International Journal of Allied Medical Sciences and Clinical Research (IJAMSCR)

ISSN:2347-6567

IJAMSCR |Volume 8 | Issue 4 | Oct - Dec - 2020
www.ijamscr.com

Research article ***Medical research***

New Advanced Analytical Methodologies Developed For Quality Control of Herbal Extracts of Cuscuta Reflexa Roxb Formulation

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ABSTRACT

The quality control of plant products is a general requirement to be fulfilled. Good quality assurance is necessary when dealing with the plant products, intended tobe released in market as drug constituents or as test substances in basic pharmacological experiments. objective of study:Quality control is crucial to ensure the safety and correct handling of herbal medicines. .The pharmaceutical approach of analyzing a single ingredient cannot be applied to discern the quality of herbal preparation. Thus, quality control methods which reflect the holistic approach of complementary medicine have to be developed in order to determine the chemical basis of herbal medicines. Plan of work: it includes, Selection of formulations, Physico-chemical evaluation of formulations, Description, Loss on drying, Moisture content determination by Karl-Fischer method, Total ash, Acid insoluble ash, Water soluble ash, pH of 1% solution, pH of 10% solution. Research methodology Selection of formulations Unani formulations to be included in Unani Pharmacopeia/Formulary. Results and discussion: Physico-chemical evaluations like organoleptic characteristics, loss on drying, ash content, extractive values etc. were carried. The HPTLC fingerprint analysis of methanol, chloroform and petroleum ether extracts of Itrifal-e-Aftimoon was carried out. Heavy metal analysis of arsenic, lead, mercury and cadmium was performed for all the test samples by using the atomic absorption spectrophometer. Calibration curve for each heavy metals were set to ensure the accuracy of the Atomic absorption spectrophotometer. Quantification of Aflatoxins in Itrifal-e-Aftimoon by HPLC-PDA By using the developed and validated extraction and HPLC-PDA methods of three different batches of Itrifal-e-Aftimoon were investigated. All the samples were successfully analyzed for the content of aflatoxins and found that the samples were free from aflatoxins and safe for the use. The mobile phase was selected using different compositions of methanol–water and acetonitrile– water; it was found that the all four aflatoxin are successfully separated by using a mixture of acetonitrile and water in 1:1, v/v composition. A simple rapid and economic simultaneous HPLC method was developed and validated for the quantification of gallic acid, ellagic acid and ascorbic acid.

Keywords: **HPLC, Unani, physicochemical evaluation, Atomic absorption, spectrophotometer**, **Quantification of Aflatoxins**

INTRODUCTION

In almost all the traditional medicine, the medicinal plants play a major role and constitute backbone of the traditional medicine. Indian Materia Medica includes about 2000 drugs of natural origin almost all of which are derived from different traditional system and folklore practices. Out of these drugs derived from traditional system, 400 are of mineral and animal origin while rest is ofvegetable origin. India has a rich heritage traditional medicine and the traditional health care system have been flourishing for many years. Lot of efforts have been taken by the government and private sectors for the development of traditional system based on these methods.

Unani medicine originated from ancient Greece. The Unani system of medicine owes, as its name suggests, its origin to Greece. The term "Unani" is derived from the work "Unan" which means Greece in Arabic. In 460 BC the Greek Philosopher and father of modern medicine, Hippocrates (Bukharath) who freed medicine from the clutches of superstition and laid the foundation of Unani medicine.

Another great scholar of Unani medicine was Galen (131-210 AD) who stabilised the foundation of thissystem. As Greek and then Roman civilisation declined, Greek medical texts survived in the Islamic courts of the medieval Near East. In the eighth and ninth centuries AD, many Greek texts were translated into Arab forming the basis of Unani medicine. Some Islamic physicians like Al-Razi (Rhazes) (850-925 AD) and Ibn Sina (Avicenna) (980-1037 AD) Al Zahravi (Albucasis) the surgeon and Ibn-Nafis etc. contributed immensely to the system. In India, Unani system of medicine was introduced by Arabs, and soon it took firm roots in the soil.

Concepts of Unani system of medicine

In Unani System of medicine the human body is considered as a single unit, made of seven components known as „Umoor-e-Tabiya". These seven components are Arkan (Elements), Mizaj (Temperament), Akhlaat (Humours), Arwaah (Life force), Aaza (Organs), Quwa (Faculties), Afa"al (Functions). According to Unani philosophy, the body is made up of the four basic elements i.e. Earth, Air, Water and Fire which have different temperaments i.e. Cold, Hot, Wet and Dry respectively. After mixing and interaction of four elements a new compound having new Mizaj (temperament) comesinto existence i.e. Hot Wet, Hot Dry, Cold Wet, Cold Dry.

The herbal formulations are of mainly plant origin, they are susceptible to contamination from different sources, detoriation and variations of chemicalcomposition which may occur due climatic and geographical changes. Therefore the development of standardization procedure for the herbal formulations is must. The standardization of herbal drugs may give acceptance by worldwide moreover it improves the therapeutic efficacy and safety of the drugs. The standardization gives a clear picture about the intrinsic value of the drug i.e. the amount of medicinal principles and constituents present, presence or absence of adulterants etc.

OBJECTIVE & PLAN OF STUDY

Rationale of the study and plan of work

Rationale of the study

Herbal medicines have a strong potential in the primary health care**.** Most Ayurvedic and Unani traditional products are marketed as dietary supplements worldwide. Although herbal medicinal products have been perceived by the public as relatively low risk, there has been more recognition of the potential risks associated with this type of product as the use of herbal medicines increases. Potential harm can occur via inherent toxicity of herbs, as well as from contamination, adulteration, plant misidentification, and interactions with other herbal products or pharmaceutical drugs.Quality control is crucial to ensure the safety and correct handling of herbal medicines. There have been numerous reports on the toxicity, the misidentification and substitution of plant species. Herbal medicines have been reported to contain heavy metals and synthetic prescriptions or non-prescription drugs. The prevalent use of herbal medicines due to their easy availability has raised concerns over their quality, efficacy and safety. Many herbal products are sold even without prescriptions and consist of a decoction of several herbal materials defined in a formula. As a result, the clinical application of a particular herbal medicine is the synergistic effectof multiple chemical compositions. In this case, the pharmaceutical approach of analyzing a single ingredient cannot be applied to discern the quality of herbal preparation. Thus, quality control methods which reflect the holistic approach of complementary medicine have to be developed in order to determine the chemical basis of herbal medicines. Current herbal standardizations are often based on the quantitative analysis of a single compound, which may not reflect the total characteristic, bioactive and toxic nature of the herbs or products. Therefore, there is aneed to establish internationally recognized methodology for quality standardization of traditional herbal medicines like multiple marker based quantification, determination of total metabolite content or application of hyphenated techniques such as LC-PDA, LC-MS or GC-MS which will give better understanding of bioactive multi component herbal formulations (Priti and Rajani, 2019; Rajani and Kanaki, 2018). Hence, in the present investigation an attempt had been made for quality control of compound traditional Unani formulations using multiple marker based analysis by HPLC/HPTLC/GC-MS methods in addition to additional quality control parameters.

PLAN OF WORK

Selection of formulations-Physico-chemical evaluation of formulations-Description-Loss on drying-Moisture content determination by Karl-Fischer method-Total ash-Acid insoluble ash-Water soluble ash-pH of 1% solution-pH of 10% solution-Extractive value determination by Successive Extraction Method-Alcohol soluble matter-Water soluble matter-Total phenolic contents by UV spectrophotometer-Determination of sugar content by Anthrone Method-Dimensional variation-isintegration test-Determination of contaminants-Chemical contaminants (Heavy metal, Pesticides residues)-Fungal contaminants (Aflatoxins)-Development of HPTLC fingerprinting-Development of fingerprint profile by GC-MS-Development of analytical methods for the quantification of marker constituents (HPLC/HPTLC/UPLC-MS/GC-MS)-Validation of the developed method.

MATERIALS USED FOR THE STUDY

Selection of formulations

Since the work carried out in present investigation was the part of an AYUSH/Govt.of India sponsored project for the development of analytical standards of some compound Unani formulations to be included in Unani Pharmacopeia/Formulary. The compound Unani formulations were selected randomly by Unani council and three batches of each was supplied to us for analysis. In brief the different batches of formulations were prepared by a qualified Hakim in 'Matab Khana' of Department of 'Ilmul Advia', Faculty of Unani Medicine, Hamdard University, under the supervision of Dr. Aftab Ahmad (Head) as per the formula and instruction given in National Formulary of Unani Medicine. The raw materials used for the preparation of first batch were collected from Delhi region, second batch from Chennai where as third batch was obtained as gift samples from CCRUM Hyderabad unit. All the components used were identified by a qualified Hakims and Botanist, which were further authenticated by Pharmacognosist. The voucher specimens of all the rawmaterials used and formulations have been procured

in Bioactive Natural Product Laboratory for further use.

Formulations chosen for the study

Itrifal-e-Aftimoon

The Itrifal-e-Aftimoon composed of twelve crude drugs namely *Terminalia chebula, Terminalia belerica, Emblica officinalis, Operculum turpenthum, Cuscuta epithymum, Cassia angustifolia, Plumbago zeylanica, Polypodium vulgare, Lavendula stoechas, Rosa damascene, Pimpinella anisum* and *Prunus amygdalus.* (NFUM) and mainly prescribed in Unani System of Medicine as brain tonic.

Reference standards used for the study

The reference standards used were obtained; ascorbic acid, rutin, quercetin, chebulinicacid (Sigma Aldrich, USA); gallic acid, ellagic acid, tannic Acid, glabridin, berberine,6-gingerol, Keto beta boswellic acid, Acetyl keto beta boswellic acid, Beta boswellic acid, Acetyl beta boswellic acid, glycyrrhizic acid (Sami Labs Ltd., Bangalore, gift samples); sennoside A, sennoside B, piperine, piperlongumine, guggul sterone E & Z (ChromaDex, Bangalore); aloe-emodin (Yucca Enterprises, Maharashtra). All the reference compounds used were having percentage purity above 98%.

EXPERIMENTAL

ITRIFAL-E-AFTIMOON

Physico-chemical standardization of Itrifal-e-Aftimoon

Physico-chemical standardization of Itrifal-e-Aftimoon was carried out for different parameters like organoleptic characteristics, loss on drying, moisture content by Karl Fischer method, total ash, acid insoluble ash, water soluble ash, pH of 1 and 10 % suspensions, petroleum ether; chloroform; acetone and methanol extractive values, total phenolic content by UV, content of sugar by Anthrone reagent method, water soluble matter and alcohol soluble matter. All the parameters has been done as per the procedures mentioned in general physico-chemical experimental section 5.1.1-5.1.12.

Determination of contaminants in Itrifal-e-Aftimoon

The Itrifal-e-Aftimoon formulation has been analyzed for the content of heavy metals,aflatoxins and pesticide as per the procedure mentioned above.

HPTLC finger printing of Itrifal-e-Aftimoon

High Performance Thin Layer Chromatography was performed to develop fingerprint profiles of Itrifal-e-Aftimoon formulation. Methanol, petroleum ether and chloroform extracts were used for the fingerprint development. The methanol (200 mg/mL), chloroform (250 mg/mL) and petroleum ether (250 mg/mL) extracts were prepared by sonicating 1.0, 1.25 and 1.25 g of Itrifal-e-Aftimoon in 20 mL of respective solvent for 30 min, followed by centrifugation to get the supernatant, which was concentrated under nitrogen and the final volume was adjusted to five mL using respective solvents.

HPTLC instrumentation and sample application

The HPTLC fingerprints of the different extracts of all drugs were established by developing the solvent systems for their separation by thin layer chromatography. Thesolvent system in which maximum and well resolved spots were found, selected for HPTLC. The samples were applied in triplicate (8.0 µL), the band width was kept to 5.0 mm and distance between tracks was 13 mm on precoated silica gel 60 F_{254} plates (E. Merck, 0.20 mm thickness) using Linomat V (HPTLC sample applicator). After sample application, the plates were developed up to 80 mm in development chamber saturated with the respective solvent system. The chromatograms were scanned at 254 and 366 nm wavelength followed by spectral analysis. Reprostar was used for taking photographs of the HPTLC plates. Plates were also scanned at visual range after spraying with visualizing reagent.

RESULTS AND DISCUSSION

ITRIFAL-E-AFTIMOON

Physico-chemical standardization of Itrifal-e-Aftimoon

Physico-chemical evaluations like organoleptic characteristics, loss on drying, ash content, extractive values etc. were carried out and the results with standard deviation and limit are tabulated in Table 1. All the findings are based on the analysis of three batches.

Table 1: Physico-chemical parameters of the Itrifal-e-Aftimoon

S. No.	Parameters	Observations (n=9)	Limits (Lower-Upper)
1.	Colour of the formulation	Dark brown	
2.	Odour	Characteristic	
3.	Taste	Sweet	
4.	Consistency	Semi solid	
5.	Loss on drying at 105° C (%w/w)	10.7±0.26	9.5-11.0
6.	Moisture content by Karl Fischer method (%w/w)	9.6±0.6%	9.5-11.0
7.	Total ash (%w/w)	1.25±0.12	1.0-1.3
8.	Acid insoluble ash (%w/w)	0.47±0.03	0.4-0.5
9.	Water soluble ash (%w/w)	0.58±0.04	0.55-0.65
10.	pH of 1 % suspension	4.16±0.03	4.0-4.5
11.	pH of 10 % suspension	3.82±0.10	3.5-4.0
12.	Petroleum ether extractive value (%w/w)	0.93±0.06	0.85-0.95
13.	Chloroform extractive value (%w/w)	0.51±0.03	0.45-0.55

14.	Acetone extractive value (%w/w)	0.54±0.02	0.50-0.60
15.	Methanol extractive value (%w/w)	53.09±0.73	50-60
16.	Total phenolic content by UV (%w/w)	3.18±0.03	2.8-3.5
17.	Content of sugar by Anthrone reagent method (%w/w)	62.78±1.5%	60-65
18.	Water soluble matter (%w/w)	53.3 ±2.11	50-60
19.	Alcohol soluble matter (%w/w)	49.3 ±1.62	45-53

Results of the heavy metal analysis performed for all batches of Itrifal-e-Aftimoon found to be satisfactory with the given limits (Table 2).

Table 2: Results of heavy metal analysis in Itrifal-e-Aftimoon

Batch	Arsenic (ppm)	Mercury (ppm)	Lead (ppm)	Cadmium (ppm)
Limits	NMT 3	NMT 1	NMT 10	NMT0.3
1	<0.1	<0.1	<1	<0.1
2	<0.1	<0.1	<1	<0.1
3	<0.1	<0.1	<1	<0.1

Quantification of Aflatoxins in Itrifal-e-Aftimoon by HPLC-PDA

By using the developed and validated extraction and HPLC-PDA methods as discussed in experimental sections 5.2.2., the content of aflatoxins (B1, B2, G1 and G2) of three different batches of Itrifal-e-Aftimoon were investigated. The chromatogram though obtained was studied for the content of aflatoxins, whichshown an average recovery of 92%, ensured the accuracy of the optimized extraction method. All the samples were successfully analyzed for the content of aflatoxins and found that the samples were free from aflatoxins and safe for the use. The mobile phase was selected using different compositions of methanol–water and acetonitrile–water; it was found that the all four aflatoxin are successfully separated by using a mixture of acetonitrile and water in 1:1, v/v composition. In the above mentioned conditions, the retention of all four aflatoxins were 10.78, 12.16, 13.35 and 16.90 min with enough resolution as per the specification mentioned in the US Pharmacopoeia (Fig. 1).

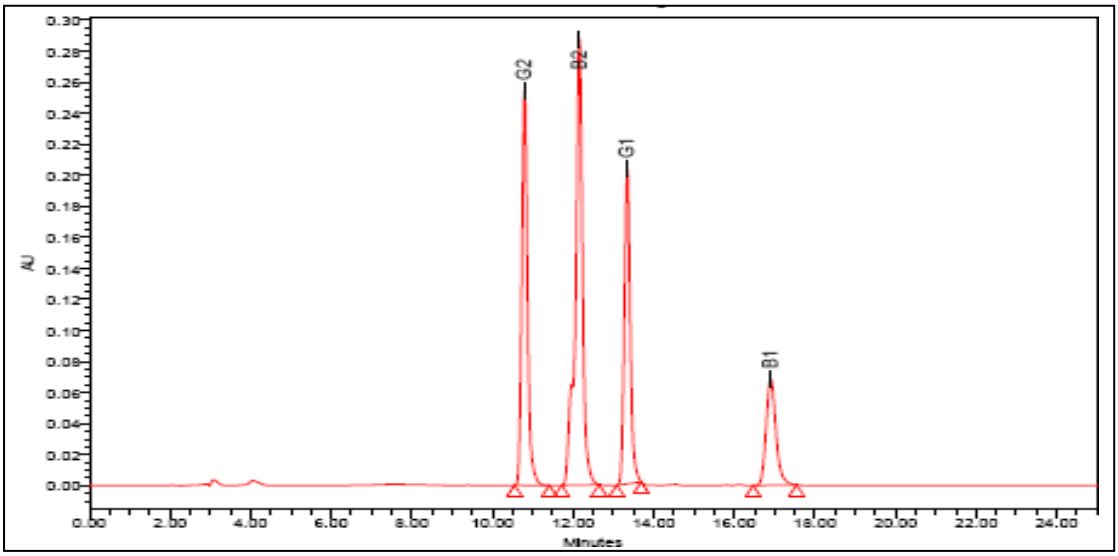

Fig 1: Representative HPLC-PDA chromatogram aflatoxins standards at 360 nm

Determination of pesticides in Itrifal-e-Aftimoon by GC-MS

The pesticides content in 3 different samples were analyzed by GC-MS method as per the procedure mentioned in experiment section 5.2.3. The peaks were identified by matching with the NIST library. The analysis proved the absence of pesticides in the Itrifal-e-Aftimoon samples. The results of pesticides analysed by GC-MS are given in the table 6.

Table 3: Results of pesticide analysis by GC-MS in Itrifal-e-Aftimoon sample

S.No	Compound name	Results
1.	9-octadecenoic acid, 12-(acetyloxy)-, methyl ester, [R-(Z)]	Not detected
2.	Ethyl (9Z,12Z)-9,12-octadecadienoate	Not detected
3.	9,12,15-octadecatrienoic acid, Methyl ester, (Z,Z,Z)	Not detected
4.	Hexadecane	Not detected
5.	Octadecanoic acid, Methyl ester	Not detected
6.	Heneicosane	Not detected

7.	Eicosane	Not detected
8.	1-eicosanol	Not detected
9.	3-pentadecylphenol	Not detected
10.	Hexatriacontane	Not detected
11.	1,2-Benzenedicarboxylic acid, mono(2-ethylhexyl) ester	Not detected
12.	Dotriacontane	Not detected
13.	9-Hexacosene	Not detected
14.	Dotriacontane	Not detected
15.	2,6,10,14,18,22-Tetracosahexaene, 2,6,10,15,19,23-hexamethyl-, (all-E)	Not detected
16.	1-eicosanol	Not detected
17.	Tetrapentacontane	Not detected
18.	Methyl commate C	Not detected
19.	(+)-cedrylacetat	Not detected
20.	Lup-20(29)-en-3-one $$ Lup-20(30)-en-3-one	Not detected
21.	9,19-Cyclolanost-24-en-3-ol, (3.beta.)	Not detected
22.	Methyl commate B	Not detected
23.	9,19-Cyclolanostan-3-ol, 24-methylene-, (3.beta.)	Not detected

Simultaneous estimation of gallic acid, ellagic acid and ascorbic acid in Itrifal-e-Aftimoon

A simple rapid and economic simultaneous HPLC method was developed and validated for the quantification of gallic acid, ellagic acid and ascorbic acid, in poly- herbal Unani formulations, containing aamla as an ingredient. The HPLC experimental method has been carried. Optimization of chromatographic conditions. Optimization of mobile phase was done after many trials using different combinations of solvents, which include acetonitrile, water, methanol, orthophosphoric acid and phosphate buffer in different ratios. The acetonitrile and 0.1% orthophosphoric acid used in the ratio of 60:40, v/v was found to give sharp peaks with poor resolutions between the peaks. To increase the resolutions between the peaks, it was decided to run a gradient elution programme using acetonitrile and 0.1% orthophosphoric acid. The wavelength 254 nm was found best for detection of all the components simultaneously. This mobile phase helped to achieve sharp peaks with good resolutionbetween the peaks (Figure 37). System suitability parameters were calculated like theoretical plates 3861, 4539 and 18317; tailing factor 2.0, 1.14 and 2.1 for ascorbic acid, gallic acid and ellagic acid, respectively, whereas resolution between ascorbic and gallic acid (8.8) and between gallic and ellagic acid (17.03) were found satisfactory. The optimized chromatographic conditions are mentioned in the below table (Table 04).

Table 4: HPLC chromatographic conditions for the analysis of gallic, ellagic and ascorbic acid in Itrifal-e-Aftimoon

Parameters	Observations
Mobile phase	0.1% orthophosphoric acid: acetonitrile (40:60 *v/v*)
Column used	C 18 (250 x 4.6 mm, particle size 5 µm)
Temperature	Ambient
Wavelength	254 nm
Flow rate	1.0 mL/minute
Injection volume	20 µl
Detector	UV visible detector
Method	Gradient elution method

Method validation

Linearity was assessed with the aid of serially diluted calibration solutions as mentioned above. Calibration graphs were plotted on the basis of triplicate analysis of each calibration solutions by using peak area against concentration. The proposed method was found to be linear over a wide range of concentration 0.1-500 µg/mL for ascorbic acid, 1-500 µg/mLfor ellagic and gallic acid with good regression coefficient of 0.997, 0.998 and 0.993, respectively. The slope and intercept was found 51964 ± 550.9, 45362 ± 383.4, 61315 ± 412.9 and 631397 ± 1311.7, 283684 ± 3009.7, 424083 ± 5559.8, respectively for gallic, ellagic and ascorbic acid (Fig. 38). The accuracy of the method was evaluated as recovery by standard addition method. The pre-analyzed samples were spiked with standard at three different concentration levels i.e. 50, 100 and 150% and the mixtures were re-analyzed by the proposed method. The results of the experiment are incorporated in (Table 6.25). The precision of the methods were carried out by doing intermediate precision. Assay for each analysis was calculated and % RSD was determined (Table 26). Robustness of the proposed method was determined at 100 µg/mL in two different ways, i.e. by changing the detecting wavelength and analyzing temperature. The % RSD of the experiment was calculated to assess the robustness of the method (Table 27). The LOD and LOQ was determined on the basis of signal-to-noise ratio. For the proposed method LOD was found to be 35 ng/mL for ascorbic acid, 0.31 µg/mL for ellagic acid and 0.38 µg/mL for gallic acid. The LOQ is the smallest concentration of the analyte, which gives response that can be accurately quantified (signal to noise ratio of 10). For the proposed method LOQ was found to be 0.1 µg/mL for ascorbic acid, 1.0 µg/mL for ellagic acid and 1.3 µg/mL for gallic acid.

Sample analysis

The amount of gallic, ellagic and ascorbic acids in Itrifal-e-Aftimoon Unani formulation was analyzed using developed and validated chromatographic method. The samples were injected in triplicates in HPLC column and peak area of all the triplicate samples were used for analysis of content by regression equation. Thedeveloped mobile phase gave optimal separation, with well-defined and well resolved sharp peaks in both standard and samples at R_t 2.2 ± 0.1 for ascorbic acid, 3.58 ± 0.3 for gallic acid and 7.67 ± 0.3 for ellagic acid, respectively. It was found contain 0.43, 0.14 and 0.09% w/w of gallic, ellgic and ascorbic acids, respectively inItrifal-e-Aftimoon polyherbal formulation.

SUMMARY AND CONCLUSION

Traditional systems of medicine have been in vogue for centuries and use of plant- based medicine has been increasing all over the world especially for conditions like cancer, high blood pressure, allergies, and for general well being. Commercialization and manufacture of these medicines to meet this increasing demand has resulted in a decline in their quality, primarily due to a lack of adequate regulations pertaining to this sector of medicine. Hence it is necessary to come up with a systematic approach to develop well-designed methodologies for the quality control of polyherbal formulations. By considering these facts, the aim of the present research work was to develop high standard quality parameters for some polyherbal formulations which are frequently used in Unani system of medicine. The work has given emphasis on the importance of the qualitative and quantitative methods for characterizing the samples, quantification of the biomarkers and/ or chemical markers and the fingerprint profiles along with the conventional parameters followed for the standardization of polyherbal Unani herbal formulations.

The Unani System of Medicine include a large number of traditional formulation and used since long time in India and abroad. This system consists of different types of formulations like Itrifal, Jawarish, Majoon, Qurs and Habbs and has been ignored for scientific validation of these formulations as well as for the quality control using modern analytical techniques.

The seven formulations have been selected (randomly) for the development of modern quality control standards with conventional parameters. The common and conventional quality control parameters such as organoleptic evaluations like colour, odour, taste and consistency; physico-chemical evaluation like loss on drying, disintegration time for tablets, friability for tablets, moisture content by Karl Fischer method, total ash, acid insoluble ash, water soluble ash, pH of 1 % solution, pH of 10

% solution, extractive values, water soluble matter, alcohol soluble matter, total phenolic/ flavanoid content etc. have been carried out in triplicate of three batches of each formulations.

The evaluation for contaminants like heavy metal content by atomic absorption specrophotometer, determination of aflatoxins by HPLC and pesticide residues, by GC-MS have been carried out in each formulation.

High Performance Thin Layer Chromatography (HPTLC) was performed to develop fingerprint profiles of the formulations. Methanol, petroleum ether and chloroform extracts were used for the fingerprint development.

The plants are composed of complex mixture of primary and secondary metabolites, which are responsible for the bioactivity known as marker compounds and hence multiple-marker based analysis has recently been gaining importance. Multiple- marker-based standardization strategy adopted to minimize batch-to-batch variation and to maintain quality and ensure safety and efficacy. More over gives a rough idea about the therapeutic efficacy of each formulation (Rajani and Kanaki, 2008). To achieve the goal different analytical methods have been developed for thequantification multiple markers present in each formulations. Chromatographic methods developed for the multiple marker analysis include HPLC/UV/PDA detectors, HPTLC and GC-MS methods. All the developed methods were validated as per the ICH guidelines for various parameters like linearity, accuracy, precision, robustness, limit of detection and limit of quantification. Summary of work done in each formulation have been mentioned separately.

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