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Standardization of herbal formulation for mastitis in Animals

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Abstract
Standardization of herbal formulation is essential in order to assess the quality of drugs for therapeutic value based on the concentration of their active principles. In the present study herbal formulation containing rhizomes of *Curcuma longa* and leaves of *Diploclisia glaucescens* were standardized according to the set guidelines from world health organization. The parameter considered were organoleptic, physicochemical, pH range, fluorescence analysis, swelling and foaming index, preliminary phytochemical analysis, heavy metal testing and acute oral toxicity studies of the formulations. The results indicated, all the pharmacognostic parameters were as per the limit set by world health organization and the formulation showed maximum phytocon students. Upon oral toxicity, the extract shown to be safer at 2000 mg/kg body weight both in terms of haematological, biochemical parameters. In conclusion, these data of herbal formulation can be used as reference monographs for further detailed study of pharmacological activity.

Keywords: Standardization, *Curcuma longa*, *Diploclisia glaucescens*, phytochemical analysis, Toxicity

1. Introduction
Standardization of the crude drugs is the confirmation of their identity and determination of purity and quality. The process of standardization is a cumbersome task as multifarious factors influence the bio efficacy and reproducibility of therapeutic effects. To obtain a quality oriented herbal products care should be taken right from the identification, extraction, purification and rationalizing the combination in case of combining one or more drugs [1]. In recent years, plant derived products are increasingly being sought out as medicinal products, nutraceuticals and cosmetics and are available in health food shops and pharmacies over the counter as self-medication or also as drugs prescribed in the non-allopathic systems [2]. According to an estimate of the World Health Organization (WHO), about 80% of the world population still uses herbs and other traditional medicines for their primary health care needs [3]. Herbal formulations have reached widespread acceptability as therapeutic agents for diabetics, arthritics, liver diseases, cough remedies, memory enhancers and adaptogens [4]. Bovine mastitis is the inflammation of the mammary glands in dairy cows, caused by invasion and destruction of the milk producing tissues by pathogenic microorganisms [5]. About 137 species of microorganisms including bacteria, yeasts and algae are known to cause mastitis [6, 7]. Despite management advancements, mastitis continues to rob the dairy industry in terms of decreased milk production, veterinary services, diagnostics, drugs, discarded milk, labour, decreased product quality, increased susceptibility to diseases, increased risk of culling and materials for prevention [8].Mastitis has remarkably rising impact on Indian economy where overall losses due to mastitis is estimated to be Rs. 7165.51 crores [9]. Subclinical mastitis was found more important in India (varying from 10 to 50% in cows and 5 to 20% in buffaloes) than clinical mastitis (1 to 10%). The incidence was highest in Purebred Holsteins and Jerseys and lowest in local cattle and buffaloes [10]. The use of synthetic antibiotics is being increasingly discouraged because their presence in dairy milk may have potential downstream effects on population health and the Agri-food chain [11]. As demand for higher milk quality standards increases, dairy producers are concerned to improve efforts to control mastitis through prevention and treatment [12].
The herbal medicines possess certain advantages being non-toxic, efficacious, cultural acceptability, lesser side effects and act selectively enhancing body resistance [13]. Multifarious reports have claimed the use of formulation containing honey suckle flower, *Chrysanthemum morifolium*, *Citrus reticulata* in preventing mastitis. Other medicinal plants like *Houttuynia cordata*, *Echium sp.*, *Leptospermum scoparium* are also studied in bovine mastitis and the therapeutic efficacy of *Withania somnifera* (Ashwagandha), *Asparagus racemosus* (Shatavari),

Garcuma amada (Amahaldi), *Ocimum sanctum* (Tulsi) have also been well exposed in bovine mastitis. *Tinospora cordifolia, Ocimum sanctum* have also been reported to possess high therapeutic efficacy, anti-inflammatory and immunomodulatory properties and used in animals suffering from mastitis [14-18].

The present study comprise of documenting and standardization of herbal formulation used by the locals of Wayanad regions of Kerala state for prevention of mastitis having equal proportion of *Diploclisia glaucescens* leaves and *Curcuma longa* rhizome respectively.

Curcuma longa L., belongs to the Zingiberaceae family, is a perennial herb that measures up to 1 m high with a short stem, distributed throughout tropical and subtropical regions of the world, being widely cultivated in Asiatic countries, mainly in India and China. Current traditional Indian medicine claims the use of its powder against biliary disorders, anorexia, coryza, cough, diabetic wounds, hepatic disorder, rheumatism and sinusitis, Several studies using the modern techniques have authenticated turmeric used as anti-inflammatory, antimicrobial, anti-fertility, anticancer, anti-diabetic, antioxidant, hypolipidemic, anti-venom, anti-hepato-toxic, nephroprotective, anticoagulant [19-20]. *Diploclisia glaucescens* (Menispermaceae) is a medicinal plant widely distributed in South Asia, Southeast Asia, China, Indonesia and the Philippines. The leaves and stems of the plant have been used as a folk medicine in the treatment of rheumatism, snake venom, biliousness, and venereal diseases [21-23].

2. Materials and methods

2.1 Plant materials

The rhizomes of *Curcuma longa* and leaves of *Diploclisia glaucescens* were collected from Wayanad regions of Kerala state, India. Identification and authentication was done by MS Swaminathan Research Foundation, Wayanad. The plants were dried in shade and grounded to a uniform powder using a blender and stored in polythene bags at room temperature.

2.2 Organoleptic evaluation

Organoleptic evaluation refers to evaluation of the formulation by color, odor, taste, texture, etc. and were evaluated based on the method described by Siddiqui *et al.* [24]

2.3 Study of Physico chemical characteristics

The physicochemical characteristics of polyherbal formulation was done according to the standard protocol described by AOAC, 2016. The homogenized parts of *Curcuma longa* rhizomes and *Diploclisia glaucescens* leaves were finely powdered and stored in an air tight container for physicochemical analysis. The moisture content, total ash, acid insoluble ash, crude protein, ether extract and crude fiber were analyzed based on the guideline numbered AOAC, 930.15, AOAC, 942.05, AOAC, IS: 14826:2000, AOAC, 2001.11, AOAC, 2003.06 and AOAC,978.10 respectively [25].

2.4 Determination of pH range

The powder sample of herbal formulation was weighed to about 2g and immersed in 40 ml of water in a beaker. The beaker was closed with aluminum foil and left behind for 24 hours in room temperature. Later the supernatant solution was decanted into another beaker and the pH of the formulation was determined using a calibrated pH meter [26-27].

2.5 Fluorescence analysis

Take about 0.5gms of plant powder into clean and dried test tubes. To each tube 5ml of different organic solvents like distilled water, acetone, ethanol, benzene, chloroform, diethyl ether, methanol, glacial acetic acid, sulphuric acid, nitric acid, hydrochloric acid, 5% FeCl3, 5% I2, picric acid, 1N NaOH and 1N NaOH + methanol were added separately. Then, all the tubes were shaken and they were allowed to stand for about 20-25 min. The solutions obtained were observed under the visible day light and UV light of short wavelength (254 nm) and UV light of long wavelength (365 nm) for their characteristic colour [28].

2.6 Determination Swelling index and foaming index

2.6.1 Swelling index

One gram of the herbal powder was taken and kept for 24hrs in a graduated, stoppered cylinder in contact with water up to mark of 25 ml, shaken repeatedly for 2 hour and allowed to stand for 24 hrs at room temperature. The volume in ml was measured which is occupied by plant materials including mucilages. Swelling index is determined as the volume in ml of taken up by the swelling of 1 g of plant material [29].

2.6.2 Foaming index

The saponins are high molecular weight compounds containing phytoconstituents having the detergent or soap like property. Many herbal drugs contain saponins that can cause persistent foam when shaken with water. The foaming ability of herbal drugs and their extracts is measured in terms of foaming index. 1 g of the plant materials transfer to a 500 ml conical flask containing 100 ml of boiling water. Maintain at moderate boiling for 30 minutes. Cool and filter into a 100 ml volumetric flask and add sufficient water through the filter to dilute. Pour the decoction in 10 ml stoppered test tube (height 16 cm, diameter 16 mm) in successive portion in 1 ml, 2 ml, 3 ml and adjust the volume of the liquid in each tube with water to 10 ml and stopper the tubes and shaken them in a length wise motion for 15 seconds, two shakes per second. Allow standing for 15 minutes and measured the height of the foam [29].

2.7 Preparation and evaluation of extracts

The extraction of dried leaves of *Diploclisia glaucescens* and rhizome of *Curcuma longa* was done by continuous hot extraction method (Soxhlet extractor) using methanol as solvent. Each extract was subsequently filtered and the filtrates were concentrated under reduced pressure in a vacuum rotary evaporator [30].

2.8 Preliminary Phytochemical analysis

Preliminary qualitative phytochemical analysis of the extract was carried out by employing standard conventional protocols [31-34].

2.9 Heavy metal testing

The heavy metal testing of herbal formulation was carried out according to the method described by [35].

2.10 Acute oral toxicity study of Herbal Formulation

The acute oral toxicity study was sanctioned to be conducted in compliance with OECD guideline

423, which stipulate the use of only three animals (OECD 423, Paragraph 23) [36-38]. The requisite permission for animal experimentation were duly obtained from Institutional Animal Ethical Committee before commencing of the experiment with no. IAEC/COVAS/PKD/4/2018 dated 13.04.2018 respectively. Three of the test animals were fasted overnight

(~12 h) and weighed. Test doses of methanolic extract of herbal formulation were calculated in relation to the body weight of every fasted animal; and administered via oral gavage at the dose of 2000 mg/kg (Figure 1). The animals were regularly and individually observed for behavioral changes and general toxicity signs after dosing for the first 24 h, with special attention being given during the first 4 h. Thereafter, observation was continued daily for a total of 14 days.

2.11 Behavioral pattern studies

For preliminarily screening of toxic effects of these herbal poly-pharmaceutical preparations, all the treated rats were kept under close observation for one week following the dosing. The symptoms including awareness, mood, motor activity, CNS excitation, posture, motor incoordination, muscle tone and reflexes were recorded for 7 days. Any mortality occurring during next two weeks were also recorded [39-40].

3. Results and Discussion

Table 1: Heavy metal testing of Herbal formulation for the presence of Cadmium

Experiment	Observation	Result
NH_4OH add in a sample solution	White ppt is absent	p/o cadmium
Potassium Ferrocyanide is added	White ppt is absent	p/o cadmium

Table 2: Heavy metal testing of Herbal formulation for the presence of Bismuth

Experiment	Observation	Result
NH_4OH add in a sample solution	White ppt is absent	p/o bismuth
H_2S gas is added	Dark brown ppt is absent	p/o bismuth

Table 3: Heavy metal testing of Herbal formulation for the presence of Lead

Experiment	Observation	Result
Dil. HCL add in a sample solution	White ppt of $CaCl_2$ is absent	p/o lead
KI is added	Yellow ppt is absent	p/o lead

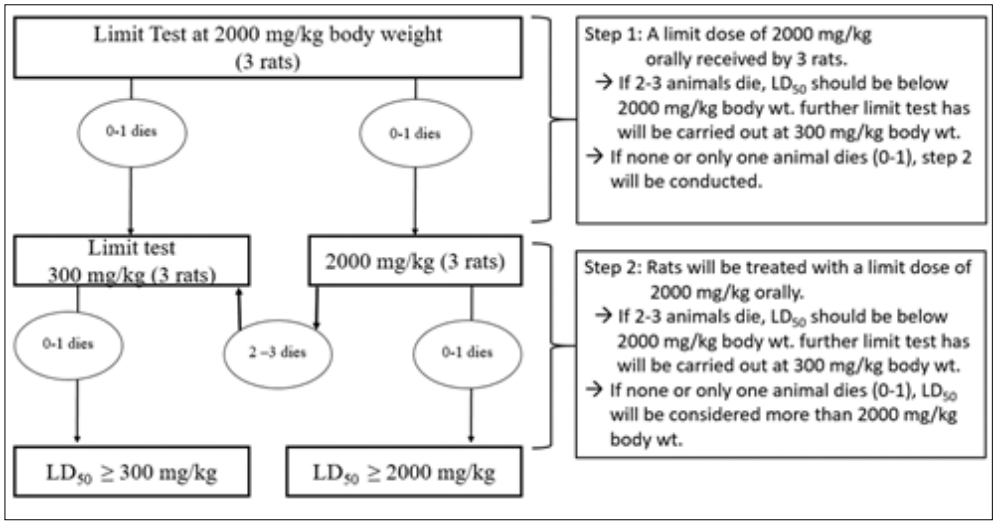

Fig 1: Flow chart of acute oral toxicity studies

Table 4: Organoleptic evaluation of Herbal Formulation

Parameters	Herbal formulation
Appearance	Powder
Colour	Yellowish green
Odors	Fragrant
Taste	Bitter
pH (*Curcuma longa* rhizome powder)	5.6
pH (*Diploclisia glaucescens* leaves)	6.3
pH (Combination of *Curcuma longa* rhizome and *Diploclisia glaucescens* leaves)	5.9
Swelling index	0.7 cm
Foaming index	< 100
Foreign organic matter	Nil

Table 5: Physicochemical characteristics of Herbal formation

S. No	Parameters	Result in % w/w
1	Moisture	14.43
2	Dry matter	85.57
3	Crude protein	16.32

4	Ether extract	2.253
5	Crude fiber	18.02
6	Total ash	7.085
7	Acid Insoluble Ash	0.362
8	Organic matter	92.915
9	Nitrogen Free Extract on Dry Matter Basis	56.322

Table 6: Fluorescence analysis of herbal formulation.

S. No.	Experiments	Visible light	UV Fluorescence	
			254 nm	365 nm
1	Powder as such	Greenish yellow	Dark brown	Grey
2	Powder + 1N Acqueous NaOH	Dark green	Brown	Brown
3	Powder +1N Alcoholic NaOH	Blackish	Brown	Greenish brown
4	Powder + 1N HCl	Light green	Dark brown	Dark blue
5	Powder + conc. H2SO4	Dark brown	Light green	Brown
6	Powder + 50% H2SO4	Green	Blue	Grey
7	Powder +conc. HCl	Dark brown	Green	Brown
8	Powder +conc. HNO3	Light green	Yellow brown	Light green
9	Powder + 50% HNO3	Dark brown	Greenish brown	Brown
10	Powder +Acetic acid	Light green	Brown	Brownish black
11	Powder +Ferric chloride	Dark brown	Dark brown	Black
12	Powder + NH3	Yellowish green	Grey	Brown
13	Powder +Benzene	Brown	Yellow brown	Greenish brown
14	Powder +Petroleum ether	Green	Green	Dark brown (F)
15	Powder + Chloroform	Dark brown	Light brown	Yellowish black
16	Powder +Acetone	Brown	Grey	Black
17	Powder +Ethyl acetate	Dark brown	Dark brown	Brownish green
18	Powder +Acetonitrile	Yellowish green	Blue	Black
19	Powder + Di ethyl ether	Light green	Green	Yellowish brown
20	Powder + Picric acid	Green	Light brown	Brownish black
21	Powder +2 propanol	Brown	Yellow brown	Black
22	Powder +Methanol	Yellowish green	Dark brown	Dark brown
23	Powder +Ethanol	Dark green	Blue	Brownish yellow (F)
24	Powder +Water	Yellowish green	Green	Dark brown

F- Fluorescence

Table 7: Behavioural pattern of Rats in extract treated (2000 mg/kg body wt.) and vehicle treated group.

Parameters	Observation of vehicle and herbal extract treated groups											
	30 mins		4 hrs		24 hrs		48 hrs		7 days		14 days	
	CG	TG	CG	TG	CG	TG	CG	TG	CG	TG	CG	TG
Mucous membrane	N	N	N	N	N	N	N	N	N	N	N	N
Sleep	N	N	N	N	N	N	N	N	N	N	N	N
Somatomotor activity & behavior pattern	N	↑	N	N	N	N	N	N	N	N	N	N
Faeces consistency	N	N	N	N	N	N	N	N	N	N	N	N
Food intake	N	N	N	N	N	N	N	N	N	N	N	N
Sedation	NE	NE	NE	NE	NE	NE	NE	NE	NE	NE	NE	NE
Urination(color)	N	N	N	N	N	N	N	N	N	N	N	N
Respiration	N	N	N	N	N	N	N	N	N	N	N	N
Salivation	N	N	N	N	N	N	N	N	N	N	N	N
Eyes	N	N	N	N	N	N	N	N	N	N	N	N
Fur & skin	N	N	N	N	N	N	N	N	N	N	N	N
Itching	P	P	NF	P	NF	NF	NF	NF	NF	NF	NF	NF
Coma	NF	NF	NF	NF	NF	NF	NF	NF	NF	NF	NF	NF
Diarrhoea	NP	NP	NP	NP	NP	NP	NP	NP	NP	NP	NP	NP
Convulsion and Tremor	NF	NF	NF	NF	NF	NF	NF	NF	NF	NF	NF	NF
Lethargy	NF	NF	NF	NF	NF	NF	NF	NF	NF	NF	NF	NF
Death	NF	NF	NF	NF	NF	NF	NF	NF	NF	NF	NF	NF

CG – Control Group, TG – Test Group, N – Normal, NE – Not Evident, NP – Not Present, NF – Not Found, P – Present

Table 8: Effect of Herbal formulation on hematological parameters of Rats in Acute toxicity

Parameters	Groups	
	Control Group	Treatment Group
Hemoglobin (g/dl)	11.80±0.60	12.33±0.60
PCV (%)	41.0±3.37	40.33±3.37
Platelet count (10^3/µl)	481.66±3.57	496.23±2.46
RBC (10^6/ µl)	8.33±0.23	9.78±0.13*
WBC (10^3/µl)	8.72±0.67	10.98±0.75

Neutrophil (%)	15.33±2.84	21.00±4.58
Eosinophil (%)	3.00±1.00	3.33±1.45
Basophil (%)	0.33 ±0.33	0.67±0.66
Lymphocyte (%)	81.33±2.72	73.67±5.54
Monocyte (%)	0.33±0.33	1.33±0.88

Values are expressed in Mean± SEM, n=3

Table 9: Organ weight of the rat's acute toxicity study of Methanolic extract of Herbal formulation

Organs	Control Group (g / 100 g of body weight)	Treatment Group (g / 100 g of body weight)
Liver	3.23±0.014	3.22±0.058
Kidney	0.49±0.02	0.46±0.01
Spleen	0.23±0.01	0.29±0.01
Heart	0.42±0.02	0.5±0.01
Lung	0.46±0.008	0.53±0.014
Testis	0.86±0.008	0.90±0.008

Values are expressed in Mean ±SEM, n=3

Table 10: Biochemical parameters of the rats treated with Methanolic extract of Herbal formulation

Parameters	Control Group	Treatment Group
SGOT (IU/L)	71.91±1.79	75.71±0.86
SGPT (IU/L)	31.88±1.30	33.05±0.77
Glucose (mg/dl)	86.21±2.31	78.67±1.73
Total Bilirubin (mg/dl)	1.83±0.06	1.91±0.05
Total Protein (g/dl)	6.94±0.15	7.33±0.09
Urea (mg/dl)	25.77±2.22	28.78±1.22
Creatinine (mg/dl)	0.53±0.01	0.54±0.00
Total Cholesterol (mg/dl)	82.00±2.34	94.62±1.96
Triglycerides (mg/dl)	57.54±1.09	63.11±0.67

Values are expressed in Mean ±SEM, n=3

The preliminary screening for the phytochemicals showed the presence of multifarious phytoconstituents viz. alkaloids, flavonoids, glycosides, tannins, saponins and phenolic compounds. The formulation has been evaluated for the presence of heavy metals like cadmium, bismuth and lead respectively. The result of the present study showed the absence of all metals tested.

Organoleptic evaluation by means of sense organs is the technique of qualitative evaluation based on the study of morphological and sensory profile of whole drugs [41]. The organoleptic evaluation of present formulation revealed bitter taste with yellowish green colour and fragrant odour respectively. The quality criteria for herbal drugs are based on a clear scientific definition of the raw materials. It is difficult to establish comprehensive quality criteria for herbal drugs due to 'professional secrecy' of herbalists, but in order to improve the purity and safety of the products, observation of basic hygiene during preparation, standardization of some physical characteristic such as moisture content, pH and microbiological contamination levels are desirable [42]. The pH analysis of the present formulation either singly or in combination are within the neutral range indicating the acidic nature of the extract. When the pH value is low (acidic), the bacterial count was observed to be equally low, but at neutral or higher pH the level of contamination of the herbal preparations were observed to be higher. This suggests that a neutral or alkaline pH favored high contamination levels of the herbal preparations [43].Most of herbal drugs are of specific therapeutic properties or pharmaceutical utility because of their swelling properties, especially gums and those containing an appreciable amount of mucilage, pectin or hemicelluloses. The technique has been accepted as an official method for evaluation by various pharmacopoeias. In the present study the swelling index is 0.7 cm and foaming index is less than 100 indicating the much swelling factors are available in the formulation and also the presence of detergent or soap like property in the present formulation.

The physico chemical constants of the formulation like moisture content, dry matter, crude protein, and crude fiber were estimated using standard protocols as per the methods described by AOAC International. In the formulation, Organic matter was found to be highest with moisture (14.43%), Dry matter (85.57), Crude protein (16.32), Crude fiber (18.02) and total ash (7.085%) respectively. Loss on drying indicates the total volatile content and moisture content of the formulation. High moisture content may affect the quality of drug and the less value of moisture content could prevent bacterial, fungal or yeast growth. The percentage of soluble constituents present in the drug is determined by the value of water and alcohol extractive. These values correlate with the metabolic reaction of the drug and helps in evaluating crude drugs. Ash value depends upon the inorganic substances present in the particular formulation. This may be useful in standardizing the drugs. The ash value of formulation is 10.4%. The acid insoluble ash value of the drug denotes the amount of siliceous matter in the plants. The quality of the drug is better if the insoluble acid value is low. It is 3.4 % for formulation [44-46].

The fluorescent characteristic of powdered drug plays a vital role in the determination of quality and purity of the drug material. Some constituents show fluorescence in the visible range in daylight. The ultraviolet light produces fluorescence in many natural products which do not visibly fluorescence in daylight. If substance themselves are not fluorescent, they may often be converted into fluorescent derivatives or decomposition products by applying different reagents [47]. Hence crude drugs are often assessed qualitatively in this way and it is an important parameter for pharmacognostic evaluation of crude drugs. In the present study, the powdered drug solutions have exhibited a wide range of fluorescence

colour under UV and Visible light. The petroleum ether and ethanol extracts have exhibited fluorescence characteristics in UV light.

The preliminary phytochemical analysis, reported the presence of multifarious phytoconstituents including flavonoids and phenolic compounds reported exerting multiple biological effects including antioxidant, free radical scavenging abilities, anti-inflammatory, anti-carcinogenic activity. Tannins are considered as superior antioxidants as they prevent cellular damages by shielding the proteins from oxidation and glycation reactions, besides their copper scavenging action [48-50]. The formulation is safe as it doesn't contains any heavy metals.

FDA and WHO emphasize the validation of efficacious and safe use of herbal therapies through conduction of scientific based studies. Preliminary toxicological evaluation is necessary for authentication of safety of herbal medications [51-53]. Although the individual plants has been evaluated for their toxicological profiling, their combination is seldom studied for acute toxicity in rats. The toxic outcomes of drugs on vital body organs are exposed by clinical signs and symptoms which are principal observations among various other toxicity indicators [54]. No animal was found dead while some changes in behavioral pattern like increased respiration, increased somatomotor activity, convulsion, tremor and itching were observed in treatment group in first 24 h (Table 2). During 14 days of acute toxicity evaluation period, it was observed that food and water intake were normal with non-significant body weight variations. It suggests the normal processing of lipids, carbohydrates and protein metabolism inside animals body because these nutrients play a major role in different physiological functions of the body [55-57]. The major vital organs of the body were remained intact w.r.t their weight and shown no major lesions upon observations. Hematological parameters are sensitive markers of the physiological changes in response to any environmental pollutant or toxic stress in animals [58]. In this study, there is no significant differences exhibited in haematological parameter among the experimental animals under study and the same is with the biochemical parameters.

4. Conclusion

The quality control of crude drugs and herbal formulation is of paramount importance in justifying their acceptability in modern system of medicine. But one of the major problems faced by the herbal drug industry is non-availability of rigid quality control profile for herbal material and their formulations. The present investigation is carried out to meet the requirements of WHO and other regulatory bodies concerning standardization of herbal formulation containing *Curcuma longa* rhizome and *Diploclisia glaucescens* leaves on the same pattern as synthetic drugs. The present studies show that organoleptic character, physiochemical, fluorescence analysis and toxicity profiles of traditional formulations falls within the permissible limits as per WHO. The formulation shows satisfactory results, but the efficacy of the products can only be judged by doing the pharmacology of which is suggested as future scope of R & D. The study shows that the contents of formulation presents within the permissible limits as per WHO, all these investigations are not specified in the standard literature such as in pharmacopoeia. The result of present study will also serve as reference monograph in the preparation of drug formulation.

Conflict of Interest: None Declared

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