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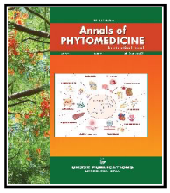

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Physicochemical standardization, evaluation of antioxidant and antimicrobial activity of a polyherbal formulation

Nehal Ashraf*, Mohammed Abdul Rasheed Naikodi, Ghali Shravan Kumar***, Tasleem Ahmed***, Younis Iftikhar Munshi***** and Mohammad Zakir******◆**

** Department of Ilmul Advia, National Research Institute of Unani Medicine for Skin Disorders (NRIUMSD), Hyderabad-500038, Telangana, India*

*** Drug Standardization Research Unit, National Research Institute of Unani Medicine for Skin Disorders (NRIUMSD), Hyderabad-500038, Telangana, India*

**** BCMB, National Research Institute of Unani Medicine for Skin Disorders (NRIUMSD), Hyderabad-500038, Telangana, India.*

***** National Research Institute of Unani Medicine for Skin Disorders (NRIUMSD), Hyderabad-500038, Telangana, India*

****** Department of Ilmul Advia, National Research Institute of Unani Medicine for Skin Disorders (NRIUMSD), Hyderabad-500038, Telangana, India*

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Abstract

The herbal drugs have been used since the human existence for the management of any disease conditions. The natural drugs are used worldwide with great acceptance especially in developing countries. Herbal drugs have some limitation when quality control aspect comes into the light. The standardization of the herbal or natural drugs is considered a challenge and WHO has laid down several guidelines for the quality control checks of herbal drugs. Physicochemical parameters and phytochemical screening along with TLC and HPTLC fingerprint are essential for standardization of the herbal drugs. Safoof-i Dama Haldiwala (SDH) is an important formulation of Unani System of Medicine. It is commonly used for the management of asthma. The standardization of this formulation was done as there was a lack of scientific data for this formulation, although, it is still used as an important drug for the respiratory ailments. Its antimicrobial and antioxidant activity was also evaluated to confirm its use in disease condition like asthma. The result of the study shown that it has showed antimicrobial activity with MIC of 200 mg/ml against *S. aureus* and *E. coli*. It has also shown the antioxidant activity and methanol extract exhibited greater antioxidant activity (IC_{50} = 415), compared to the hydroethanolic (IC_{50} = 473) and aqueous extracts (IC_{50} >500). The results of physicochemical, phytochemical parameters, TLC and HPTLC will serve as the finger printing for the standardization of SDH. The clinical trial may be conducted to validate its therapeutic efficacy in bronchial asthma.

1. Introduction

Since the beginning, people have relied on and used herbal remedies due to their efficacy, safety, cultural acceptability, and lack of adverse actions. People have used plants and plant-based items to cure and prevent diseases throughout history, with varying degrees of success. Medicinal plants serve as a plentiful reservoir of therapeutic compounds which may be used to cure and prevent various ailments (Sharma *et al*., 2008). Aligned with the global trend towards natural remedies, the utilization of herbal treatments has experienced a significant increase. If, the medicine under scrutiny has not undergone authentication and characterization procedures to guarantee consistency in its manufacturing, then it is not possible to scientifically validate the authenticity of an herbal product. The therapeutic efficacy of an herbal medicine is dictated by its phytochemical constituents (Choudhary and Sekhon, 2011). Assessing the efficacy of multi-ingredient Unani drug is essential in determining their suitability for use in contemporary medicine (Ajazuddin, 2010).

Corresponding author: Dr. Mohammad Zakir
Department of Ilmul Advia (Unani Pharmacology), National Research Institute of Unani Medicine for Skin Disorders (NRIUMSD), Hyderabad-500038, India
E-mail: urzakir@rediffmail.com
Tel.: +91-9776355133

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The herbal sector has encountered a challenge of the lack of stringent quality control measures for finished products. The WHO has recognized the importance of herbs for the management of primary healthcare in underdeveloped nations. In response, the WHO has developed guidelines to all nations in creating national policies on natural medicine. These guidelines aim to explore the potential benefits, assess safety and effectiveness, and promote research in this field (De La Sante, 1992).

India serves as a centre for traditional medicines and boasts a wealth of natural medicinal practices. The Ayush system encompasses Ayurveda, Unani, and Siddha medicine, which collectively offer a vast array of medications for diverse therapeutic purposes. There is a significant demand and urgency at present for the creation of standardized pharmaceuticals that are therapeutically effective. It is necessary to investigate the pharmacologically significant plants and their preparations. Accomplishing this goal requires the analysis and evaluation of herbal products utilizing advanced modern techniques of standardization (Sriwastava *et al*., 2010). One of the most important requirements for the pharmaceutical sector working with Ayush medications is quality evaluation of herbal preparations. Safoof-i Dama Haldiwala (SDH) is a mixture of herbs that is advised to use to treat Diq al-Nafas (bronchial asthma) in the Unani System of Medicine (Hafiz, 2005; Kabiruddin, 1997 and 2008). Although,

the drug has been used since long but there was no scientific data for its physicochemical, phytochemical and HPTLC fingerprinting for its quality control. Quality control parameters were not available for SDH so its quality may not be checked by the pharmacy and its efficacy and safety may be compromised. Although, there are several challenges in quality control of polyherbal formulations due to lack of proper specific test but some specific test as per WHO guidelines may be carried out to make some fingerprinting data for future reference. Therefore, current investigation was conducted to produce quality control data as well as evaluate its antioxidant and antibacterial properties.

2. Materials and Methods

2.1 Procurement of raw drugs

The Botanist at the SMP Unit of NRIUMSD, Hyderabad, identified and verified the constituents of the product, SDH, which was purchased from a local market in Hyderabad. The voucher specimens for Gehun SMPU/CRIHyd-143666 and Haldi SMPU/CRIHyd-143667 were assigned and stored for future reference in the herbarium.

2.2 Preparation of safoof (Powder)

The Gehun (wheat) was placed in a new clay pot and roasted to form ash. The Haldi (*Curcuma longa* L. - rhizome) was also slightly roasted separately at low flame. Both roasted components were ground individually and then sifted through a No. 100 sieve to obtain a uniform powder. Then, the powder of Gehun two parts was mixed with one part of Haldi powder thoroughly and preserved in an airtight glass container (Hafiz, 2005).

2.3 Organoleptic evaluation

The organoleptic characteristics of SDH was evaluated and recorded as described in section 3.1 (Anonymous, 2007).

2.4 Bulk density and tapped density

A dried 25 ml measuring cylinder previously weighed was taken and was filled with SDH up to the mark. The primary volume was recorded and the sample tapped till no additional decrease in volume was seen. The original volume provides the measurement for bulk density, while the volume after tapping provides the measurement for tapped density (Anonymous, 2022).

2.5 Physicochemical evaluation

The physicochemical parameters of SDH were carried out as per the procedures mentioned by Mukherjee, 2019; Rasheed *et al*., 2012, Chaithra *et al*., 2022; Khatoon *et al*., 2022; Naaz *et al*., 2021 and Kokate *et al*., 2017.

2.5.1 Determination of total ash

Total ash measures incineration residue. Five grams of SDH powder was put in a tared silica crucible and burned at 450^{0}C in the muffle furnace till it become white. Placed in desiccator for cooling and weighed, the difference in weight of empty tared crucible and crucible with ash gives total drug ash (Anonymous, 2016).

2.5.2 Determination of acid-insoluble ash

Acid insoluble ash is the remaining of the ash after boiling the total ash with 10% dilute HCl. It determines the presence of silica in the total ash. Total ash was filtered on ash less filter paper after boiling with 10% dilute HCl, again incinerated at 450^{0}C and cooled in desiccator and weighed (Anonymous, 2016).

2.5.3 Extractive value

The secondary metabolites present in the raw material of the formulation may be extracted by different solvents. The solvent used for extraction depends upon various polar and nonpolar components present in the plant materials. The commonly used solvent includes water, alcohol, and hexane (Anonymous, 2016).

2.5.3.1 Determination of alcohol soluble, hexane soluble and water-soluble extractive

In a conical flask with 100 ml ethanol add five grams of coarsely crushed air-dried sample powder of SDH powder. After 6 h of shaking in a bench top orbital shaker, the flask was left for 18 h followed by filtration using Whatman filter paper No. 1 and then 25 ml transferred to a tared beaker and kept on water bath for drying. After cooling in a desiccator and weighing, the ethanol soluble extractive percentage was determined. The same process was used for hexane and water soluble percentage extractives was determined (Anonymous, 2016; Beg *et al*., 2022; Naikodi *et al*., 2012 and 2023).

2.5.4 Estimation of pH

The pH of 1% and 5% aqueous solutions (w/v) of SDH was measured by digital pH meter according to the method described in Anonymous (2016).

2.5.5 Loss in weight on drying at 105ºC

Ten gm of SDH powder was placed in a tared petri dish and heated for 6-8 h at 105^{0}C. The procedure was repeated till constant weight was reached. The weight loss, which is expressed as a percentage, indicates the moisture present in the sample (Anonymous, 2016; Moin *et al*., 2023).

2.5.6 Phytochemical screening of SDH

The preliminary phytochemical analysis was done in methanolic (Me), chloroform (Ch), and aqueous (Aq.) extracts of SDH (Alam *et al.*, 2020; Naikodi *et al*., 2011 and 2012; Das *et al.*, 2014; Anonymous, 2006).

2.6 HPTLC fingerprinting analysis

HPTLC fingerprint is an essential element as part of Standardization of herbal drugs. It provides us the fingerprint pattern which enables us to correct identification and authentication of the product (Singh *et al*., 2023). The ethanol extract of SDH was applied to a siligca gel TLC plate (60 F254) for the purpose of conducting HPTLC analysis. The mobile phase consisted of a solvent system of chloroform, methanol, and formic acid at a volumetric ratio of 9.6:0.4:0.1, *v/v/v* in ml.

2.6.1 Preparation of sample extracts

To each 10 g of fine grounded powder of SDH separately in a flask 100 ml of methanol, hydro-alcohol (50:50), and distilled water was added. The flasks were kept on shaker for shaking six hours and left untouched for 18 h. The extracts were collected by filtration through Whatman No. 1 filter paper and dried on water bath at or below 40ºC. The dried extracts were dissolved in methanol, hydro-alcohol, and distilled water. Each solution was prepared at the concentration

of 200, 250, 300, and 350 mg/ml, respectively (Rasheed *et al*., 2012 and 2017; Venkatesham *et al*., 2021; Manandhar *et al*., 2019; Bouyahya *et al*., 2016).

2.7 Pesticide residue

The pesticide residue analysis was carried out at Bureau Veritas Testing Laboratory in Hyderabad as per WHO issued guidelines and UPI (Anonymous, 2016).

2.8 Analysis of antimicrobial activity

The antibacterial activity of the methanol (Me), hydro-alcoholic (HA), and aqueous (Aq) extracts of SDH was assessed. The investigation encompassed various strains of *Staphylococcus aureus, Escherichia coli*, and *Klebsiella* species. The reference strains were acquired from the Clinical Microbiology Laboratory at ESIC Medical College Hyderabad. The positive control utilised in the experiment was Ceftriaxone (30 µg, Himedia Company). Mueller Hinton The agar was prepared and sterilised by subjecting it to autoclaving at a pressure of 15 lbs per square inch and a temperature of 121ºC for 15 min (Gupta *et al*., 2016; Gupta *et al*., 2015). The agar well diffusion method was used to carry out antimicrobial activity and zones of inhibition was calculated. The wells were filled with 100 µl of the extracts from each solvent ranging from 200 to 350 mg/ml concentrations. The reference medicine used was ceftriaxone (30 µg) (Mirtaghi *et al*., 2016).

2.8.1 Minimum inhibitory concentration (MIC) estimation

The measurement of the MIC was conducted utilising an innovative dilution tube technique. The technique was executed for each concentration, specifically 200, 250, 300, and 350 mg/ml. 1 millilitre of nutrient broth was added, followed by the addition of 100 microliters of the test organism into pre-diluted 0.5 McFarland turbidity standard tubes. A tube holding only the nutrient broth was placed next to the test organism as a control. The tubes were incubated at a temperature of 37°C for a period of 24 h. Afterwards, the presence of growth was evaluated by observing the turbidity (Khan *et al*., 2009).

2.9 DPPH radical scavenging activity

The scavenging behaviour of DPPH, was evaluated using a modified approach previously described (Abeysekera, 2019). An aliquot of 200 µl of a 0.1 mM DPPH solution was rapidly combined with 50 µl of various concentrated test extracts (ranging from 7.8-500 µg/ml) in a test plate. Subsequently, the reaction mixture was kept in a dark environment for 30 min. Subsequently, the OD measurements were taken at 517 nm using the Tecan Multimode Microplate reader. The positive control used was gallic acid. The IC_{50} value, which represents the sample concentration that results in 50% inhibition, was calculated by plotting the percentage of inhibition against the concentration of the extract on a graph. The results are quantified as the concentration of plant extract, measured in µg/ml, required to provide a 50% reduction in DPPH radical signal (Abeysekera, 2019).

3. Results

3.1 Organoleptic evaluation and physicochemical parameters

The appearance of SDH was fine powder, yellowish brown colour, aromatic odour and slightly bitter taste. The bulk density was found to be 0.57 ± 0.005 to 0.58 ± 0.004 and tapped density was 0.8932 ± 0.03 to 0.8939 ± 0.01. Total ash of SDH was found to be 4.03 ± 0.01 to 4.18 ± 0.10% and acid insoluble ash was 0.45 ± 0.02 to 0.52 ± 0.02%. Alcohol soluble extractive was found to be 4.38 ± 0.22 to 4.77 ± 0.33%, hexane soluble extractive 2.18 ± 0.31 to 2.31 ± 0.20 and water-soluble extractive 22.53 ± 0.23 to 22.89 ± 0.72%. The pH of 1% aqueous solution of SDH was found to be 6.01 ± 0.02 to 6.03 ± 0.02 and for 5% aqueous solution was 5.89 ± 0.02 to 5.93 ± 0.01. The loss of weight on drying at 105^{0}C was found in the range of 1.92 ± 0.07 to 2.24 ± 0.10. An analysis of the secondary metabolites present in the formulation has been conducted, and the findings are shown in Table 1.

The phytochemical screening (Anonymous, 2007b) was done in methanol extract, chloroform extract, and aqueous extract to determine the nature of phytoconstituents were present in the SDH are in Table 1.

Table 1: Phytochemical analysis of SDH

Phytochemicals	Test	ME extract	CH extract	AQ extract
Alkaloids	Dragendorff's test	+++	+	+
	Mayer 's test	++	-	-
	Wagner's test	++	+	+
	Hager 's test	++	+	+
Carbohydrates	Fehling 's test	++	-	+
	Benedict's test	++	+	-
	Molisch 's test	++	+	+
Tannins	Ferric chloride test	++	-	++
	Lead acetate test	++	-	++
Phenolic compounds	Ferric chloride solution	++	-	++
	Liebermann's test	++	-	++
	Lead acetate test	++	-	++
Glycosides	Glycoside test	-	-	++

Protein	Biuret's test	+++	+	+
	Millon's test	+++	+	+
Starch	Iodine test	-	-	+++
Flavonoids	Magnesium ribbon test	+	-	-
Saponins	Froth test	-	-	+
Resins	-	-	-	-
Fixed oils	-	-	-	-
Steroids and Triterpenes	Liebermann-Burchard test	++	++	+
	Salkowski reaction test	++	++	+

ME: Methanolic; CH: Chloroform; AQ: aqueous

3.2 HPTLC fingerprinting analysis

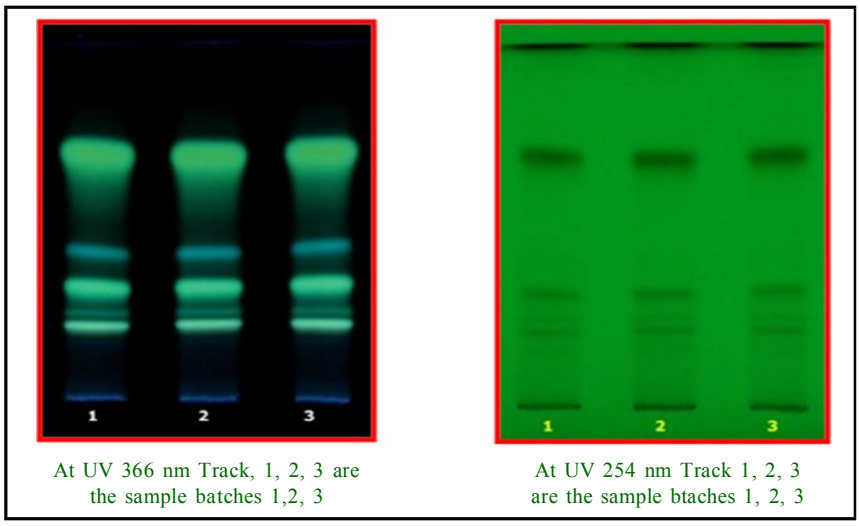
HPTLC fingerprint analysis of the ethanol extract of SDH showed five prominent spots upon detection under UV 366 nm at Rf values 0.21, 0.24, 0.33, 0.43, 0.71 (all pale yellow); and six major spots detected under UV 254 nm at Rf values 0.02, 0.22, 0.25, 0.32, 0.68, 0.98 (all black) as shown in Figure 1; and under visible region shows seven prominent spots at Rf values 0.02, 0.16, 0.21, 0.25, 0.32, 0.42, 0.68 (all yellow) as shown in Figure 2 the corresponding HPTLC densitograms showed six peaks at UV 254 nm and seven peaks at UV 580 nm respectively (Tables 2 and 3, Figures 3 and 4).

Table 2: Peak list of ethanol extract of SDH at UV 254 nm

Peak No.	Y-Pos	Area	Area%	Height	Rf value
1	10.7	226.80	4.43	111.22	0.02
2	24.5	501.84	9.81	217.05	0.22
3	27.0	202.57	3.96	112.40	0.25
4	32.0	923.87	18.06	319.41	0.32
5	58.2	3218.64	62.94	657.35	0.68
6	79.5	40.50	0.79	24.50	0.98

Table 3: Peak list of ethanol extract of SDH at visible 580 nm

Peak No.	Y-Pos	Area	Area%	Height	Rf value
1	10.6	53.09	8.63	23.90	0.02
2	20.7	15.86	2.58	9.42	0.16
3	24.4	34.38	5.59	21.92	0.21
4	27.2	6.96	1.13	5.12	0.25
5	31.9	105.17	17.10	39.17	0.32
6	39.0	24.65	4.01	9.92	0.42
7	58.3	374.73	60.95	67.46	0.68



At UV 366 nm Track, 1, 2, 3 are the sample batches 1,2, 3

At UV 254 nm Track 1, 2, 3 are the sample btaches 1, 2, 3

Figure 1: TLC plate of alcoholic extract of SDH at UV 366 nm and UV 254 nm.

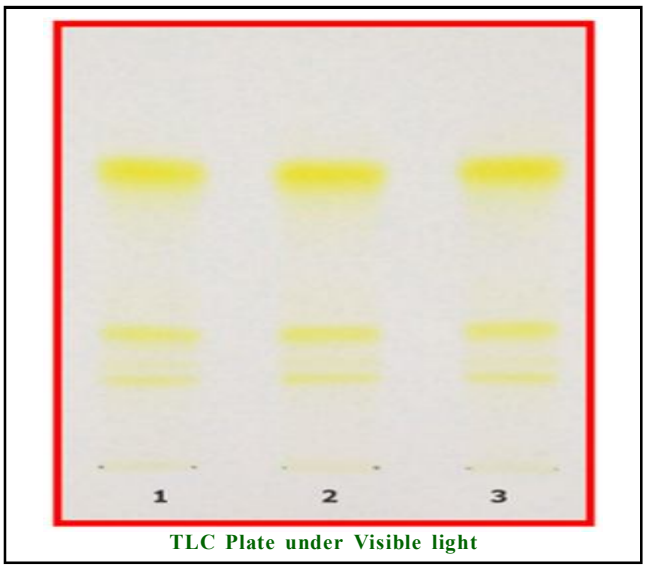


Figure 2: TLC plate of alcoholic extract of SDH at visible light.

3.3 Minimum inhibitory concentration (MIC) and antimicrobial activity

The methanolic and hydroalcoholic extracts exhibited a significant zone of inhibition against *Staphylococcus aureus*, measuring 20.33 ± 1.52 to 22.33 ± 0.57 mm and 19.33 ± 0.57 to 21 ± 0.66 mm, respectively. *E. coli* exhibited a significant zone of inhibition ranging from 19.33 ± 0.57 to 21.33 ± 0.57 mm and from 17.66 ± 1.54 to 20.33 ± 0.66 mm in the methanolic and hydroalcoholic extracts, respectively. *Klebsiella pneumoniae* displayed resistance to all concentrations of the three extracts, and the aqueous extract showed no activity against any of the tested organisms, as evidenced by the absence of a zone of inhibition. The ethanolic and hydro-alcoholic extracts of SDH exhibited a minimum inhibitory concentration (MIC) of 200 mg/ml against *S. aureus* and *E.coli*. However, no results were observed against *K. pneumonia* when using methanolic, hydroalcoholic, and aqueous extracts of SDH.

3.4 DPPH radical scavenging activity

The methanol extract exhibited greater antioxidant activity compared to the hydroethanolic and aqueous extracts (Table 4; Figure 5).

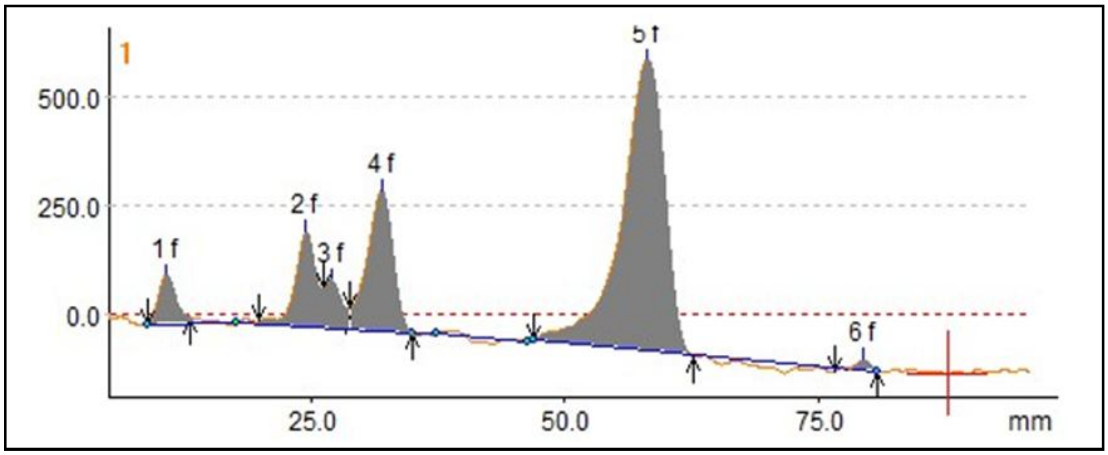


Figure 3: HPTLC densitogram of ethanol extract of SDH at UV 254 nm.

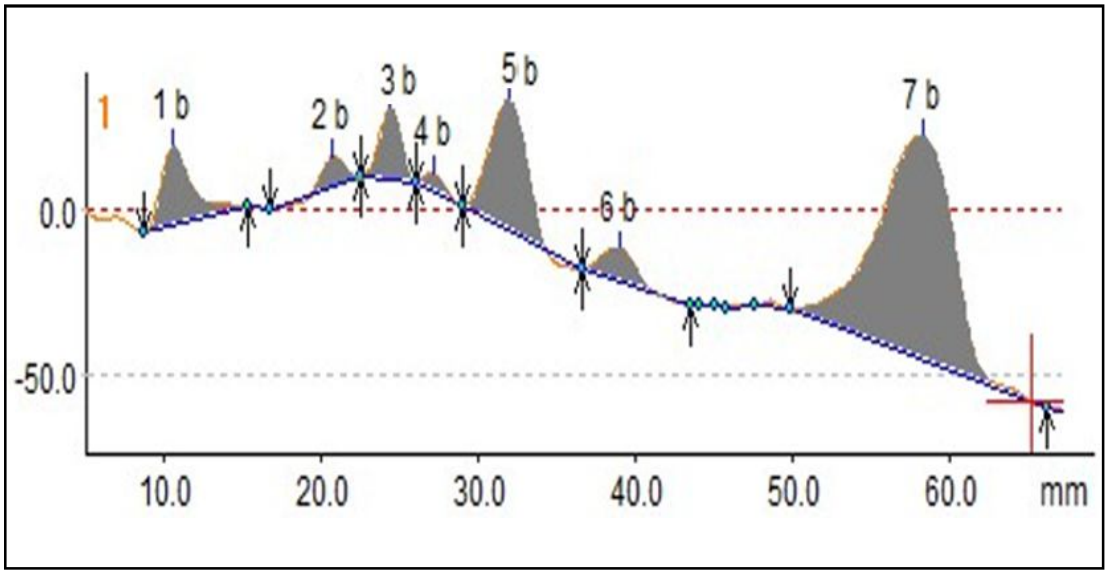


Figure 4: HPTLC densitogram of ethanol extract of SDH at UV 580 nm.

Table 4: DPPH scavenging activity of AQ, HE, and ME extract of SDH

Formulation (SDH) extracts	Mean ± SD	IC_{50} µg/ml
Aqueous	0.19 ± 0.08	473
Hydro-ethanol	0.29 ± 0.05	>500
Methanol	0.32 ± 0.04	415

Data expressed as mean ± SD of samples (p<0.05).

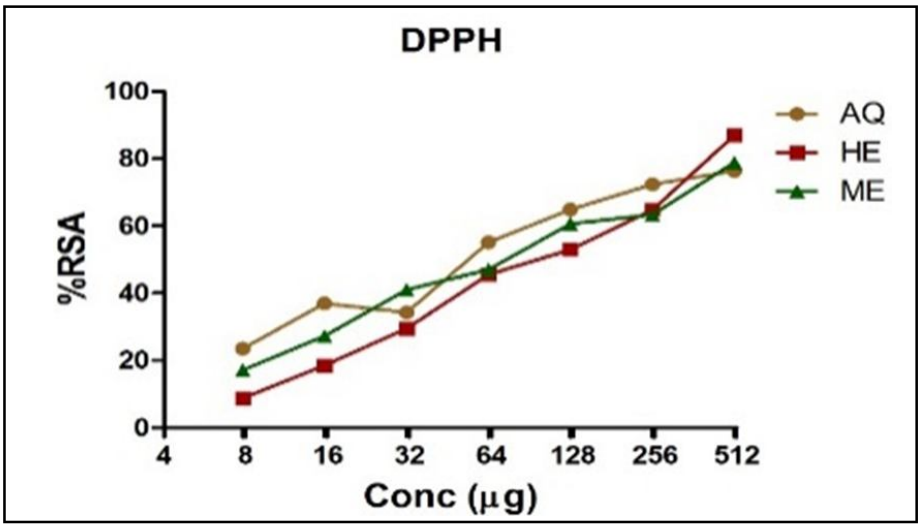


Figure 5: DPPH scavenging activity of AQ, HE, and ME extract of SDH.

4. Discussion

According to the WHO, herbal remedies are the most ancient and dependable approach of managing diseases. Traditional medicines have significantly contributed to the overall health and wellness of humanity, with herbal pharmaceuticals serving as the fundamental component of these traditional remedies. Herbal medications are widely relied upon by people worldwide, particularly in under developed nations (Mehta and Dhapte, 2016; Mehta *et al*., 2015). Standardising herbal formulations is essential for assessing the quality and purity of pharmaceuticals by analysing the concentration of their active ingredients. Standardisation is an essential technique for evaluating the quality and purity of a medication, as well as guaranteeing its efficacy (Yadav and Dixit, 2008).

The extractive values are crucial for identifying the chemical components or therapeutically active secondary metabolites present in crude and compound medications. They also help in estimating the precise components that are soluble in a given solvent. The variation in the material that can be extracted using various solvents suggests that a variety of internal and external factors influence the bioactive principle of medicinal plants. Polar compounds, including phenols, tannins, and glycosides, are visible in samples with ethanol and aqueous extractive. Consequently, the extraction values are vital in determining the standard for any medicine (Baravalia *et al*., 2011).

The moisture and volatile matter percentage is calculated by quantifying the loss on drying (LOD) content. This provides information about the extent to which the medicine may be adulterated with moisture. The LOD concentration of medications varies due to the hygroscopic nature of most vegetable drugs. An abundance of moisture fosters an ideal habitat for the proliferation of bacteria and fungi. The qualitative assessment of phytoconstituents in SDH is an initial analysis that helps identify bioactive compounds. It contains details regarding the phytoconstituents present in different solvent extracts. A plant extract has the potential to contain multiple secondary metabolites, however the phytochemical examination will only uncover a limited range of the extract's components. The current investigation on SDH involved conducting phytochemical screening and qualitative analysis on different solvent extracts, including alcoholic, chloroform, and aqueous solutions, to determine the types of phytoconstituents present in the formulation. The analysis revealed the existence of alkaloids, glycosides, carbohydrates, resin, saponins, starch.

Thin layer Chromatography (TLC) is the process of physically separating distinct components from a combination of herbs. It is a crucial step in order to identify and evaluate the bioactive component from the drug. HPTLC is a powerful analytical method that can efficiently separate and measure a wide range of compounds, even from a complex mixture. The HPTLC technique is also frequently used for product identification, adulterant detection, and quality control. The benefit of HPTLC is that multiple samples can be performed at once while requiring a less mobile phase. Therefore, HPTLC technique is essential for determining the quantity of chemical entities present in the extract and can give quantitative peak characteristics.

Pesticide is any agent used to prevent, eliminate, or deter pests from causing damage. A pesticide can be an insecticide, fungicide, molluscicide, *etc*., depending on the target pest. Most pesticides' principal areas of action are the neurological and endocrine systems, making them potentially harmful to people with substantial direct or indirect health consequences. Herbal remedies can potentially contain pesticide residues that have built up *via* agricultural practices

like spraying, soil treatment during production, and fumigant administration during storage. As a result, these residues should be within acceptable limits (Grover *et al*., 2014).

Antimicrobial agents are powerful medicines that treat various infectious ailments caused by microorganisms and have successfully saved millions of lives. As safe and effective antimicrobial agents evaluated from herbal products can be used as a scientific action plan to control the problem that occurs due to drug-resistant pathogens. Therefore, a systematic and scientific screening of antimicrobial activity of compound formulation SDH was done.

Antioxidants are compounds that impede the process of oxidation in other molecules, and they are extensively employed in dietary supplements. Antioxidant activity refers to the overall ability of antioxidants to remove free radicals from cells and food. Polyphenols exhibit remarkable antioxidant effects due to the inclusion of a 32-42 dihydroxy group in their B ring and the galloyl ester in the C ring of flavonoids. These structures are essential for the process of metal ion chelation (Hur *et al*., 2014).

5. Conclusion

The SDH was standardized in this investigation. The standardisation procedure commenced with the acquisition of the raw pharmaceuticals and the establishment of Standard Operating Procedures (SOPs) for the manufacture of the SDH. Following the preparation of the formulations, various quality parameters were assessed, including organoleptic characteristics, bulk density, tapped density, and physicochemical evaluation such as ash values, pH values, loss in weight at 105^{0}C, and extractive values in different solvents based on their polarity. HPTLC fingerprinting was conducted to present the fingerprint pattern for SDH which will be used as a reference for standardization and identification purposes. The pesticide residue was also examined to ensure that it did not exceed the permissible limit. The antimicrobial and antioxidant activity of the SDH was also conducted. The results of the standardisation are documented and can be utilised by the pharmacy as a quality control measure in the future. The formulation's antioxidant activity supports its efficacy and potential use in treating various ailments. The outcomes of this study will offer initial data for quality control. However, it is recommended to standardise SDH by using particular parameters for quantitative assessments of secondary metabolites with modern methodologies. A clinical trial can be established to study the scientific basis for proving the antioxidant activity and other therapeutic effects.

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Conflict of interest

The authors declare no conflicts of interest relevant to this article.

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