Journal of Pharmaceutical Research International

33(59B): 1-7, 2021; Article no.JPRI.79945
ISSN: 2456-9119
(Past name: British Journal of Pharmaceutical Research, Past ISSN: 2231-2919, NLM ID: 101631759)

Phytochemical Screening and *In-vitro* Free Radical Scavenging Activity of Unani Formulation Habb-e-Asgand

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

Article Information

DOI: 10.9734/JPRI/2021/v33i59B34344

Open Peer Review History:
This journal follows the Advanced Open Peer Review policy. Identity of the Reviewers, Editor(s) and additional Reviewers, peer review comments, different versions of the manuscript, comments of the editors, etc are available here: https://www.sdiarticle5.com/review-history/79945

Original Research Article

Received 10 October 2021
Accepted 14 December 2021
Published 17 December 2021

ABSTRACT

Aim: In the present work we aimed to perform phytochemical Screening and In-vitro free radical scavenging activity of Unani formulation Habb-e-asgand.
Study Design: The present work was designed to determine the total antioxidant capacity (TAC), Ferrous reducing antioxidant capacity assay (FRAC), DPPH radical scavenging assay, hydroxyl radical scavenging activity, and nitric oxide scavenging assay.
Place and Duration of the Study: The present work has been carried out at Ali-allana College of Pharmacy, Akkalkuwa, from February-2020 to November-2020.
Methodology: Habb-e-asgand (HEA), a Unani polyherbal drug used in arthritis, gout, and joint pain, is a blend of many herbal medicinal plants. Scientific attempts to test and validate its effectiveness are scarce. The selected Unani formulation was evaluated for DPPH radical scavenging activity, hydroxyl radical scavenging activity, and nitric oxide scavenging assay. The given formulation also evaluated the total antioxidant capacity and the reducing antioxidant capacity of ferrous metals.
Results: It was found that DPPH radical scavenging activity of HEA and BHT at a concentration of 100 µg/ml was 95±0.74 and 59±0.94 µg/ml. Hydroxyl radical scavenging activity of HEA, at the concentration of 100µg/ml, was 55±0.64, whereas at the same concentration catechin was 63±0.84 µg/ml. The IC_{50} value for nitric acid scavenging activity was found to be 49.60±1.57 for HEA, and

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186.34 ± 3.28 µg/ml for ascorbic acid. The result obtained from TAC and FRAC activity demonstrates that HEA possessed the highest TAC and FRAC values in comparison to standards catechin and ascorbic acid.

Conclusion: From the above results it was concluded that HEA has a potent antioxidant activity. HEA showed moderate to high TAC and FRAC in dose-dependent manner compared to standards catechin and ascorbic acid. HEA shows dose-dependent DPPH and hydroxyl radical scavenging activity. Nitric oxide generation was observed after the incubation of solutions of sodium nitroprusside in PBS at 25°C for 150 min. HEA effectively reduced the generation of nitric oxide.

Keywords: Habb-e-asgand; DPPH scavenging activity; polyherbal; phytochemical screening; unani; etc.

ABBREVIATIONS

AA : Ascorbic Acid
BHT : Butylated hydroxytoluene
CC : Catechin
DPPH : 2, 2-Diphenyl-1-Picrylhydrazyl
FRAC : Ferrous Reducing Antioxidant Capacity
HEA : Habb-e-asgand
NO : Nitric Oxide
TAC : Total Antioxidant Capacity

1. INTRODUCTION

Most of Unani's medications are herbal, mineral, or a combination of the two. Most of these drugs are developed for specific indications, but some have general tonic and rejuvenating properties [1,2]. In the absence of effective treatment for liver ailments. Unani drugs have been extensively studied to develop innovative therapies for liver disease and drug-induced liver toxicity [3,4]. Most Unani drugs are safe, but toxicity, including liver injury, has also been observed [5,6]. Furthermore, there are very few scientific attempts to certify and validate the safety of Unani's drugs [7,8]. Therefore, the numerous studies reported have evaluated the hepatoprotective and antioxidant potential of many Unani drugs.

Habb-e-asgand is a popular herbal preparation prescribed for arthritis, gout, and joint pain [9]. It is also known to have aphrodisiac properties [9]. Habb-e-asgand contains *Withania somnifera* (L) Dunal (Solanaceae) (English name: winter cherry, Hindi name: ashwagandha) as the main constituent of Table 1, known for its various medicinal uses [10]. Briefly, Habb-e-asgand therapy comes in small, round, uniformly shaped pills made with the ingredients of the formulation composition shown in Table 1. The blend included Ajwain Desi [*Ptychotis ajowan* DC (Apiaceae) seeds], Asgand Nagauri (*W. somnifera*), Chob Bidhara [wood of *Gmelina Asiatica* L. (Lamiaceae)], Pippl Kalan-Desi [Fruit of *Piper longum* L. (Piperaceae), dried immature], Pipla Mool (the root of *P. longum*) Moosli Siyah [*Curculigo orchioides* Gaertn. (Hypoxidaceae) stem], Satawar [*Asparagus racemosus* Willd. (Asparagaceae)), Zanjabeel-Khushk [rhizome of *Zingiber officinalis* Roscoe (Zingiberaceae)) and Qand Siyah Kohna [*Saccharum officinarum* L. (Poaceae)] as basic ingredients [9]. The high content of Qand Siyah Kohna is attributed to its use as a coating material in the preparation of pills.

Table 1. Constituents of Habb-e-asgand

Sr. No	Plant Common Name	Plant Botanical Name
1	Ajwain Desi	*Ptychotis ajowan*
2	Asgand Nagauri	*Withania somnifera*
3	Chob Bidhara	*Gmelina Asiatica*
4	Moosli Siyah	*Curculigo orchiodes*
5	Pippl Kalan (Desi)	*Piper longum*
6	Pipla Mool	*Piper longum*
7	Satawar	*Asparagus racemosus*
8	Zanjabeel (Khushk)	*Zingiber officinale*
9	Qand Siyah Kohna	*Saccharum officinarum*

A review of the literature revealed that the scientific study of the *In-vitro* free radical scavenging activity of Unani formulation Habb-e-asgand was not previously conducted to evaluate the formulation traditional claim as an antioxidant. The main objective of the present research work is to study phytochemical screening and *in-vitro* free radical scavenging activity of Unani formulation Habb-e-asgand.

2. MATERIALS AND METHODS

2.1 Chemicals and Reagents

Folin Ciocalteu reagent, 2, 2-diphenyl-1-picryl hydrazyl radical (DPPH), phenazine methosulfate, nicotinamide adenine dinucleotide, sodium nitroprusside (SNP), trichloro acetic acid (TCA), thio barbituric acid (TBA), and L-ascorbic acid were purchased from Lab Trading Laboratory Aurangabad. Catechin and rutin were purchased from Sigma Chemicals, India. All other chemicals and solvents used were of analytical grade available commercially. The Habb-e-asgand formulation was procured from local Hamdard chemist shop with batch number: OKM0067.

2.2 Physico-chemical Analysis of Habb-e-asgand

The specification of the Habb-e-asgand mixture was evaluated by performing physicochemical analyzes of appearance, color, taste, odor, pH value, friability, hardness, weight change, disintegration time, etc.

2.3 Preparation of Aqueous Extracts

Water-soluble extract: The Habb-e-asgand tablets were triturated and the 5 g of the air-dried mixture was macerated and coarsely sprayed with 100 ml of water in a closed flask for 24 h with frequent agitation for 6 h and allowed to stand for 18 h. The extract was quickly filtered with precautions due to loss of solvent. A 25 ml aliquot of the filtrate was evaporated to dryness on a flat-bottomed tarred dish at 105^0C. The percentage of water extraction was calculated concerning the air-dried drug [10,11].

2.4 Preliminary Phytochemical Screening of the Extract

The extract of Habb-e-asgand leaves obtained during the extraction process was subjected to preliminary phytochemical examination for the presence of various phytoconstituents using reported methods [12].

2.5 *In vitro* Models

2.5.1 Determination of total antioxidant capacity (TAC)

TAC of samples was determined by the previously described method [13]. This assay is mainly based on the reduction of Mo (VI) to Mo (V) by the drugs/samples and thus the formation of green-colored phosphate/ Mo (V) complex at acidic pH. Samples/standard approx. 0.5 ml at different concentrations of 12.5-150 µg/ml was mixed with 3 ml of a mixture containing 0.6 M sulphuric acid, 28 mM sodium phosphate, and 1% ammonium molybdate. The test tubes containing the above mixtures were incubated at 95^0C for 10 min so that the reaction can be completed. After cooling the reaction mixture at room temperature, the absorbance of the sample was measured at 695 nm using a spectrophotometer against a blank solution. Catechin was used as a standard. A blank solution was prepared with 3 ml of the reaction mixture and the same volume of the solvent was added which was used for samples/standard. The blank was also incubated for 10 min at 95^0C followed by measurement of absorbance at 695nm. Increased absorbance indicates increased total antioxidant capacity. Standard/samples were used at five different concentrations ranging from 12.5 to 150 µg/mL for each antioxidant assay. Concentrations were selected based on trial and error to fit the concentration that can represent the rational change of antioxidant activity with increasing concentration of samples.

2.5.2 Ferrous reducing antioxidant capacity assay (FRAC)

FRAC of samples was evaluated by the previously described method [13]. The Fe^{2+} can be monitored by measuring the development of Perl's Prussian blue at 700 nm. 0.25 ml of standard/sample solutions at different concentrations of 12.5-150 µg/ml, 0.625 ml of potassium buffer (0.2 M), and 0.625 ml of 1% potassium ferricyanide solution were added into the test tubes. The above reaction mixtures were incubated for 20 min at 50^0C to complete the reaction. Then 0.625 ml of 10% trichloroacetic acid solution was added to the test tubes. The above mixture was centrifuged at 3000 rpm for

10 min, later which 1.8 ml supernatant was withdrawn from the test tubes and mixed with 1.8 ml of distilled water and 0.36 ml of 0.1% ferric chloride solution. The absorbance was measured at 700 nm using a spectrophotometer against blank. A blank solution contained the same reaction mixture without the sample/standard and was incubated under the same conditions and the absorbance was measured at 700 nm. Increased absorbance of the reaction mixture indicates increased reducing capacity. The experiment was repeated three times at each concentration.

2.6 Free Radical Scavenging Activity

2.6.1 DPPH radical scavenging assay

The free radical scavenging ability of the drugs was tested by DPPH radical scavenging assay as previously described method [13]. The hydrogen atom donating ability of the drugs was determined by the decolorization of methanol solution of 2, 2-diphenyl-1-picrylhydrazyl (DPPH). DPPH produced purple/violet color in methanol solution and fades in presence of antioxidants to shades of yellow color. A solution of 0.1 mM DPPH in methanol was prepared, and 2.4 ml of this solution was mixed with 1.6 ml of extract in methanol at varied concentrations of 12.5 to 150 µg/ml. The solution mixture was mixed thoroughly and left in dark at room temperature for 30 min. The absorbance was measured spectrophotometrically at 517 nm. Butylated hydroxytoluene (BHT) was used as a reference. Percentage of DPPH radical scavenging activity was calculated by the following equation:

$$\%\text{DPPH radical scavenging activity} = [(A_0-A_1)/A_0] \times 100 \quad (1)$$

Where A_0 is the absorbance of the control, and A_1 is the absorbance of the drugs/standard. Then % of inhibition was plotted against concentration, and from the graph, IC50 was calculated. The experiment was repeated three times at each concentration.

2.6.2 Hydroxyl radical scavenging activity

Hydroxyl radical scavenging activity of the drugs was determined by the previously described method [13]. Hydroxyl radical was generated by the Fe^{3+}-ascorbate-EDTA-H_2O_2 system (Fenton reaction). The assay is based on the quantification of the 2-deoxy-D-ribose degradation product, which forms a pink chromogen upon heating with TBA at low pH. The reaction mixture contained 0.8 ml of phosphate buffer solution (50 mmol L^{-1}, pH 7.4), 0.2 ml of drug/standard at different concentrations of 12.5-150 µg/ml, 0.2 ml of EDTA (1.04 mmol L^{-1}), 0.2 ml of Fecl3 (1 mmol L^{-1}) and 0.2 ml of 2-deoxy-D-ribose (28 mmol L^{-1}). The mixtures were kept in a water bath at 37^0C and the reaction was started by adding 0.2 ml of ascorbic acid, AA (2 mmol L^{-1}), and 0.2 ml of H_2O_2 (10 mmol L^{-1}). After incubation at 370c for 1 h, 1.5 ml of HCL (25%). The reaction mixture was heated at 100^0C for 15 min and then cooled down with water. The absorbance of the solution was measured at 532 nm with a spectrophotometer. The hydroxyl radical scavenging capacity was evaluated with the inhibition of the percentage of 2-deoxy-D-ribose oxidation on hydroxyl radicals. The percentage of hydroxyl radical scavenging activity was calculated according to the following formula:

$$\%\text{ hydroxyl radical scavenging activity} = [A_0-(A_1-A_2)] \text{ X } 100/A_0 \quad (2)$$

Where A0 is the absorbance of the control without a sample. A_1 is the absorbance after adding the sample and 2-deoxy-D-ribose. A_2 is the absorbance of the sample without 2-deoxy-D-ribose. Then % of inhibition was plotted against concentration, and from the graph, IC_{50} was calculated. The experiment was repeated three times at each concentration.

2.6.3 Nitric oxide scavenging assay

The method was performed according to the process previously described [14]. 2 ml of 10mM sodium nitroprusside solution which was prepared in phosphate buffer saline (PBS) pH 7.4 was mixed with 0.5 ml of drug solutions at various concentrations ranging from 10 to 50 µg/ml and ascorbic acid at concentrations ranging from 25 to 200 µg/ml. This mixture was incubated at 25 ^{0}C. After 150 min, 0.5 ml of incubation solution was collected which was then mixed with 0.5 ml of Griess reagent [1.0 ml sulfanilic acid reagent (0.33% prepared in 20% glacial acetic acid at room temperature for 5 min 1 ml of naphthyl ethylene diamine dihydrochloride (0.1% w/v)]. The mixture was incubated at room temperature for 30 min, followed by the measurement of absorbance at 540 nm using a spectrophotometer (Shimadzu UV-1800).

3. RESULTS AND DISCUSSION

3.1 Physico-chemical Analysis of Habb-e-asgand

The Habb-e-asgand pills were brown and spherical (solid) with a distinctive Asgand taste and smell. The pills were stored in a cool, dark place in tightly closed containers, protected from moisture, light, and temperature. Physico-chemical analysis showed a slightly basic pH of 6.5 as shown in Tables 2 and 3.

Table 2. Organoleptic character

Sr. No	Parameters	Observation
1	Size	Round
2	color,	Light Brown
4	Taste,	Pungent
5	Appearance	Habb (Pills)

Table 3. Physiochemical character

Sr. No	Parameters	Habb-e-agand
1	Friability Test	0.219%
2	Hardness Test	12.5 kg/cm ^{2} (Monsanto)
3	Weight Variation	0.0668
4	Disintegration time	55 min
5	the pH of 1% Solution	6.5
6	10% Solution	5.2

3.2 Preliminary Phytochemical Screening of the Extract

The medicinal properties of Habb-e-asgand pills are perhaps due to the presence of various secondary metabolites such as alkaloids, tannins, flavonoids, proteins, and mucilages. Therefore, preliminary screening tests can be useful in the detection of bioactive ingredients and can subsequently lead to drug discovery and development. Furthermore, these tests facilitate their quantitative estimation and qualitative separation of pharmacologically active chemical compounds [15]. Preliminary phytochemical screening with various qualitative chemical tests revealed the presence of carbohydrates, reducing sugars, protein, amino acid, alkaloids, glycosides, flavonoids, saponins, fats & oils, steroids, and tannins in the Habb-e-asgand extract.

3.3 Determination of TAC and FRAC

The TAC and FRAC of HEA are shown in Table 4. HEA showed a considerable antioxidant activity compared to standard catechin. At the concentration of 100µg/ml, the absorbance of HEA was in the range of 0.584±0.053 to 1.89±0.076; while at 150µg/ml the range was 0.874±0.043 to 2.394±0.064. Total antioxidant activity was increased with an increase in the dose of the drugs.

HEA showed moderate to high FRAC with increased concentrations of the drugs. At 100µg/ml, the absorbance was in the range of 0.975±0.056 to 3.409±0.065, while at 150 µg/ml, the range was 1.345±0.086 to 3.225±0.067. These results demonstrate that HEA possessed the highest TAC and FRAC values, thus closely resembling that of the standards catechin and ascorbic acid.

3.4 Determination of DPPH Radical Scavenging Activity of HEA

Table 5 shows the free radical scavenging activity of HEA. At a concentration of 100 µg/ml, the scavenging activity of HEA and BHT was 95±0.74 and 59±0.94µg/ml. The extract showed concentration-dependent antioxidant activity by inhibiting the DPPH radical with a good inhibition value. The method is based on the reduction of the DPPH solution in the presence of a hydrogen donor antioxidant, due to the formation of the non-radical form DPPH-H by the reaction. The extract was able to reduce the stable DPPH radical to the yellow diphenyl picrylhydrazine. Ascorbic acid has been found to reduce and discolor 1,1-diphenyl-2-picrylylhydrazyl due to its ability to donate hydrogen. HEA extract appears to have the ability to donate hydrogen and act as an antioxidant. The elimination effect increases with the increasing concentration of the extract.

3.5 Determination of Hydroxyl Radical Scavenging Activity of HEA

Table 6 shows the hydroxyl radical scavenging activity of HEA and present drugs have shown a dose-dependent activity. The hydroxyl radical is a highly reactive oxygen-centered radical formed by the reaction of various hydroperoxides with transition metal ions. Attacks proteins, DNA, polyunsaturated fatty acids on membranes, and most biological molecules. At the concentration of 100 µg/ml, the scavenging activity of HEA was 55±0.64, whereas at the same concentration catechin was 63±0.84 µg/ml.

Table 4. Absorbance HEA at two different concentrations (n=3, X ± SEM)

Drugs	TAC		FRAC	
	At 100 µg/ml	At 150 µg/ml	At 100 µg/ml	At 150 µg/ml
HEA	1.378 ± 0.036[a]	1.784 ± 0.019	2.235 ± 0.137	2.986 ± 0.107
CA	1.89 ± 0.076	2.394 ± 0.064	-	-
AA	-	-	3.409 ± 0.065	3.225 ± 0.067

Where, CA and AA represent catechin and ascorbic acid; [a]n each value is the average of three analyses ± standard deviation

Table 5. Determination of DPPH radical scavenging activity of HEA

Concentration (µg/ml)	BHT	HEA
0	0	0
12.5	80	65
25	93	73
50	95	79
100	95	90
150	95	90

Table 6. Determination of hydroxyl radical scavenging activity of HEA and CA

Concentration (µg/ml)	CA	HEA
0	0	0
12.5	16	36
25	22	40
50	37	50
100	63	55
150	84	63

3.6 Determination Nitric Oxide Scavenging Assay

The extract showed moderate nitric oxide scavenging activity in a dose-dependent manner (IC50 = 49.60 µg/ml). The plant/plant products may have the property of counteracting the effect of NO formation and, in turn, may be of considerable interest in preventing the harmful effects of excessive NO production in the human body. Furthermore, the elimination activity can also help stop the chain of reactions initiated by the excessive generation of NO harmful to human health. The extract showed moderate nitric oxide scavenging activity [16]. The% inhibition increases with increasing concentration of the extract. NO generation was observed after the incubation of solutions of sodium nitroprusside in PBS at 25 ^{0}C for 150 min. HEA effectively reduced generation NO. The IC50 was found to be 49.60±1.57 for HEA, and 186.34±3.28 µg/ml for ascorbic acid Table 7.

Table 7. IC_{50} value for *in-vitro* nitric acid scavenging activity

Sr. No	Sample	Nitric oxide (µg/ml)
1.	HEA	49.60±1.57
4.	Ascorbic acid	186.34±3.28

4. CONCLUSION

To characterize the antioxidant activity of HEA extracts, it is desirable to subject it to a battery of tests evaluating the range of activities such as hydroxyl radical scavenging activity test, nitric oxide scavenging activity test, free radical scavenger DPPH in formulation by Habb-e-asgand. The *in-vitro* antioxidant activities of the aqueous extract have indicated the effectiveness of the formulation as a source of natural antioxidants that will be used to reduce oxidative stress with consequent health benefits. The activity of Habb-e-asgand could be due to the presence of different polyherbal constituents, which is a known potent antioxidant. Further research is needed for the isolation and characterization of the phytoconstituents responsible for its antioxidant property.

DISCLAIMER

The products used for this research are commonly and predominantly use products in our area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. Also, the research was not funded by the producing company rather it was funded by personal efforts of the authors.

NOTE

The study highlights the efficacy of "sigma, Unani" which is an ancient tradition, used in some parts of India. This ancient concept should be carefully evaluated in the light of modern

medical science and can be utilized partially if found suitable.

CONSENT

It is not applicable.

ETHICAL APPROVAL

It is not applicable.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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Peer-review history:
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