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MICROBIAL LOAD, PESTICIDES RESIDUE, AFLATOXIN ESTIMATION AND HEAVY METALS ANALYSIS OF A SINGLE UNANI DRUG BADRANJBOYA (MELISSA OFFICINALIS)

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ABSTRACT

The recent study was aimed to evaluate safety parameters of Badranjboya (*Melissa officinalis*) as a very common drug used in Unani System of Medicine for its cardiotonic and exhilarant effect in palpitation and syncope. The study revealed the presence of heavy metals lead, cadmium, mercury and arsenic within the permissible limit as per WHO guidelines, while aflatoxins, pesticides and microbial load were found to be absent in the crude drug sample. It could be said that the drug was free from toxicity.

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Introduction

Melissa officinalis(Family-Labiatae) known as Malsoonan, Malitanain in Unani [1] and as Badrunjbuyeh in persian [2, 3,4], is commonly known as Balm . Melissa is a Greek word means honeybee, as the honeybees are attracted towards the flowers. This species is native to the Mediterranean region [5]. This is an evergreen herbaceous perennial plant, growing to a height of 60 cm, is pubescent, and has lemon scented odour [6]. It was formerly valued as a corroborant in hypochondriacal affections, and as a Persian drug, it is still used for this purpose by Indian Hakims. In Europe, Balm tea is still a domestic remedy, and is used as a grateful diluent in febrile affections. It also has a place in French codex [7]. In Unani literature, its actions were described as Cardiotonic, Exhilarant [1,4,8,9], brain tonic, deobstruent ,memory enhancer, stomachic, demulcent, aphrodisiac, appetiser, blood-purifier, and sedative. It has been used in palpitation, syncope, hiccup, eczema, pruritus, scrofula [8], Alzheimer disease [10], loss of appetite, vertigo, hysteria, nervous complaints, general atony [5].

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Phytochemical investigations revealed that this plant contains volatile compounds, triterpenoids, phenolic acids, and flavonoids. Crude extracts and pure compounds isolated from M. officinalis exhibited numerous pharmacological effects, from which only anxiolytic, antiviral and antispasmodic activities of this plant as well as its effects on mood, cognition and memory have been shown in clinical trials. AChE inhibitory activity, stimulation of the acetylcholine and $GABA_A$ receptors, as well as the inhibition of matrix metalloproteinase-2 have been the main mechanisms proposed for the widely discussed neurological effects of this plant [11].

Current practices of harvesting, production, transportation and storage of herbal drugs have caused additional contamination and microbial growth proliferation of microorganisms that may result from the failure to control the moisture levels of herbal medicines during transportation and storage [12]. Aflatoxin B1, G1,B2,G2, are fungal secondary toxic metabolites produced by Aspergillus flavus, Aspergillus parasiticus and Aspergillus nomius. Aflatoxins are the strongest naturalcarcinogens, and their main targetorgan is the liver. The International Agency for Research on

Cancer (IARC) has classified aflatoxin B1 in the group1as a human carcinogen, and aflatoxin G1, B2 and G2 in the group 2 as possible carcinogens to humans [13]. Contamination of herbal materials with toxic substances such as arsenic can be attributed to many factors. These include environmental pollution (i.e. contaminated emissions from factories, leaded petrol, and contaminated water including run off water which finds its way into rivers, lakes and seas, and some pesticides), soil composition, and fertilizers. The contamination of the herbal material leads to the contamination of the products during various stages of the manufacturing process [12]. The worldwide consumption of herbal medicines is enormous, so in terms of population exposure alone, it is essential to identify the risks associated with their use as safety of herbal medicines as an important public health issue [14]. The present study was an attempt to assess these safety parameters in a well known herbal drug used in Unani System of Medicine Badranjboya (Melissaofficinalis).

Fig A. Market sample of Melissa officinalis

2.Material and Methods

2.1 Sample preparation: The test drug Badranjboya (Melissa officinalis)(Fig.A) was procured fromlocal market of Aligarh city in the month of April 2017, and was properly identified according to the morphological features mentioned in botanical and Unani literature, confirmed in Pharmacognosy section of department of Ilmul Advia,A.M.U., Aligarh. It was also authenticated by National Institute of Science Communication and Information Resources, New Delhi (NISCAIR /RHMD/ Consult/ 2017/ 3064-13). The sample of the test drug was submitted to Mawalid-e-Salasa Museum of the Department after the identification, for future reference with the Voucher No. SC-0219/17.

The drug was cleaned from the earthy material, washed with double distilled water, and dried at 45ºC in hot air oven, and powdered in an electrical grinder. There, the drug was passed through sieve no. 80 to confirm its fineness and uniformity of particle size. Finally, the powdered drug was stored in an air tight container for the experimental study.

The powder of aerial part of Badranjboya M.officinalis was studied to evaluate the presence of microbial load, pesticides residue, aflatoxins estimation, and heavy metals analysis at Delhi Test House, Azadpur, Delhi-110033 [QR-0302 Report No 25521709251M-80010 Sample Dated 25/09/2017 Reported on 03/10/2017].

2.2.Microbiological determination tests:

2.2.1 Total viable aerobic count (TVC):

For detection of the anti-bacterial activity of the test drug, the total viable aerobic count (TVC) of the test drug was carried out, as specified in the test procedure, using plate count results.

2.2.2 Pre-treatment of the test drug :

Depending on the nature of the herbal drug, the sample was dissolved using a suitable method, and any antimicrobial property present in the sample was eliminated by dilution or neutralization. Buffered Sodium Chloride-Peptone Solution, pH 7.0 (MM1275-500G, Himedia Labs, Mumbai, India) was used to dilute the test sample.

2.2.3 Plate count for bacteria:

1 ml of the pre-treated test sample was added to about 15 ml of the liquefied casein-soybean digest agar in a petri-dish of 90 mm diameter at a temperature not exceeding 45 ^{0}C. Alternatively, the test sample was spread on the surface of the solidified medium. Two dishes were prepared with the same dilution, they were inverted and incubated at 30-35^{0}C for 48-72 hrs, unless a more reliable count was obtained in a short period of time. The formed colonies were counted, and the results were calculated using the plates with the largest number of colonies, up to a maximum of 300.

2.2.4 Plate count for For fungi:

1 ml of the pre-treated test sample was added to about 15 ml of the liquefied Sabouraud glucose agar with antibiotics in a petri-dish of 90 mm diameter at a temperature not exceeding 45^{0}C. Alternatively, the test sample was spread on the surface of the solidified medium. Two dishes were prepared with the same dilution; they were inverted and incubated at 20 - 25^{0}C for 5 days, unless a more reliable count was obtained in a short period of time. The formed colonies were counted, and the results were calculated using the plates with not more than 100 colonies [15]

2.3 Heavy metals

Heavy metals including Arsenic, Mercury, Cadmium and lead were determined in the test sample using Atomic Absorption Spectroscopy (AAS).

2.4 Estimation of Aflatoxins Sample preparation

The test for the determination of aflatoxins B_1, B_2, G_1, and $G_2$was carried out using LC-MS/MS.2gm of the test drug which was blended at a high speed with 20 ml of 60% acetonitrile/water for two minutes. The blended sample was centrifuged for ten minutes using 1600 rpm (av.), and the supernatant was retained and diluted with 2 ml of filtrate with 48 ml of phosphate buffered saline (PBS, pH 7.4) to give a solvent concentration of 2.5% or less; methanol/water was prepared by taking 2 ml of sample, and diluted with 14 ml of PBS (pH 7.4) to give a solvent concentration of 10% or less. The sample diluent was passed through the immunoaffinity column at a flow rate of 5 ml/ min. The column was then washed by passing 20 ml of distilled water through the column at the flow rate of approximately 5 ml/ min, and dried by rapidly passing air through the column. 1.5 ml of distilled water was added to the sample elute. 500 µl of sample was injected onto the LCMS-MS (LC-Perkin, MS Applied Bio System, Model No.2000, Mobile Phase). A- Water 100%, B-ACN 100%, Column oven temperature = 30, Column ZORBAX Rx c18, narrow base 2.1×150 mm - 5 micron, Flow = 0.750 ml) were used. The aflatoxin concentration was quantified by comparing sample peak heights or areas to the total aflatoxin standard (R-Biopharm) [15].

2.5 .Pesticidal residue:

The test for the assessment of specific pesticide residues like Organochloride compounds, Organophosphorous compounds and Pyrethroids compound was conducted using GC-MS/MS [16].

3. Results

The results of this study demonstrated that microbial load (Bacterial, yeast and Mould) as listed in Table 1 and 2, and heavy metals as depicted in Table 3, were found below the permissible concentration, while aflatoxins as shown in Table 4 , and pesticide residue as listed in Table 5, were not found at all.

Table 1. Microbial load in Mellissa oficinalis

S.No.	Microbes	Result	Permissible Limit
1.	Total Bacterial Count	4300	Not more than 1x10^5 CFU/g
2.	Total Yeast & Mould	170	Not more than 1 x10^3 CFU/g

CFY/g: colony-forming units per gram

Table 2. Test for Specific Pathogens in Mellissa oficinalis

S.No.	Pathogens (/gm)	Result (gm)	Permissible limits as
1.	E-coli	Absent	Absent
2.	Salmonella	Absent	Absent
3.	S. aureus	Absent	Absent
4.	P. aeruginosa	Absent	Absent

E.Coli=Escherichia coli

S.aureus =Staphylococcus aureus

P. aeruginosa=Pseudomonas aeruginosa

Table 3. Heavy Metal in Mellissa oficinalis

S.No.	Test parameter (mg/kg)	Result (mg/kg)	LOQ(mg/kg)	Permissible limit (mg/kg)	Method
1.	Lead (Pb)	Not detected	2.50	Not more than 10	AAS
2.	Mercury (Hg)	Not detected	0.5	Not more than 1	AAS
3.	Arsenic (As)	Not detected	1.25	Not more than 3	AAS
4.	Cadmium (Cd)	Not detected	0.25	Not more than 0.3	AAS

LOQ = Limit of Quantification
BLQ = Below the limit of Quantification
AAS = Atomic Absorption spectroscopy

Table 4. Aflatoxin in Mellissa oficinalis

S.No.	Aflatoxin(mg/kg)	Result	LOQ	Permissible Limit (mg/kg)	Method
1	Aflatoxin B_1	Not detected	0.001	Not more than 0.5	LCMSMS
2.	Aflatoxin G_1	Not detected	0.001	Not more than 0.5	LCMSMS
3.	Aflatoxin G_2	Not detected	0.001	Not more than 0.1	LCMSMS
4.	Aflatoxin B_2	Not detected	0.001	Not more than 0.1	LCMSMS

LOQ = Limit of Quantification
BLQ = Below the limit of Quantification
LC-MS/MS = Lliqiuid chromatography Mass Spectrometry

Table 5. Pesticidal residue in Mellissa oficinalis

S.No.	Pesticides residue (mg/kg)	Result	LOQ	Permissible Limit (mg/kg)	Method
1.	Alachlor	Not Detected	0.02	0.02	GCMSMS
2.	Aldrin & Dieldrin	Not Detected	0.04	0.05	GCMSMS
3.	Azinophos-methyl	Not Detected	0.04	1.0	GCMSMS
4.	Bromopropylate	Not Detected	0.08	3.0	GCMSMS
5.	Chlordane	Not Detected	0.04	0.05	GCMSMS
6.	Chlorfenvinphos	Not Detected	0.04	0.5	GCMSMS
7.	Chlorpyrifos	Not Detected	0.04	0.2	GCMSMS
8.	Chlorpyrifos-methyl	Not Detected	0.04	0.1	GCMSMS
9.	Cypermethrin (and isomers)	Not Detected	0.10	1.0	GCMSMS
10.	DDT (Sum of pp-DDT, pp-DDE and pp-TDE)	Not Detected	0.04	1.0	GCMSMS
11.	Deltamethrin	Not Detected	0.10	0.5	GCMSMS
12.	Diazinon	Not Detected	0.04	0.5	GCMSMS
13.	Dichlorvos	Not Detected	0.04	1.0	GCMSMS
14.	Dithiocarbamates	Not Detected	0.01	2.0	UV-VIS Spectrophotometry
15.	Endosulfan (Sum of Isomer and Endosulfan sulphate)	Not Detected	0.04	3.0	GCMSMS
16.	Endrin	Not Detected	0.04	0.05	GCMSMS
17.	Ethion	Not Detected	0.04	2.0	GCMSMS
18.	Fenitrothion	Not Detected	0.04	0.05	GCMSMS
19.	Fenvalerate	Not Detected	0.10	1.5	GCMSMS
20.	Fonofos	Not Detected	0.04	0.05	GCMSMS

21.	Heptachlor (Sum of Heptacholar & Heptachlor epoxide	Not Detected	0.04	0.05	GCMSMS
22.	Hexachlorobenzene	Not Detected	0.04	0.1	GCMSMS
23.	Hexachlorocyclohexane isomer (other than ý)	Not Detected	0.04	0.3	GCMSMS
24.	Lindane (ý-Hexachlorocylohexane)	Not Detected	0.04	0.6	GCMSMS
25.	Malathion	Not Detected	0.04	1.0	GCMSMS
26.	Methidathion	Not Detected	0.04	0.2	GCMSMS
27.	Parathion	Not Detected	0.04	0.5	GCMSMS
28.	Parathion Methyl	Not Detected	0.04	0.2	GCMSMS
29.	Permethrin	Not Detected	0.04	1.0	GCMSMS
30.	Phosalone	Not Detected	0.04	0.1	LCMSMS
31.	Piperonyl butoxide	Not Detected	0.04	3.0	LCMSMS
32.	Primiphos Methyl	Not Detected	0.04	4.0	LCMSMS
33.	Pyrethrins	Not Detected	0.10	3.0	GCMSMS
34.	Quintozen (Sum of Quintozene, pentachloroaniline and methyl pentachlorophenyl sulphide)	Not Detected	0.10	1.0	LCMSMS

DDT=Dichloro diphenyl trichloroethane
DDE=Dichloro diphenyl dichloroethylene
GCMS=Gas Chromatography Mass Spectrometry
LCMS/MS = Lliqiuid chromatography Mass Spectrometry

4. Discussion

Safety studies of Unani drugs provided a scientific justification for their traditional use, and proved that they are safe and efficacious. The presence of heavy metals in a drug beyond the permissible limits would cause serious side effects on brain, kidney, developing foetus, vascular and immune system [17]. Similarly, aflatoxins have emerged as a major threat to human health, because a number of serious side effects such as hepatotoxicity, carcinogenicity and immune suppression are associated with them. Therefore, WHO has set a permissible limit of their concentration in the plant. Although, their absence in product is desirable but in case detected, their concentration must be limited to the permissible limits because if the limit exceeds, the drug will not be allowed to be used in the management of diseases. Standardization of Unani medicine using such practiced scientific techniques may help in building confidence for their possible use as a therapeutic medicine, and their global acceptance.

5. Conclusion

All four parameters undertaken in the study were considered instrumental to determine the safety/toxicity of drugs. Theresults of the study demonstrated that microbial count (Bacterial, yeast and Mould) was found below the permissible limit, which was unable to produce any toxicity. Heavy metals (Arsenic, Mercury, Cadmium and Lead) were not found to be present. Aflatoxin (B1, B2, G1and G2) caused serious side effects such as hepatotoxity, carcinogenicity etc. The absence of these toxic elements in the test drug made it safe and free from serious toxic effects. Badranjboya (Mellissa officinalis) was also free from pesticide residue contamination. The findings of the present study revealed that all the safety parameters carried out on the Badranjboya were found within the permissible limits. It indicated that the test drug can be used safely .

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