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RESEARCH ARTICLE

STANDARDIZATION OF MARKETED HERBAL PRODUCT KAWACH BEEJ CHURNA

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ABSTRACT

Herbal medicines are the most ancient form of health care known to human beings. It consists of plant or its part to treat injuries, disease or illnesses and is used to prevent and treat diseases and ailments or to promote health and healing. It is a drug or preparation made from a plant or plants and used for any of such purposes. Now a day, approximately 70% of "synthetic" medicines are derived from plants. Organoleptic evaluation revealed that Kawach churna MK-3 was observed to be whitish brown in color. Results of Extractive value demonstrated that water soluble extractive value and alcohol soluble extractive value of product MK-3 was lesser as compared to products MK-1, MK-2 and LB-1. Phytochemical studies exhibited presence of Alkaloids, Steroids, Saponins, and Tannins in all the four products. Ash value determination revealed that the acid insoluble ash of all the products was within range < 1%, but acid insoluble ash of product MK-3 was more as compared to products MK-1, MK-2 and LB-1, which indicates presence of sand or earth material in product MK-3. Moisture content of all the products were within acceptable ranges < 5 % w/w. But as product MK-3 contained more moisture it is more prone to microbial contamination and enzymatic degradation. When all the four products were subjected for Microbial Limit test, all the compounds demonstrated growth of non-pathogenic micro-organisms but growth of the pathogenic micro-organisms was not observed. Product MK-2 may be better than products MK-1 and MK-3 in view of safety and efficacy, as it contains higher quantity of active markers. TLC showed yellow spot having Rf value 0.73 in n-butanol: Glacial Acetic Acid: Water (8:2:2) (v/v/v) solvent system. UV and IR spectra of isolated and standard marker compound (Levodopa) showed similar results.

INTRODUCTION

Medicinal plants are the nature's gift to human being to make disease free healthy life. It plays a vital role to preserve our health. Plants and their products are being used as a source of medicine since long. They provide basic compounds affording less toxic and more effective drug molecules Popularity among the common people increased the usage of medicinal plants/herbal products [(1)]. World Health Organization (WHO) has defined herbal medicines as finished, labeled medicinal products that contain active ingredients, aerial or underground parts of the plant or other plant material or combinations. World Health Organization has set specific quidelines for the assessment of the safety, efficacy, and quality of herbal medicines [(4)]. In drug industry, herbs are used for following reasons [(1)]: Source of phyto-pharmaceuticals: To isolate pure compounds. Herbal raw materials: To formulate herbal, Ayurvedic, Homeopathic and Unani medicines. Medicinal herbs: To prepare plant extracts and single ingredient powders. Dietary supplements: Sources of vitamins and enzymes. The emergent use of herbal drug by the human is forcing moves to evaluate the health claims of these agents and to develop standards of quality, purity, safety and efficacy of the drug. The mostly herbal drugs are effective but lack of standardization. So there is need to develop standardization parameters.

Herbal medicines make up an important part of the trend toward alternative medicine.

In many countries herbal drugs are lunched into the market without standard scientific evaluation.

Herbal Drugs [2, 3] Traditionally, herbal drugs have played a significant role in the management of both minor and major medical illnesses. Herbal drugs means a ***"dosage form consisting of one or more herbs or processed herb in a specified quantities to provide specific nutritional, benefits, and/or other benefit meant for use to diagnosis treat, mitigate diseases of human*** *beings or animal and/or alter the structure or physiology of human beings or animals"*. Herbal drugs are obtained by whole plant, cut plant part, powdered plant part, extract, essential oil, expressed juices and plant parts to treatment such as distillation, extraction, expression, fractionation, purification and concentration. **Herbal medicinal product** is any medicinal product, exclusively containing as active substances one or more herbal substances or one or more herbal preparations, or one or more such herbal substances in combination with one or more such herbal preparations. **Herbal substances** are mainly whole, fragmented or cut plants, plant parts, algae, fungi and lichen in an unprocessed, usually dried form but sometimes fresh. Certain exudates that have not been subjected to a specific treatment are also considered to be herbal

1.2 Advantages of Herbal Drugs [4]

•They have low risk of side effects.
•They have more effective than any synthetic drug.
•They have lower cost.
•They have large amount of availability.
•They have better patient tolerance as well as acceptance.
•They have large therapeutic activity.
•The medicinal plants have renewable source of cheaper medicines.
•Many natural medicines are being shown to produce better results than drugs or surgery without the side effects.
•They have a strong traditional or conceptual base and the potential to be useful as drugs in terms of safety and effectiveness whereas modern medicines have a very strong experimental basis for their use but are potentially toxic.
•They can deliver mixtures of multi-functional molecules with potentiating and synergistic effects.
•They are well suited for long-term disease prevention in an era of genetic testing and increased life expectancy.
•Herbal products have better compatibility with the human body.
•A prolong therapy dose not showed any side effect by use of most of the herbal products.
•Herbal products have long history of use and better patient tolerance as well as acceptance.
•The cultivation and processing of medicinal herbs and herbal products is environmental friendly.
•Medicines are available in both crude as well as a pure chemical form.
•They also provide additional vehicles for delivering health and wellness.

1.3 Standardization

1.3.1General Introduction:

Traditional medicine implies knowledge and practice of herbal therapy for the prevention, diagnosis and elimination of physical, mental, or social imbalance. The costs for health care are rising at an alarming rate throughout the world. At the same time, the world market for phytopharmaceuticals is growing steadily. Standardization involves adjusting the herbal drug preparation to a defined content of a constituent or a group of substances with known therapeutic activity by adding excipients or by mixing herbal drugs or herbal drug preparations[5]. Standardization of herbal drug is necessary to confirm its identity and to determine its quality, purity, safety, content, physical, chemical, biological properties. But the most important challenges faced by these herbal drugs are the lack of complete standardization by physiochemical. The standardization of herbal drugs,

moisture content, ash values, extractive values, volatile oil content and fluorescence study were carried out. Chromatography study, heavy metalcontent, microbial contamination and pesticide residues were also carried out to assure the quality, purity and safety of herbal drugs.

Standardization is the process by which one or more active ingredients of an herb are identified, and all batches of the herb produced by a single manufacturer contain the same amount of active ingredient. Consumers expect the component ingredients of their nonprescription and prescription drug products to be standardized to ensure that each dose contains the requisite amount to elicit the desired effect. However, consumers either may not expect the same level of standardization for herbal products, or they assume they are standardized. One reason they may not expect the same level of product quality may be the commonly held perception by consumers that "natural is always good," and Phytochemical standardization [(11)]:

Methods of standardization should take into consideration all aspects that contribute to the quality of the herbal drugs, namely correct identity of the sample, Organoleptic evaluation, pharmacognostic evaluation, volatile matter, quantitative evaluation phytochemical evaluation, test for the presence of xenobiotics, microbial load testing, toxicity testing, and biological activity. Of these, the phytochemical profile is of special significance since it has a direct bearing on the activity of the herbal drugs. The fingerprint profiles serve as guideline to the phytochemical profile of the drug in ensuring the quality, while quantification of the marker compounds would serve as an additional parameter in assessing the quality of the sample. Phytochemical standardization encompasses all possible information generated with regard to the chemical constituents present in an herbal drug.

1. Preliminary testing for the presence of different chemical groups.
2. Establishment of fingerprint profiles.
3. Quantification of important chemical constituents.

1.4 CONCEPT OF MARKERS [(5)]

The purpose of quality control is to ensure that each dosage unit of the drug product delivers the same amount of active ingredients and is, as far as possible, free of impurities. As herbal medicinal products are
complex mixtures which originate from biological sources, great efforts are necessary to guarantee a constant and adequate quality. By carefully selecting the plant material and a standardized manufacturing process the pattern and concentration of constituents of herbal medicinal products should be kept as constant as possible as this is a prerequisite for reproducible therapeutic results. Selection of chemical markers is crucial for the quality control of herbal medicines.

Fig.no1-Kawach (Mucuna pruriens) plant

Fig.no.2- Dried seeds of Kawach

Scientific classification-
Kingdom: Plantae.
Division: Magnoliophyta.
Class: Magnoliopsida
Order: Fabales
Family: Fabaceae

Subfamily: Faboideae.
Tribe: Phaseoleae
Genus: Mucuna.
Species: M. pruriens.
Botanical Name: Mucuna pruriens
Other Names: Mucuna pruriens, Velvet Bean, Cowhage, Cow-itch, Buffalo bean, Kaunch, Atmagupta, Kapikachhu. Mucuna pruriens or Kaunch is one of the popular medicinal plant of India. It is widespread over most of the subcontinent and is found in bushes and hedges and dry-deciduous, low forests throughout the plains of India. parts of Mucuna posses valuable medicinal properties and there is a heavy demand of Mucuna in markets. The main constituent of Mucuna pruriens seeds is L-dopa or Levadopa which acts as a natural dopamine. The seeds of Mucuna pruriens are high in protein (20-29 %), lipids (6-7%), dietary fiber (8-10%), ash (3%), carbohydrates (50-60%) and minerals. Also they are extremely rich in alkaloids, saponins, and sterols.
Main use of the plant are following:
•It contain L-Dopa which is used as anti-Parkinson's, help increases testosterone
•Aphrodisiac - increases libido and it is very good for impotency and erectile dysfunction
•It is used to stimulate growth of hormone - anabolic/androgenic •It help reduces spasms and relieves pain - analgesic/ antispasmodic
•Help lowers blood sugar and blood pressure (hypo tensive)
•It increases urination (uterine stimulant), help reduces fever (febrifuge) and it is a good cough suppressant
•It help lowers cholesterol (hypocholesterolemic)
•it act as a weight loss aid
•It reduces inflammation - anti-inflammatory

3.2 OBJECTIVES:

Mucuna is an annual twinning plant. Leaves are trifoliate, gray-silky beneath; petioles are
long and silky, 6.3-11.3 cm. Leaflets are membranous, terminal leaflets are smaller, lateral
very unequal sided. Dark purple flowers (6 to 30) occur in drooping racemes. Fruits are
curved, 4-6 seeded. The longitudinally ribbed pod, is densely covered with persistent pale brown or grey trichomes that cause irritating blisters. Seeds are black ovoid and 12 mm long. Mucuna seeds (as well as the seeds of all Mucuna species) contain high concentration of L-dopa (7-10%) - a direct precursor of the neurotransmitter dopamine. The hairs of the seed pots contain the phytochemical mucunain, the very constituent that irritates the skin when in contact. The serotonin is also has been found in the pod (as well as in the leafandfruit).

Other important Mucuna properties:
• It help prevents or eliminates kidney stones (antilithic)
•It help kills parasites (antiparasitic) and expels worms (vermifuge)
•It expels gas (carminative)
•It is a menstrual stimulant
•It is a central nervous system stimulant

Benefits of Mucuna pruriens in nervous system disorder:
It is used as a nervine tonic for nervous system disorders. Because of the high
concentration of L-dopa in the seeds. It has been studied for its possible use in Parkinson's disease. It was found to slow the progression of Parkinson's symptoms (such as tremors, rigidity, slurring, drooling, and imbalance), and to have none of the side-effects of the current pharmaceutical L-dopa.

The main objectives of the studies are:

Evaluation of procured product as per the various standardization parameters, which
includes
1. To carry out organoleptic parameters.
2. To carry out physico-chemical parameters.
a) To determine solvent extractive values: includes water, alcohol soluble extractives.
b) To determine ash values: includes total ash, acid insoluble ash and water soluble ash.
c) To determine the moisture content.
d) To carry out microbial limit tests.
e) To carry out phytochemical tests.
3. Extraction and isolation of the marker compound from marketed herbal medicine.
4. To compare isolated marker compound with pure substances (marker compounds)
using chromatographic and spectroscopic methods.

4. EXPERIMENTAL WORK

4.1. Organoleptic evaluation [6]:

Colour, Odour and Taste - All the four samples were analyzed for their morphological characteristics. All the samples were visually analyzed; a small portion of the drug was taken, slowly and repeatedly inhaled the air over the material and examined
the odour. A small portion of drug was taken on the tongue and the taste of formulation was found.

4.2. Determination of solvent extractive values [7]:

Solvent extractive value is the amount of active constituent in a specified weight of
medicinal plant material when extracted with specific solvent. The extraction of any crude drug with a particular solvent yields a solution containing different phytoconstituents. The composition of these phytoconstituents in that particular solvent depends upon the nature of the drug and solvent used. The solvent extractive value was determined by following methods to measure the water soluble extractive value and alcohol soluble extractive value using water and ethanol as solvent for extraction respectively.

4.2.1. Determination of water soluble extractives:

5 g of churna of each manufacturer were weighed and taken into the four conical flasks in which 100 ml of water were added separately and allowed to macerate for 24 h, shaking frequently for first 6 h then allowed standing for 18 h. Which was then filtered rapidly and the 25 ml of the filtrate was allowed to evaporate to dryness in a tared 250 ml beaker at 105^0C, the difference in weight of the beaker is indication of water soluble extractive value of that drug with respect to the amount of drug (5 g) taken for extraction.

4.2.2. Determination of alcohol soluble extractive value:

5 g of churna of each manufacturer were weighed and taken into the conical flask in which 100ml of ethanol were added separately and allowed to macerate for 24 hours, shaking frequently for first 6 hours, then allowing to stand for next 18 hours, which was then filtered rapidly and 25ml of the filtrate was allowed to evaporate to dryness in a tared 250ml beaker at105^0C. The difference in weight of the beaker is an indication of alcohol soluble extractive value of that drug with respect to the amount of drug (5 g) taken for extraction.

4.3 Determination of ash values [7]:

The ash of any organic material is composed of their non-volatile inorganic components. Controlled incineration of crude drug results in an

ash residue consisting of an inorganic material (metallic salts and silica) in certain drugs, the percent variation of the weight of ash from sample to sample is very small and any marked difference indicates a change in quality. The ash value can be determined by three different methods to measure the total ash, the acid insoluble ash and the water soluble ash.

4.3.1. Determination of total ash content:

The total ash was determined by placing 2 g of accurately powdered material into the tared crucible. Then samples were placed in muffle furnace and the samples were ignited by gradually increasing the heat up to 500-600^{0}C until it is white, indicating the absence of carbon. The crucible was kept in desiccator and weighed. Initially carbon free ash was not obtained thus the cooled crucible was removed and the residue was moistened with about 2ml of water, dried on water bath, and ignited to constant weight. The residue was allowed to cool in desiccator for 30 minutes. Content of total ash was calculated with reference to the amount of sample taken.

4.3.2. Determination of acid insoluble ash value:

The total ash which was obtained in the previous step was boiled with 25ml of 2 M HCl for 5 minutes, then it was filtered through ash-less filter paper, the insoluble matter was collected in a previously tared crucible, then the residue was washed with hot water, then ignited in muffle furnace at about 450^{0}C for 30 minutes, cooled in desiccator and weighed. The percent of acid insoluble ash was calculated with reference to the amount of sample taken.

4.3.3. Determination of water soluble ash:

In the crucible containing total ash, 25ml of water was added and boiled for 5 minutes. The insoluble matter was collected on an ash less filter paper. This was then washed with hot water and ignited in a crucible for 15 minutes at a temperature not exceeding 450^{0}C separately. Then the weight of this residue was subtracted from the weight of total ash. Finally the content of water soluble ash with reference to the amount of sample taken was calculated.

4.4. Determination of moisture content [8]:

Moisture is an inevitable component of crude drug, which must be eliminated as far as practicable. Drying of crude drug is important during collection of drug and is also important for preservation, preventing hydrolytic degradation of active constituents and for easy size reduction of crude drug. Excess moisture or insufficient drying is responsible for spoilage of drug due to growth of microbes. There for drying process should reduce the moisture content of drug below the critical level. Moisture content determination is use to check the total water content in given weight of crude drug. Excess moisture content in a crude drug sample suggests not only that the purchaser could be paying a high price due to unwanted water, but also that the drug has been incorrectly prepared or subsequently incorrectly stored. Method of determination of moisture content includes the loss on drying. It can be carried out either by heating at 100^0-105^{0}C or in a desiccator over phosphorus pentoxide under atmospheric pressure at room temperature for specific period of time.

Method: - Loss on drying, Azeotropic distillation method and Karl fisher

method use for moisture content determination in crude drug.

- **Loss on drying:**

Loss on drying is the loss of mass expressed as percent w/w. To estimate the loss on drying 2-5 g of air dried drug is accurately weighed in dried and tared flat weighing bottle. The substance is dried to a constant mass or for the prescribed time as specified. 5 g of sample was accurately weighed in separate dried and tared petridish. Sample was placed in an oven, and drying of sample was carried out at 105^0C until a constant mass of the sample was not observed. The difference in weight before and after heating in oven was recorded and loss on drying was expressed as percent w/w.

4.5 Determination of physical parameters

4.5.1 Determinations of angle of repose and flow rate :[8] -

Good flow properties are critical for the development of any pharmaceutical product including powder formulations. It is essential that an accurate assessment of flow porters to be made as early in the development process so that an optimum formulation can be quickly identified. Interparticle forces between particles as well as flow characteristics of powders are evaluated by angle of repose.

Angle of repose is defined as the maximum angle possible between the surface of pile of sample and horizontal plane. Angle of repose is affected by presence of moisture, size and shape of sample, percent fines, and amount of glidant and lubricant. More the moisture in sample, more it becomes sticky. Similarly smaller the size more is the flow of powder. Angle of repose is a helpful parameter to monitor quality of powdered of granular harmaceutical formulations. For good flowing powders angle of repose should be less than 30^0.

Method: -

1. Take a clean and dry funnel with a round stem of 20 to 30 mm diameter with flat tip and attached it to the burette stand.
2. Place a graph paper sheet below the funnel, on clean and dry platform.
3. Adjust the distance between lower tip of the funnel and sheet to some specified height.
4. Gentles pour sample in funnel from top till a heap of powder forms and touches of lower tip of the funnel.
5. Repeat the procedure four times to obtain average reading.
6. Find out average diameter and radium of the each drawn circle.

Angle of repose Calculation

1. Radius determination - r = diameter of heap (d) / height of heap (h)
2. Average radius determination – r = r1 + r1 + r1 + r1/4
3. Angle of repose (θ) - tan-1 (h/r)
4. Flow rate calculation = mass of powder / time required for flow = W2/t (gm/sec)

4.5.2 Determination of Bulk and Tap Density and Compressibility Index [9]

Requirement: - Graduated measuring cylinder and bulk density apparatus and weighing balance.

Method: -

1. Weigh accurately 25 g of powder (W1).
2. Place it in dried graduated measuring cylinder and note the volume as V1 ml.
3. Place the cylinder containing sample in bulk density apparatus for 100 tapping and operate it. Record the volume occupied by the powder as V2 ml.

Bulk and tap density Calculation

1. Bulk density = Mass / Bulk volume = W1 / V1 (g/ml)
2. Tap density = Mass / Tap volume = W1 / V2 (g/ml)
3. Compressibility index = tap density - Bulk density / tap density

4.6. Microbial limit tests [3, 4]:

4.6.1 Preliminary testing:

The methods given are invalid unless it is demonstrated that the test specimens to which they are applied do not, of themselves, inhibit the multiplication under the test condition of microorganisms that can be present.

Preparation of sample for preliminary testing:

1gm of sample was taken to prepare 50ml of fluid lactose medium. But this medium was not suitable for sufficient growth of *Escherichia coli* and *Pseudomonas aeruginosa*. Hence the relevant medium was prepared by taking 1gm of sample to prepare 100ml of fluid lactose medium. Prior to doing the test, diluted specimen of substance being examined was inoculated with separate viable cultures of *Escherichia coli, Salmonella species, Pseudomonas aeruginosa* and *Staphylococcus aureus.* This was done by adding 1ml of 24 hours cultures of the microorganisms to the first dilution (in buffer solution pH 7.2, fluid lactose medium) of the test material.

Initially *Escherichia coli* and *Pseudomonas aeruginosa* organisms failed to grow in the relevant medium. The procedure was modified by increasing the volume of diluents with the quantity of test material remaining the same, i.e. 1gm of sample was taken to prepare 100 ml of fluid lactose medium. After this modification growth of the strains was observed.

4.6.2 Tests for specified micro-organisms:

A. Test for E. coli:

1 g of sample was added to the MacConkey broth and incubated at 43-45^{0}C for 24 hours. A subculture with the same media was made and incubated at the same temperature for the same time. Growth of red, generally no mucoid colonies of gram negative rods, sometime surrounded by a reddish zone of precipitation, indicates the possible presence of *E. coli.* Therefore, the material will pass the test if no such colonies are grown.

B. ***Test for S. typhi:***

The solution of the pretreated sample was prepared and incubated as above for 24 hours. 10ml of the enrichment medium was transferred to 100ml of tetrathionate bile brilliant green broth and incubated at 42-43^{0}C for 24 hours. Subculture was prepared by streaking method, with the agar media like, Xylose lysine agar. Incubated at 35-37^{0}C for 24 hours and look out for well-developed red with or without black centered colonies, indicates the possible presence of *S. typhi*. Therefore, the material will pass the test if no such colonies are grown.

C. *Test for P. aeruginosa*

The 1 gm of sample was pretreated with buffered sodium chloride peptone solution, pH 7.0. Inoculated 100ml of soyabean casein digest broth the solution, suspension or emulsion was obtained. After mixing it was incubated at 35-37^{0}C for 48 hours. Subculture was prepared on a Cetrimide agar plate and incubated at the same condition. If there is no growth of the microorganisms then the material passes the test. If growth of colonies of gram negative rods occurs usually with greenish fluorescence, then it indicates the presence of the *Pseudomonas species*.

D. *Test for S. aureus*

The pretreated material of all four samples were used and inoculated. It has to be incubated in Vogel- Johnson agar at 35-37^{0}C for 24 h. If there is no growth of the microorganism then the material passes the test. The presence of *Staphylococcus aureus* will be detected with the presence of black colonies

of gram positive cocci often surrounded by a clear zone.

4.7 Phytochemical tests [5]:

The alcoholic extracts of churna were subjected to qualitative chemical investigation. The following procedures were adopted to test the presence of various chemical constituents in the extracts.

Test for alkaloids:

1. Mayer's test: Treat the extract with Meyer's reagent (potassium mercuric iodide) gives cream colored precipitate.
2. Wagner's test: Treat the extract with Wagner's reagent (iodine in potassium iodide) gives brown precipitate.
3. Dragendorff's test: Treat the extract with Dragendorff's reagent (potassium bismuthiodide) gives reddish brown precipitate.
4. Hager's test: Treat the extract with Hager's reagent (saturated solution of picric acid) gives yellow colour.

Test for steroids:

1. Salkowski test: Treat the extract with few drops of concentrated sulphuric acid; red colour at the lower layer indicates presence of steroids.
2. Libermann-Burchard test: Treat the extract with few drops of acetic anhydride, boil and cool. Then added concentrated sulphuric acid from the side of the test tube, brown ring is formed at the junction of two layers and upper layer turns green which indicates presence of steroids.

Test for flavonoids:

1. Shinoda test: To the extract, add few magnesium turnings and concentrated hydrochloric acid dropwise, crimson red or green to blue colour appears after few minutes which indicates presence of flavonoids.
2. Alkaline reagent test: To the extract, add few drops of sodium hydroxide solution, intense yellow colour is formed which turns to colourless on addition of few drops of dilute acid indicates presence of flavonoids.

Test for saponins:

1. Foam test: Small quantity of churna treated with water and shaken for sometime which shows the formation of foam which remained stable for 15 minutes.
2. Bromine water test: The test solution treated with bromine water separately, which gives yellow precipitate.

Test for tannins:

1. Ferric chloride test: Test solution with few drops of Ferric chloride solution gives dark colour.
2. Gelatin test: Test solution when treated with gelatin solution forms white precipitate.

4.8 Extraction and isolation of marker constituents from marketed Kawach churna:

4.8.1 Isolation of Levodopa from Marketed Kawach Churna [10]:

The dried, milled seeds (100 g) were defatted with acetone (300ml) by shaking for 48 h at room temperature. Defatted material was extracted with water-ethanol (1:1) with 0.1% ascorbic acid by shaking overnight. The residue was removed by filtration and filtrates were pooled and concentrated. The concentrate after crystallization yeilded crude L dopa, which on further recrystallization in hot water gave pure crystals.

4.8.2 Identification of isolated Levodopa :

For identification, isolated samples were subjected to Qualitative tests, IR spectroscopy were determined and compared with standard sample of Levodopa.

a) Qualitative Tests for Levodopa[10] :

Dissolve 2 mg in 2 ml of water and add 0.2 ml of Ferric chloride solution Green colour develops which changes in bluish violet on addition of 0.1 g of hexamine.

b) I R spectroscopy [10]

The IR analysis of standard levodopa was carried out using pressed pellet technique [f, and presence of probable functional groups were interpretated.

Similarly the isolated levodopa of all the four samples were analyzed for their IR spectra, and the IR spectra of the isolated levodopa of all the four samples were Compared (overlaid) with that of standard levodopa

4.9 Qualitative and Quantitative Estimation of isolated levodopa by spectroscopic analysis:

For Estimation, isolated levodopa was subjected to UV spectroscopy and results were compared with standard levodopa (RS).

UV spectroscopy :[11]

Determination of λ max for standard levodopa (RS) and isolated levodopa:

Preparation of stock solution of standard levodopa (RS) and their dilutions:

An accurately weighed quantity of about 10 mg of standard levodopa was taken in 100 ml volumetric flask dissolved in sufficient quantity of 0.1 N HCL then sonicated for 15 min and diluted up to 100 ml of 0.1 N HCL to yield a concentration 100 µg/ml. Further dilutions were made, 0.5 ml of stock solution was separately transferred to 10 ml volumetric flask and volume was made up to 10ml in volumetric flask with 0.1 N HCL to yield a concentration of 5µg/ml likewise 1 ml of stock solution in 10 ml of 0.1 N HCL, 1.5 ml of stock solution in 10 ml of 0.1 N HCL, 2 ml of stock solution in 10 ml of 0.1 N HCL and 2.5 ml of stock solution in 10 ml of 0.1 N HCL to yield a concentration 10µg/ml, 15µg/ml, 20µg/ml and 25µg/ml.

The prepared dilutions of standard levodopa were scanned separately in a wavelength range of 200-400 nm against 0.1 N HCL as blank to get maximum absorption for the standard levodopa. And above same procedure was followed for preparation of stock solution of isolated levodopa and their dilutions.The prepared dilutions of isolated levodopa were scanned separately in a wavelength range of 200-400 nm by using UV spectrophotometer against 0.1 N HCL as blank to get maximum absorption for the isolated levodopa.

5. Result and Discussion

5.1 Organoleptic Evaluation[11]:

Organoleptic properties of Kawach churna of different manufacturers were similar i.e. Brown colour, characteristic odour and sweet taste. The only difference was that Kawach churna of MK-3 was whitish brown in colour.

Organoleotic evaluation of marketed kawacha churna

Sample	Colour	odour	Taste
MK-1	Brown	characteristics	Sweet
MK-2	Brown	Characteristics	Sweet
MK-3	Whitish Brown	Characteristics	Sweet
LB-1	Brown	Characteristics	Sweet

2 Extractive Value [12]

The water soluble extractive of kawach churna of different manufacturers MK-1, MK-2, MK-3 and LB-1 was found to be 31.5%, 32.18%, 30.68% and 32.62% w/w respectively. .

The alcohol soluble extractive of kawach churna of different manufacturers MK-1, MK-2, MK-3 and LB-1 was found to be 15%, 15.38%, 14.94% and 15.7% w/w respectively

Water soluable extraction of different manufacturing of Kawach Churana

Sample	Wt. of sample gm	Mean water soluable extraction	%w/w water soluable extraction	**± SD**
MK-1	5	1.575	31.50	0.129
MK-2	5	1.609	32.18	0.114
MK-3	5	1.534	30.68	0.068
LB-1	5	1.631	32.62	0.084

Alcohol soluable extraction of different manufacture of kawach

Sample	**Wt of sample**	**Mean soluable extraction (g**	**%w/w alcohol extract**	**± SD**
MK-1	**5**	**0.750**	**15.00**	**0.097**
MK-2	**5**	**0.769**	**15.38**	**0.100**
MK-3	**5**	**0.747**	**14.94**	**0.093**
LB-1	**5**	**0.785**	**15.70**	**0.086**

5.3 Ash Value[12] :

The total ash value of kawach churna of different manufacturers MK-1, MK-2, MK-3 and LB-1 was found to be 4.04%, 3.9%, 4.06% and 3.16% w/w respectively. The water soluble ash value of kawach churna of different manufacturers MK-1, MK-2, MK-3 and LB-1 was found to be 1.68%, 0.62%, 1.72% and 1.27% w/w respectively. The acid insoluble ash value of kawach churna of different manufacturers MK-1, MK-2, MK-3 and LB-1 was found to be 0.53%, 0.39%, 0.58% and 0.36% w/w respectively.

Determination of total ash value of different manufactures of kawach

Sample	Wt of sample	Mean soluable extraction (g	%w/w alcohol extract	± SD
MK-1	5	0.202	4.04	0.036
MK-2	5	0.198	3.90	0.023
MK-3	5	0.203	4.06	0.043
LB-1	5	0.158	3.16	0.019

Determination of water soluable ash of different manufacturs of kawach churna

Sample	Wt of sample	Mean soluable extraction (g	%w/w alcohol extract	± SD
MK-1	5	0.0842	1.68	0.011
MK-2	5	0.0313	0.62	0.022
MK-3	5	0.0863	1.72	0.011
LB-1	5	0.0635	1.27	0.049

Determination of acid insoluable ash value of different manufacturing of kawach churna

Sample	Wt of sample	Mean soluable extraction (g	%w/w alcohol extract	± SD
MK-1	5	0.0265	0.53	0.0003
MK-2	5	0.0196	0.39	0.0010
MK-3	5	0.0294	0.58	0.0007
LB-1	5	0.0183	0.36	0.0005

.4 Moisture Content[(1)]

The moisture content of kawachchurna of different manufacturers MK-1, MK-2, MK-3 and LB-1 was found to be 4.30%, 3.76%,5.06% and 3.71% w/w respectively .

Determination of moisture content manufactures of kawach churne

Sample	Wt of sample	Mean soluable extraction (g	%w/w alcohol extract	± SD
MK-1	**5**	**0.2153**	**4.30**	**0.0010**
MK-2	**5**	**0.1880**	**3.76**	**0.0055**
MK-3	**5**	**0.2533**	**5.06**	**0.050**
LB-1	**5**	**0.1856**	**3.71**	**0.0011**

Physical parameter
Determination of moisture Kawach churna
N=3

Sample	Wt. of sample (g)	Mean Bulk Density (gm/)	Mean tap density (gm/cm^3)	Hausner ratio	Carr's Index	Mean angle of repose
LB	**20**	**0.59±0.006**	**0.70±0.087**	**1.18±0.03**	**15.1±2.25**	**26.56±1.23**
MK-1	**20**	**0.61±0.008**	**0.70±0.111**	**1.13±0.02**	**12.5±1.65**	**24.70±1.3**
MK-2	**20**	**0.61±0.056**	**0.74±0.85**	**1.2±0.04**	**16.8±2.85**	**30.11±0.59**
MK-3	**20**	**0.62±0.055**	**0.76±0.1**	**1.21±0.055**	**17.6±3.70**	**20.55±1.12**

5.6 Microbial Limit test [13]:

i. Preliminary test: Microbial growth was observed in all the three kawachchurna from different manufacturers MK-1, MK-2, MK-3 and LB-1 when incubated on luid Lactose medium.

Table no. 10: Observation for preliminary testing of microbial limit test of different manufacturers of kawach churna

Sample	Nutrient	Incubation condition	Observation	Conclusion

MK-1 MK-2 MK-3 LB-1	Fluid lactose medium	35.37^{o} for 24h	Microbial growth was observed	Sample pass the preliminary test

a) Test for Specified micro-organisms:

***E. coli*:** Red colored colonies were not observed in any of the three kawach churna when incubated on MacConkey agar medium. While red colored colonies were observed on MacConkey agar medium when standard strain of *E. coli* was inoculated on it.

Table no. 11: Observation for growth of standard strain of E. coli and of different manufacturers of kawach churna on MacConkey broth.

Sample	Nutrient	Incubation condition	Observation	Conclusion
MK-1 MK-2 MK-3 LB-1	MacCoey broth	35.37^{o} for 24h	Growth of red colonies was observed	No formation of characteristic colonies

Figure No. 3: Comparison showing growth of standard strain of E. coli (left) and of kawach churna from different manufacturers (right) on MacConkey broth.

S. typhi:White colored colonies were observed in all the three kawachchurna when incubated on Xylose Lysine agar medium. While red colored colonies with black central portion were observed on Xylose Lysine agar medium when standard strain of *S. typhi* was inoculated on it.

Table no. 12: Observation for growth of standard strain of S. typhi and of different manufacturers of kawach churna on Xylose; lysine agar.

Sample	Nutrient	Incubation condition	Observation	Conclusion
Standard of stain of S. typhi MK-1 MK-2 MK-3 LB-1	Xylose lysine agar	35.37^{o} for 24h	red with colonies centred colony	Culture media is suitable
			No characteristic growth was observed	Absence of S. typhi

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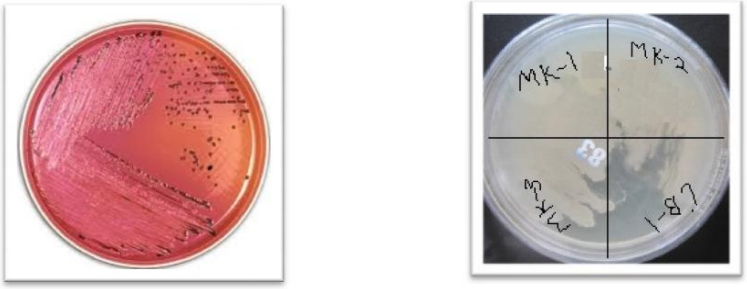


Figure no. 4: Comparison showing growth of standard strain of S. typhi (left) and of kawach churna from different manufacturers (right) on Xylose; lysine agar.

***P. aeruginosa*:** White colored colonies were observed in all the three kawachchurna when incubated on Cetrimide agar medium. While growth with green fluorescence was observed on Cetrimide agar medium when standard strain of *P. aeruginosa* was inoculated on it.

Table no. 13: Observation for growth of standard strain of P. aeruginosa and of different manufacturers of kawach churna on Cetrimide agar.

Sample	Nutrient	Incubation condition	Observation	Conclusion
Standard of stain of P. aeruginosa MK-1 MK-2	Cetrimide agar	35.37^{o} for 24h	Microbial growth with green fluorescence	Culture media is suitable
MK-3 LB-1			White colonies but no characterise growth was observed	Absence of P. aeruginosa

Figure No. 5: Comparison showing growth of standard strain of P. aeruginosa (left) and of kawach churna from different manufacturers (right) on Cetrimide agar.

***S. aureus*:** Black colored colonies were not observed in any of the three kawach churna when incubated on Vogel Johnson agar medium. While growth with black colonies was observed on Vogel Johnson agar medium when standard strain of *S. aureus* was inoculate on it.

Table no. 14: Observation for growth of standard strain of S. aureus and of different manufacturers of kawach churna on Vogel- Johnson agar.

Sample	Nutrient	Incubation condition	Observation	Conclusion
Standard of stain of S. aeruginosa MK-1 MK-2 MK-3 LB-1	Vogel Johnson agar	35.37° for 24h	Formation of black colour colonies	Culture media is suitable
			No Formation of black colour colonies	Absence of S. aeruginosa

Figure No. 6: Comparison showing growth of standard strain of S. aureus (left) and of kawach churna from different manufacturers (right) medium.

5.7 Phytochemical Investigation[14]

Phytochemical investigation revealed that kawach churna of all the four manufacturers possesses Alkaloids, Steroids, Flavonoids, Saponins and Tannins.

Table no. 15: Preliminary phytochemical investigation tests of different manufacturers of kawach churna.

Note: + indicates positive test, - indicates negative test

Sr. no	Chemical test	MK-1	Mk-2	MK-3	LB-1
1	Test for alkaloid 1.Mayers test	+	+	+	+
	2.Wagner's test	+	+	+	+
	3.Dragendroff test	+	+	+	+
2	Test foe steroid 1.salkowski test	+	+	+	+
	2.liberman test	+	+	+	+
3.	Test for flavanoid 1.shinoda test	-	-	-	-
	2. alkaline reagent test	-	-	-	-
4.	Test for saponin				

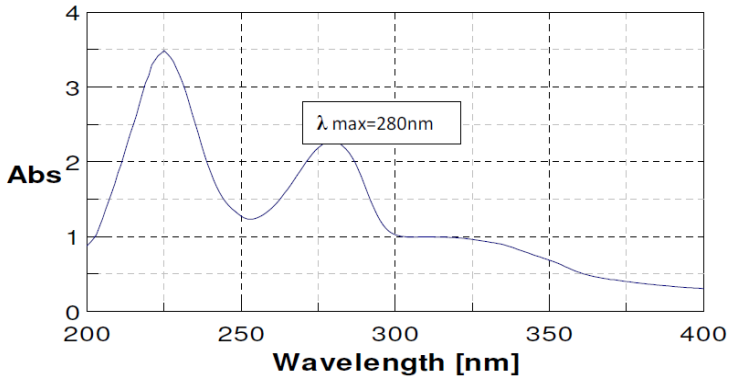
	1.foam test	+	+	+	+
	2.Bromine water test	+	+	+	+
5.	Test for tannins	+	+	+	+
	1.ferric choloride test				
	2.gelatin test	+	+	+	+

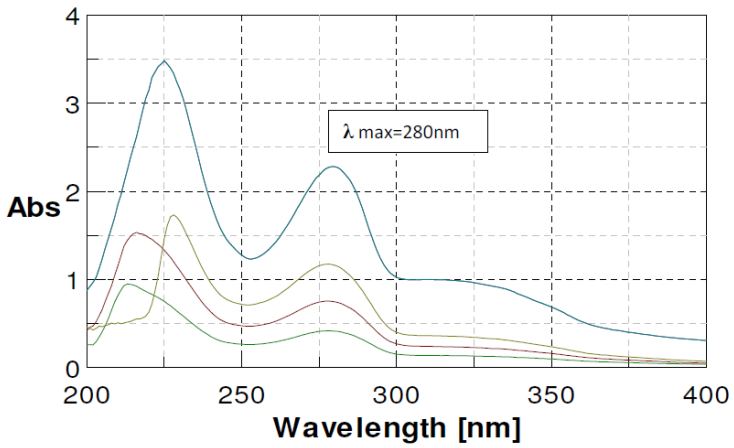
5.8 Qualitative and Quantitative determination of Marker Constituents[15]:

Isolated levodopa was identified by comparison of chemical tests and IR spectroscopy with standard levodopa.

Table no. 16: Qualitative Tests for Levodopa

Test	Sample	observation	Conclusion
Dissole 2 mg in 2 ml of water and add 0.2 ml of ferric chloride	MK-1 MK-2 MK-3 LB-1	Gren colour develop which are changes in bluish violet on a addition of 0.1 g hexamine	Levodopa may be present.





Wavelength [nm]

Figure no. 8: Overlain of UV spectra of isolated levodopa from different manufacturers of Kawach churna.

- **Determination of content of Levodopa by UV**

Table no. 17: Determination of content of Levodopa by UV of different manufacturers of kawach churna.

*** n=3**

sample	Mean absorance	±SD	% levodopa present in Kawacha churna
MK-1	**0.155**	**0.0035**	**2.75**
MK-2	**0.1563**	**0.0023**	**2.77**
MK-3	**0.1493**	**0.0016**	**2.65**
LB-1	**0.1997**	**0.0040**	**3.4**

n=3

Table no. 18: Probable functional groups of standard levodopa

Sr.no	Wave number(cmn^{1})	Type of vibration	Functional group
1	3.397	O-Hstr	Alcolol
2	1654	C=Ostr	Ketone
3	1569	C=Cstr	Aromatic
4	1063	C-Nstr	Aliphatic

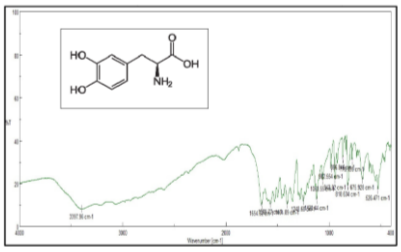


Figure no. 9: IR Spectra of standard Levodopa

Figure no. 9: IR Spectra of standard Levodopa

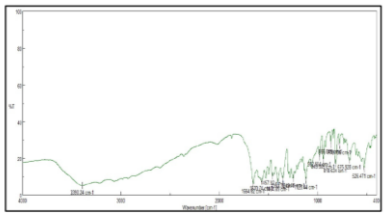


Figure no. 10: IR Spectra of isolated Levodopa from MK-1 formulation.

Figure no. 10: IR Spectra of isolated Levodopa from MK-1 formulation.

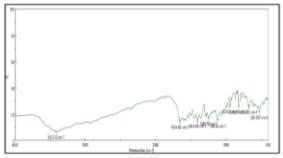


Figure no. 11: IR Spectra of isolated Levodopa from MK-2 formulation.

Figure no. 11: IR Spectra of isolated Levodopa from MK-2 formulation.

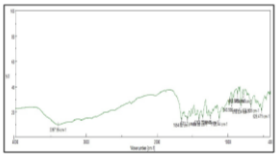


Figure no. 12: IR Spectra of isolated Levodopa from MK-3 formulation.

Figure no. 12: IR Spectra of isolated Levodopa from MK-3 formulation.

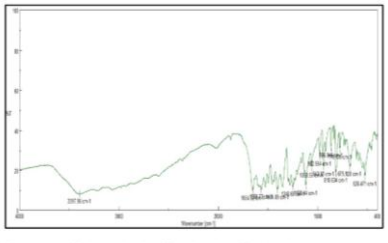


Figure no. 13: IR Spectra of isolated Levodopa from LB-1 formulation.

Figure no. 13: IR Spectra of isolated Levodopa from LB-1 formulation.

CONCLUSION

Organoleptic evaluation revealed that Kawach churna MK-3 was observed to be ightish brown in color. Results of Extractive value demonstrated that water soluble extractive value and alcohol soluble extractive value of product MK-3 was lesser as compared to products MK-1, MK-2 and LB-1. Phytochemical studies exhibited presence of Alkaloids, Steroids, Saponins, and Tannins in all the four products. Ash value etermination revealed that the acid insoluble ash of all the products was within range < 1%, but acid insoluble ash of product MK-3 was more as compared to products MK-1, MK-2 and LB-1, which indicates presence of sand or earth material in product MK-3. Moisture content of all the products were within acceptable ranges < 5 % w/w. But as product MK-3 contained more moisture it is more prone to microbial contamination and enzymatic degradation. When all the four products were subjected for Microbial Limit test, all the compounds demonstrated growth of non-pathogenic micro-organisms but growth of the pathogenic micro-organisms was not observed. Product MK-2 may be better than products MK-1 and MK-3 in view of safety and efficacy, as it contains higher quantity of active markers. TLC showed yellow spot having Rf value 0.73 in n-butanol: Glacial Acetic Acid: Water (8:2:2) (v/v/v) solvent system. UV and IR spectra of isolated and standard marker compound (Levodopa) showed similar results. Chemical test of Levodopa showed the positive result. All above observation and results confirm the isolated compound was Levodopa

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