScPR /RHMD/ Consult/ 2023/ 4319-20-4) | SC-0308/22 |
| *Lavendulastoechas*  (Lamiaceae) | *Ustukhudoos* | Flower | 80 | (NIScPR /RHMD/ Consult/ 2023/ 4319-20-5) | SC-0312/22 |
| *Polypodium vulagre* Linn.  (Polypodiaceae) | *Bisfaij* | Rhizome | 120 | (NIScPR /RHMD/ Consult/ 2023/ 4319-20-3) | SC-0310/22 |

**Table 2: Microbial load determination in PBF**

|  |  |  |
| --- | --- | --- |
| **Test Parameter** | **Result (cfu/g)** | **Permissible Limit (cfu/gm)** |
| Total Bacterial Count | 1270 | Not more than 1x105 |
| Total Yeast & Mould | 80 | Not more than 1x103 |

cfu/gm: colony-forming units per gram

**Table 3: Determination of specific pathogens in PBF**

|  |  |  |
| --- | --- | --- |
| **Pathogens** | **Result** | **Permissible Limit** |
| *Escherichia coli*/gm | Absent | Absent |
| *Salmonella*/gm | Absent | Absent |
| *Staphylococcus aureus*/gm | Absent | Absent |
| *Pseudomonas aeruginosa/*gm | Absent | Absent |

**Table 4: Determination of heavy metals inPBF**

|  |  |  |  |
| --- | --- | --- | --- |
| **Test Parameter** | **Result** | **LOQ** | **Method** |
| Lead (Pb) | BLQ | 0.1 | ICP-MS |
| Mercury (Hg) | BLQ | 0.1 | ICP-MS |
| Arsenic (As) | BLQ | 0.1 | ICP-MS |
| Cadmium (Cd) | BLQ | 0.1 | ICP-MS |

LOQ = Limit of Quantification

BLQ = Below the limit of Quantification

ICP-MS=Inductively Coupled Plasma Mass Spectrometry

**Table 5: Determination of aflatoxin in PBF**

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **Aflatoxins** | **Result** | **LOQ** | **Permissible Limit** | **Method** |
| Aflatoxin B1 | Not detected | 0.001 | Not more than 0.5 | LC-MS/MS |
| Aflatoxin G1 | Not detected | 0.001 | Not more than 0.5 |
| Aflatoxin B2 | Not detected | 0.001 | Not more than 0.1 |
| Aflatoxin G2 | Not detected | 0.001 | Not more than 0.1 |

LOQ = Limit of Quantification

LC-MS/MS = Liquid chromatography Mass Spectrometry

**Table 6: Determination of pesticide residue in PBF**

|  |  |  |  |
| --- | --- | --- | --- |
| **S. No.** | **Pesticidal Residue** | **Result (mg/kg)** | **LOQ (mg/kg)** |
|  | Alachlor | Not Detected | 0.01 |
|  | Aldrin &Deildrin (Sum of) | Not Detected | 0.01 |
|  | Azinophos- methyl | Not Detected | 0.01 |
|  | Bromopropylate | Not Detected | 0.01 |
|  | Chlordane (Sum of cis, trans and oxychlordane) | Not Detected | 0.01 |
|  | Chlorfenvinphos | Not Detected | 0.01 |
|  | Chlorpyrifos | Not Detected | 0.01 |
|  | Chlorpyrifos-methyl | Not Detected | 0.01 |
|  | Cypermethrin (and isomers) | Not Detected | 0.01 |
|  | DDT-Dichloro diphenyl trichloroethane (Sum of p,p-DDT, p,p-DDE and p,p-TDE) | Not Detected | 0.01 |
|  | Deltamethrin | Not Detected | 0.01 |
|  | Diazinon | Not Detected | 0.01 |
|  | Dichlorvos | Not Detected | 0.01 |
|  | Dithiocarbamates (as CS2) | Not Detected | 0.01 |
|  | Endosulfan (Sum of Isomer &Endosulfansulfate) | Not Detected | 0.01 |
|  | Endrin | Not Detected | 0.01 |
|  | Ethion | Not Detected | 0.01 |
|  | Fenitrothion | Not Detected | 0.01 |
|  | Fenvalerate | Not Detected | 0.01 |
|  | Fonofos | Not Detected | 0.01 |
|  | Heptachlor (Sum of Heptachlor &Heptachlor epoxide) | Not Detected | 0.01 |
|  | Hexachlorobenzene | Not Detected | 0.01 |
|  | Hexachlorocyclohexane isomer (other than y) | Not Detected | 0.01 |
|  | Lindane (y- Hexachlorocylohexane) | Not Detected | 0.01 |
|  | Malathion | Not Detected | 0.01 |
|  | Mathidathion | Not Detected | 0.01 |
|  | Parathion | Not Detected | 0.01 |
|  | Parathion Methyl | Not Detected | 0.01 |
|  | Permethrin | Not Detected | 0.01 |
|  | Phosalone | Not Detected | 0.01 |
|  | Piperonyl butoxide | Not Detected | 0.01 |
|  | Primiphos Methyl | Not Detected | 0.01 |
|  | Pyrethrins (Sum of isomers) | Not Detected | 0.01 |
|  | Quintozene (Sum of Quintozene, pentachloroaniline and methyl pentachlorophenyl sulphide) | Not Detected | 0.01 |

LOQ = Limit of Quantification

DDE=Dichloro diphenyl dichloroethylene

**Fig. 1.** Dried specimen of all single drugs-**(A)***Terminalia chebula*Retz. (fruit); **(B)** *Emblica officinalis*(fruit);**(C)** *Cuscutareflexa*Roxb. (whole plant)**; (D)** *Rosa damascena* Linn.(flower); **(E)***Lavendulastoechas*(flower);**(F)** *Polypodium vulagre* Linn. (rhizome).

**F**

**E**

**D**

**C**

**B**

**A**

****

****