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Pharmacognostical, Physicochemical and Microbiological Standardisation of *Tiryaq-E-Arba* (An Unani Polyherbal Formulation)

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Abstract
Background: *Tiryaq-e-Arba* is a famous semi-solid compound preparation of Unani medicine and useful in various medical conditions. Before this study, Standardization of this formulation has not been carried out.
Aims: The present study was undertaken to standardise the *Tiryaq-e-Arba*.
Material &Methods: Standardisation performed from the organoleptic evaluation, physico-chemical studies, estimation for organic and inorganic constituents, heavy metal toxicity, microbiological evaluation.
Results & Discussion: *Tiryaq-e-Arba* was evaluated for organoleptic evaluation and found in confirmity. Physico-chemical parameters were evaluated on three samples and mean was determined. Bulk density, specific gravity, pH of 1% and 10% solution, moisture content, alcohol and water-soluble content were determined. Percentage of total ash, acid insoluble ash, water soluble ash were determined. Estimation for organic constituents viz. alkaloid, reducing sugars, phenols, volatile oils were done. In inorganic constituents, calcium, iron, copper were evaluated and the values were recorded. Heavy metal estimation revealed lead content but within limit set by WHO, while mercury, arsenic, cadmium were absent. Total bacterial count and total fungal count also within limits and absence of specific pathogens. Results were within limits set by WHO.
Conclusions: By this study, we have developed standards for various parameters which can be used for reference for future studies. It provides information and controls, necessary to produce material of reasonable consistency. This study is not only helpful for the researchers, analysts, scientists but also strengthen the Unani System of Medicine.

Keywords: Standardisation, *Tiryaq-e-Arba*, Physicochemical, microbiological, Pharmacognostical

1. Introduction

According to WHO, Standardisation is a measurement for ensuring the quality and is used to describe all measures, which are taken during the manufacturing process and quality control leading to a reproducible quality. Standardisation is not an easy task as numerous factors influence the bio efficacy and reproducible therapeutic effect [1]. Standardization also refers to the body of information and controls, necessary to produce material of reasonable consistency. *Tiryaq* is a semisolid poly-pharmaceutical preparation of Unani medicine where one or more single drugs of plant, origin are mixed in powder or liquid form in base (*qiwam*) of sugar, honey or jaggary. *Tiryaq-e-Arba* is a wonder drug belonging to Unani system of medicine. It has important actions like *dafe samoom* (antidote to poison), *dafe tashannuj* (anti convulgent) and *Idrar-e-bol* (diuretic) and used in diseases conditions like *tashannuj* (convulsions), *qoolanj* (colitis) and *istisqa* (ascites). The *Tiryaq-e-Arba* is a reputed *Unani Tiryaq* i.e. antidote containing *Habb-ul-Ghar (Laurus nobillis* Linn), *Juntiana Roomi (Gentiana lutea* Linn), *Mur Makki (Commiphora myrrha* (Nees Engl.) and *Zarawand Mudahraj (Aristolochia rotunda* Linn). The therapeutic dose and mode of administration are 6gm twice daily with water [2].

2. Methodology

The ingredients of *Tiryaq-e-Arba* with their botanical name, part used and ratio of quantities has been described in Table 1.

Table 1: The Ingredients of *Tiryaq-e-Arba* [3]

S.No.	Drug name	Botanical name	Part used	Ratio
1.	*Habb-ul-Ghar*	*Laurus nobilis* Linn	Fruit	1 part
2.	*Juntiana Roomi*	*Gentiana Lutea* Linn	Root	1 part
3.	*Mur Makki*	*Commiphora myrrha* Ness Engl.	Gum	1 part
4.	*Zarawand Mudahraj*	*Aristolochia rotunda* Linn	Root	1 part
5.	*Shehad*	Honey	Secretion	3 part

2.1 Method of preparation

All the ingredients from 1-4 are taken in equal quantity and honey three times. Ingredients were obtained from Dawakhana Tibbiya College, A.M.U., Aligarh, except for *Habb-al-Ghar (Laurus nobilis* Linn), which was obtained from Riyadh, Kingdom of Saudi Arabia. The authenticity of

the drugs was confirmed botanically. The drugs were first crushed and then powdered separately and sifted from 60 mesh sieve to obtain a fine powder. The powder of 4 drugs was taken in equal proportion mixed thoroughly. Further prepare *qiwam* by mixing them in the suspension of honey into 1:3 ratio. Discontinue the heat as the *qiwam* was prepared and add the above powders and mix thoroughly. Allow it to cool to room temperature and all the powdered ingredients in equal weight mixed in the *qiwam* as in *Majoon*. Then it was stored in tightly closed containers [4]. It was prepared in AMU, department of Ilmul advia laboratory.

2.2 Identification

Mix 5 gm of sample in 50 ml of water by applying heat till the sample got completely dispersed in water. Centrifuge the mixture and decant supernatant, wash the sediment with distilled water, centrifuge and decant again. Take a few mg of sediment and mount it in glycerine. Then take a few mg out of it in a watch glass and add few drops of phloroglucinol and concentrated HCl, mount in glycerine to locate lignified cells. Observe the following characters in different mounts [5].

2.3 Physicochemical evaluation

2.3.1 Organoleptic evaluation:[7]

1. **Appearance**
   Appearance of four samples was recorded according to the consistency whether semisolid, semiliquid etc.
2. **Determination of color**
   The color of the drug formulation was noted by naked eye. Colour of four samples were noted.
3. **Determination of odor**
   The description of this feature sometimes may not be accurate because it depends on individual perception. So, to get accuracy of results various samples were examined by three different individuals. The strength of the odor like weak, distinct, strong is first determined and then the odor sensations were determined.
4. **Determination of taste**
   First of all, the depth of organoleptic capacity should be tested. This can be done by asking the volunteer to taste serial dilutions of drugs. It should be noted that the volunteers do not taste in ordinary sense. In so doing they would have to score the degree of flavouring, e.g., was it less than present originally, i.e., was the flavour being lost? They would also have to be able to describe the flavour well originally.
5. **Physico-chemical evaluation:**
   The present study was undertaken to standardise the *Tiryaq-e-Arba*, prepared in our laboratory. The chemical studies include the determination of extractive values in different solvents, specific gravity, alcohol and water-soluble content, moisture content, ash value and pH value.

1. Determination of extractive values:

The extractive values of *Tiryaq-e-Arba* in different solvents petroleum ether, benzene, chloroform, alcohol and water, were carried out by percolation in soxhlet's apparatus. The heat was applied for six hours on a water bath for each solvent except water which was heated directly on a heating mantle. Fresh sample of *Tiryaq-e-Arba* (10 gm) was taken for each solvent (200 ml) and subjected to extraction. The extracts were filtered and after evaporation of the solvents, the extractive value was determined with reference to the weight of *Tiryaq-e-Arba* subjected to percolation. The procedure was repeated four times and the mean value for each extract was calculated [8]. (Anonymous 1968).

2. Determination of specific gravity:

A tarred beaker of known volume (10 ml.) was carefully filled with *Tiryaq-e-Arba* and weighed. The weight of tarred beaker from combined weight of beaker and *Tiryaq-e-Arba*. The weight of an equal volume of distilled water was similarly determined by taking the water into the said beaker and subtracting the weight of beaker from total weight of beaker and water. This procedure was done for five times. The specific gravity was determined by dividing the weight of *Tiryaq-e-Arba* by the weight of distilled water.[9]
Formula:

$$\text{Spec. Gravity} = \frac{\text{Weight of } \textit{Tiryaq-e-Arba}}{\text{Weight of an equal volume of distilled water}}$$

3. Determination of water and alcohol soluble content:

5 gm. of *Tiryaq-e-Arba* was taken into 100 ml of distilled water in separating funnel. The mixture was carefully shaken and then filtered in conical flask of known weight. The residue was further taken in separating funnel containing 100 ml of distilled water and the mixture was vigorously shaken to make sure that no soluble matter left behind and then filtered in the same conical flask. The water was evaporated to dryness. The residue left after evaporation was weighed. The percentage of soluble matter was calculated with reference to the amount of *Tiryaq-e-Arba* taken. The percentage of alcohol soluble content was determined as above by using alcohol in place of water.[9]

4. Determination of moisture content:

Toluene distillation method (Jenkins, *et al*, 1957) was used for the determination of moisture content. The apparatus employed consists of a flask of 250 ml capacity connected by means of a tube receiver with a straight condenser. The tube is graduated in tenths of ml. The apparatus was carefully and thoroughly washed and well dried before use. 10 gm of *Tiryaq-e-Arba* was taken in the flask of apparatus of 75 ml of the distilled toluene was added to it. Distillation was carried out for six hours and five times. The volume of water collected in the receiver-tube, graduated in ml was noted and the percentage of the moisture content was determined with reference to the weight of the *Tiryaq-e-Arba* taken.[8,9]

5. Determination of total ash:

2 gm. of *Tiryaq-e-Arba* was taken in a silica crucible previously ignited and weighed to a constant weight. The Tiryaq-e-*Arba* was incinerated at a temperature between 450 C to 500 C until free from carbon. The heating was done until the charred mass after cooling gave a constant weight. The quantity of the ash was determined by subtracting the weight of crucible from the weight of the crucible and ash (Anonymous, 1968) [8, 9].

6. Determination of pH

1 gm. of *Tiryaq-e-Arba* was dissolved in 2 ml of distilled water. The pH was determined by Ph of 1% aquous solution and Ph of 10% aquous solution.[9]

7. Loss of weight on drying at 105°C:
Loss of weight on drying at 105°C is a method to measure the loss in mass of the sample. This is done to determine the amount of water or volatile matter in the sample, which is removed during drying.
Two gm of drug should be taken, spread uniformly and thinly in a shallow petridish. It should be heated at a regulated temperature of 105°C, cooled in a desiccator and weighed. The process should be repeated many times till two consecutive weights were constant. The percent loss in weight should be calculated with respect to initial weight [10].

8. Ash values
Ash value is an essential parameter for detection of impurities and adulteration. It represents the inorganic salts naturally occurring or adhering to the drug or added for the purpose of adulteration.
Total ash: About 2 gm of dried powdered drug should be incinerated in a silica dish at a temperature not exceeding 450°C until free from carbon, cooled and weighed and the percentage should be calculated with reference to air dried drug.
Acid insoluble ash: Total ash should be boiled with 25 ml of dilute hydrochloric acid for 5 minutes. The insoluble matter should be collected on an ash less filter paper, washed with hot water, ignited at a temperature not exceeding 450°C, and weighed after cooling. The percentage of acid insoluble ash should be calculated with reference to the air-dried drug.
Water soluble ash: Total ash should be boiled with 25 ml of distilled water for 5 minutes. The insoluble matter was collected on an ash less filter paper, washed with hot water and ignited. The weight of insoluble ash was subtracted from the weight of the total ash, giving the weight of the water-soluble ash. The percentage of water-soluble ash was calculated with reference to air-dried drug.[10]

9. Determination of water and alcohol soluble matter by cold maceration
The amount of drug soluble in a given solvent is an index of its purity. Water and alcohol soluble matter is the amount of extract that a drug yields to a given solvent which is an approximate measure of the number of constituents that the drug contains.
Accurately weighed 4 gm. of drug should be placed in a glass stoppered conical flask. Macerated with 100 ml of water for six hours shaking frequently, and then allowed standing for 18 h, then shaked well and filtered rapidly through dry filter. About 25 ml of the filtrate should be transferred to a previously weighed and tarred flat-bottomed dish and evaporated to dryness on a water bath, then dried at 105°C for six hours, cooled in a desiccator for 30 minutes and weighed without delay. The percentage of water-soluble matter should be calculated with reference to the amount of drug taken. The percentage of alcohol soluble content should be determined as above by using alcohol in place of water. [11]

10. Determination of aqueous extractive value
4gm of drug material was accurately weighed, in a glass-stoppered conical flask. Macerated with 100 ml of the water for 6 hours, shaking frequently, was then allowed to stand for 18 hours. Then rapidly filtered taking care not to lose any solvent and then 25 ml of the filtrate was transferred to a tarred flat-bottomed dish and evaporated to dryness on a water-bath, then dried at 105°C for 6 hours, cooled in a desiccator for 30 minutes and was weighed without delay. The content of extractable matterwas calculated in mg/ gm of material [13].

11. Determination of alcohol extractive value
Four gm of drug material, accurately weighed, was placed in a glass-stoppered conical flask. Macerated with 100ml of the Ethanol for 6 hours, by shaking frequently, and then was allowed to stand for 18 hours. Then rapidly filtered taking care not to lose any solvent then 25 ml of filtrate was transferred to a tarred flat-bottomed dish and evaporated to dryness on a water-bath. Dried at 105°C for six hours, cooled in a desiccator for 30 minutes and weighed without delay. The content of extractable matter in mg / gm of material was calculated [12].

12. Estimation of total alkaloids
Five gm of the drug was taken in a 250 ml beaker and 200 ml of 10% acetic acid in ethanol was added to the sample. The mixture was covered and allowed to stand for four hours. The mixture was then filtered and the extract was allowed to become concentrated in a water bath until it reached 1/4th of the original volume. Concentrated ammonium hydroxide was added until the precipitation was completed. The whole solution was allowed to settle and the precipitate was collected and washed with dilute ammonium hydroxide and then filtered. The residue, so obtained was alkaloid, which was dried and weighed [13].

13. Thin layer chromatography
Thin layer chromatography (TLC) is one of the important parameters used for detecting the adulteration for judging the quality of the drugs. The Rf values of the spots were calculated by the following formula.
Rf value =Distance travelled by the spot Distance travelled by the solvent
Extract two gm of sample with 20 ml of petroleum ether at 60-80^{0}C by refluxing on water bath for 30 minutes. Filter and concentrate to five ml and carry out thin layer chromatography. Apply the petroleum ether extract on TLC plate [14].

1. Microbial evaluation
In the present study microbial analysis was done for total bacterial count, total fungal count and presence of specific pathogenic viz; *E. coli, Salmonella*, *Staphylococcus aureus, Pseudomonas aeruginosa* samples of test drug formulation [15].

Result and Observations
1. Organoleptic evaluation
Appearance: Semi-solid
Colour: Dark Brown
Smell: Characteristic
Taste: Sweetish Bitter
2. Identification (Microscopical)
Lignified, pitted stone cells of endocarp (40-65 micron wide) and tannin filled mesocarp cells of the fruitsare found in large number that shows presence of *Habbul Ghar (Laurus nobilis* Linn). Corrkceels filled with tannin, few

lignified oval stone cells, abundant starch grains (5-15 micron) size single as well as in groups and few xylem vessels with spiral thickening from roots also seen in *Juntiana* (*Gentiana Lutea* Linn). Thick walled parenchymatous cells often containing needles of calcium oxalate from 3-10 micron long with some oil droplets of root are most abundant in feature, large vessels with reticulate thickening also seen frequently in *Juntiana* (*Gentiana Lutea* Linn).

3. Physico-chemical evaluation

Table 1

S.No.	Petroleum Ether	Chloroform	Benzene	Alcohol	Water
1.	17.25	0.94	14.23	61.82	57.06
2.	15.82	1.13	15.67	51.70	51.59
3.	15.12	0.92	14.94	44.52	54.79
4.	14.58	0.51	19.58	49.08	65.92
Total	62.77	3.6	64.42	207.13	229.36
Mean	15.69	0.9	16.105	51.78	57.34
SD+-	1.0	0.186	2.068	6.339	5.321
SE+-	0.5	0.093	1.034	3.169	2.66

Table 2: Percentage of extracts of *Tiryaq-e-Arba* in different organic solvents.

S.No.	Specific gravity	Bulk density
1.	1.43	1.30
2.	1.49	1.32
3.	1.45	1.30
4.	1.43	1.28
5.	1.43	1.28
Total	7.23	6.48
Mean	1.44	1.296
SD +-	0.022	0.01673
SE+-	0.011	0.00836

Table 3: Specific gravity and Bulk density of *Tiryaq-e-Arba*

S. No.	Water Soluble content (%)	Alcohol Soluble content (%)
1.	86.060	23.960
2.	74.040	23.900
3.	87.500	25.200
4.	82.200	28.000
Total	329.8	101.06
Mean	82.45	25.265
SD +-	5.227	1.662
SE+-	2.613	0.831

Table 4: Percentage of water soluble and alcohol soluble content of the *Tiryaq-e-Arba* at 25^0 C

S.No.	Moisture (%)
1.	15
2.	17
3.	15
4.	20
5.	15
Total	82
Mean	16.4
SD +-	1.95
SE+-	0.874

Table 5: Percentage of moisture content of the *Tiryaq-e-Arba* at 25^0 C

S.No.	Ash Value (%)	Acid insoluble ash (% *w/w*)	Water soluble ash (%*w/w*)
1.	2.90	0.78	0.23
2.	2.85	0.75	0.21
3.	3.25	0.76	0.25
4.	3.00	0.77	0.20
Total	12.00	3.06	0.89
Mean	3.00	0.765	0.22249
SD+-	0.154	0.0129	0.02217
SE+-	0.077	0.00645	0.011085

Table 6: Percentage yield of the ash values of the *Tiryaq-e-Arba* at 25^0 C

S.No.	Ph (1% aquous Solution)	Ph (10% aquous Solution)
1.	5.00	4.20
2.	5.30	4.40
3.	5.50	4.60
4.	5.20	4.30
Total	21.00	17.50
Mean	5.250	4.375
SD +-	0.20816	0.17078
SE+-	0.10408	0.08539

Table 7: Estimation of Ph of 1% and 10% aquous solution of the *Tiryaq-e-Arba* at 25^0 C

S. No.	Reducing sugars (%)	Alkaloids (%)	Total phenols (%)	Volatile oils (%)
1.	46.84	0.12	0.35	1.00
2.	47.57	0.10	0.40	0.98
3.	45.28	0.13	0.32	0.96
4.	46.00	0.13	0.34	0.98
Total	185.69	0.48	1.41	3.92
Mean	46.42	0.12	0.3525	0.98
SD +-	0.9958	0.01414	0.03403	0.01632
SE+-	0.4979	0.00707	0.017015	0.00816

Table 8: Estimation of phytochemical constituents of the *Tiryaq-e-Arba*

S.No.	Calcium (mg/gm of ash)	Iron (mg/gm of ash)	Copper (mg/gm of ash)
1.	29.30	0.081	0.058
2.	28.66	0.067	0.052
3.	27.41	0.093	0.060
4.	30.82	0.080	0.056
Total	116.19	0.321	0.226
Mean	29.0475	0.0802	0.0565
SD +-	1.41857	0.01062	0.00341
SE+-	0.709285	0.00531	0.001705

Table 9: Estimation of important minerals of medical importance of the *Tiryaq-e-Arba*

S.no	Metal	Value	Limit / Inference
1	Lead	0.01 mg/gm of ash	Within limit
2	Mercury	Absent	Within limit
3	Cadmium	Absent	Within limit
4	Arsenic	Absent	Within limit

Table 10: Estimation of heavy metals in *Tiryaq-e-Arba*
Organic and inorganic constituents estimation

Organic constituents viz. alkaloid, phenols were quantitatively estimated. The results are Alkaloids: 0.12%, Total phenols: 0.35%, Reducing sugars: 46.84 %, Calcium: 29.3 mg/gm of ash, Iron: 0.081 mg/gm of ash, Copper: 0.058 mg/gm of ash. Heavy metal estimation as is the important part of Standardisation so there are estimated and found as Lead value was 0.01 mg/gm of ash. While Mercury and Cadmium were absent. (Table 10).

Thin layer chromatography:
Petroleum ether extract on precoated silica gel G plate using mobile phase in the combination of petroleum ether: Diethyl ether (8:2). It showed three spots under UV light at 254 nm and at Rf 0.10 (blue), 0.17 (blue) and 0.19 (blue).

Table 11: Rf values of *Tiryaq-e-Arba* under UV light at 254 nm

Solvent system	Rf values under UV light at 254 nm
petroleum ether: Diethyl ether (8:2)	0.10 (blue), 0.17 (blue) 0.19 (blue)

Table 12: Microbial load of *Tiryaq-e-Arba*

S. no.	Parameter Analysed	Results	WHO Limit
1.	Total bacterial count	12000 cfu/ gm	10^5 CFU /gm
2.	Total fungal count	350 cfu/ gm	10^3 CFU /gm
3.	Enterobacteriaceae	Absent / gm	10^3 CFU /gm
4.	Salmonella	Absent/ gm	Nil
5.	Staphylococcus aureus	Absent/ gm	Nil
6.	E.coli	Absent/ gm	Nil

Discussion
The compound drug *Tiryaq-e-Arba* was prepared according to the method prescribed in National Formulary of Unani Medicine Part- I for its chemical standardisation.[4] Standardisation performed from the organoleptic evaluation, physico-chemical studies, Estimation for organic and inorganic constituents, Heavy metal toxicity, Microbiological evaluation. Prepared *TiryaqeArba* sample was evaluated for Organoleptic evaluation e.g., color, odor, taste and found in confirmity. Physico-chemical parameters evaluated and the mean values of three samples with standard error are discussed here [14] The extractive values of the drug, *Tiryaq-e-Arba* were determined by successive extraction with different organic solvents, viz. petroleum ether, benzene, chloroform, alcohol and water using a soxhlet's apparatus. The percentage were found to be 15.69+-0.5 in petroleum ether, 16.105+-1.034 in benzene, 0.9+-0.093 in chloroform, 51.78+-3.169 in alcohol and 57.34+-2.66 in distilled water.The specific gravity of *Tiryaq-e-Arba* was determined and found to be 1.44+-0.01. The percentage yield of water soluble content of drug, *Tiryaq-e-Arba* at 25^0 C was found to be 82.45+- 2.613. The pH of 1% and pH of 10% solution was 5.250 and 4.375 respectively. Bulk density was 1.296+-0.00836 and specific gravity was 1.44+-0.011. The alcohol soluble content and water soluble content of *Tiryaq-e-Arba* was determined and found to be 25.265+-0.831and 82.45+-2.613 respectively at 25^0 C. The percentage of moisture content present in the drug, *Tiryaq-e-Arba* was determined and found to be 16.4+-0.874.The percentage of total ash, acid insoluble ash, water soluble ash were 3.00+-0.077, 0.76+-0.00645 and0.21 +-0.011085 respectively.Estimation for organic constituents viz. alkaloid, reducing sugars, phenols, volatile oils was done 0.12+-0.007, 46.42+-0.4979, 0.3525+-0.01701, 0.98+-0.00816respectively. Inorganic constituents viz. calcium, iron, copper were evaluated and the values are 29.0475+-0.709285,0.0802+-0.001705, 0.0565+-0.00531 respectively. Heavy metal estimation as is the important part of Standardisation so they are evaluated for lead which was found 0.01 mg/gm of ash while mercury, arsenic, cadmium were absent.
Tiryaq-e-Arba was evaluated for total bacterial and found 12000 cfu/ gm, total fungal count was 350 cfu/ gm and absence of specific pathogens i.e. *Escherichia coli, Salmonella spp., Pseudomonas aeruginosa and Staphylococcus aureus.* Results were within limits set by WHO [16].

Conclusion
Tiryaq-e-Arba is an important formulation of Unani system of Medine but before this study, Standardization of this formulation has not been carried out. By this study we have develop standards for various parameters which can be used for reference for future studies. It has provided information and controls, necessary to produce material of reasonable consistency. This study is not only helpful for the reseachers, analysts, scientists but also strenghthen the Unani System.

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