Annals of Phytomedicine 10(2): 448-455, 2021

DOI: http://dx.doi.org/10.21276/ap.2021.10.2.59

Annals of Phytomedicine: An International Journal

http://www.ukaazpublications.com/publications/index.php

Print ISSN : 2278-9839 Online ISSN : 2393-9885

Original Article : Open Access

Extraction, phytochemical analysis and *in silico* antidepressant studies of aqueous extract of leaves of *Hibiscus sabdariffa* L.

Mohd Mohiuddin Shareef and E. Bhavya◆

School of Pharmaceutical Sciences, Vels Institute of Science, Technology and Advanced Studies, Pallavaram, Chennai-600117, Tamil Nadu, India

Article Info

Article history

Received 1 October 2021

Revised 15 November 2021

Accepted 16 November 2021

Published Online 30 December 2021

Keywords

Delphinidin

FTIR

HPLC

GC-MS

2z5y protein

Abstract

The aim of study is to extract *Hibiscus sabdariffa* L. leaves, evaluate their phytochemical properties, and screen *in silico* antidepressant effects. It is commonly called as roselle belongs to family Mallows or Malvaceae. This family has more than 200 genera and more than 4000 species. *H. sabdariffa* leaves are collected, and extracted by using different solvents, *viz.*, petroleum ether, chloroform, ethyl acetate, ethanol and water. Extracts obtained are explored for their characteristic features. Among these extracts, AEHS was selected for further analysis by FTIR, HPLC and GC-MS techniques. Further, four phytoconstituents from *H. sabdariffa*, *viz.*, beta-pinene, cyanidin, delphinidin, P-cymene are subjected for *in silico* antidepressant evaluation by using MAO-A, 2z5y protein. All the extracts obtained are brown to black in color and semisolid in nature. % Yield was highest for AEHS (12.4%). Phytochemical analysis showed the presence of various constituents like alkaloids, glycosides, tannins, flavonoids, anthocyanins, and phytosterols. AEHS FTIR spectrum showed the functional groups, *viz.*, 1111 cm^{-1} and 1053 cm^{-1} corresponding to anthocyanins. HPLC analysis of AEHS also revealed the delphinidin and cyanidin related peaks with retention time of 22.81 min and 24.97 min, respectively. GC-MS analysis of AEHS showed the presence of different phytoconstituents. Docking studies reported ligand protein interactions. Beta-pinene and p-cymene has not showed interaction with amino acids of 2z5y protein. Therefore, these molecules are reported with weak dock score and low EModel energy. Whereas, cyanidin and delphinidin showed interaction with GLN (215), MET (445), and TYR (69) amino acids of MAO-A, 2z5y protein indicating their inhibitory action. Delphinidin with highest EModel energy, shows maximum *in silico* antidepressant effect by inhibiting MAO-A, 2z5y protein.

1. Introduction

For thousands of years, plants have been utilised to treat human ailments. It appears that Neanderthal man valued herbs as medical agents about 60,000 years ago; this conclusion is based on pollen grains from eight medicinal plants discovered in a cemetery in Iran (Solecki and Shanidar, 1975). The usage of medicinal herbs is not a relic of a bygone era. Approximately, 90% of the world's population still uses raw herbs and unprocessed extracts as their sole source of medication (Duke, 1985). According to a 1997 survey, 23% of Canadians have taken herbal medications. Furthermore, plant components are found in up to 25% of modern pharmaceutical medications (Duke, 1983). Around 8,000 plant species are used in Indian medicine, including trees (33%), herbs (32%), shrubs (20%), climbers (12%), and epiphytes, grasses, lichens, ferns, and algae combined (3%).

H. sabdariffa belonging to family Malvaceae, is one of the plants that has been used in traditional medicine for a long time, indicating that it contains bioactive components. This plant contains different constituents like, hydroxycitric acid, hibiscus acid, malic acid, tartaric acid (Yamada *et al*., 2007), cyanidin-3-sambubioside, cyanidin-3diglucoside, delphinidin-3sambubioside (hibiscin), gossypin, hibiscitrin (hibiscetin-3 glucoside), kaempferol-3-Orutinoside, myricetin, sabdaritrin, chlorgenic acid, pelargonidic acid, protocatechuic acid. Their pharmacological and toxicological properties, as well as their chemical composition, have all been extensively explored in recent years, indicating a growing interest in the characteristics of plants in human-dominated environments. This plant, which originated in tropical and subtropical regions, has spread throughout the globe, appearing in Asia (China, Thailand), Africa (Senegal, Egypt, Mali), Central America (Mexico, Jamaica, Panama), and even Europe (Da Costa Roca *et al*., 2014; Mohamed *et al*., 2012).

They are high in anthocyanins, flavonoids, vitamins, and microelements, according to studies conducted by Allarcon *et al*. (2012) and Peng *et al*. (2011). The genus contains approximately 300 species that thrive in all parts of the world and can withstand temperatures of over 20°C. When ingested as medication or as part of a diet, the chemical content of the plant has therapeutic benefits. The plant has good effects on artery pressure, contributes to faster wound healing, is diuretic, antidiabetic, depressive, solves a variety of genital issues, plays a role in cholesterol stability, and is anticancerogenic when consumed as tea (Frank *et al*., 2012; Lin *et al*., 2012). Delphinidin-3-sambubioside and cyanidin-3-sambubioside, anthocyanins extracted from the calyces of *H. sabdariffa*, exhibit substantial antioxidant and chemoprotective

Corresponding author: Dr. E. Bhavya

Associate Professor, School of Pharmaceutical Sciences, Vels Institute of Science, Technology and Advanced Studies, Pallavaram, Chennai-600117, Tamil Nadu, India

E-mail: mohd_masud@yahoo.com

Tel.: +91-9502039980

activities against hepatotoxicity caused bytertiary-butyl hydroperoxide (Wang *et al*., 2000). Flavonoids and anthocyanins have been linked to this extract's cardioprotective properties (Jonadet *et al*., 1990). By considering all these beneficial values present study is designed to extract, screen phytochemical analysis and *in silico* antidepressant activity of *H. sabdariffa*.

2. Materials and Methods

2.1 Materials

H. sabdariffa leaves were collected from in and around of Hyderabad in the month of Jan 2020. Plant material was subjected to authentication at Botanical Survey of India, Deccan region, Hyderabad. It was stored in Pharmacognosy Department with a Specimen No. MESCO_PCOG_2020_015. Chloroform, ethyl acetate, ethanol, petroleum ether are procured from SD Fine Chemicals Limited. All chemicals used in the study are of LR grade.

2.2 Methods

2.2.1 Extraction of *H. sabdariffa* leaves

As mentioned above, collection and authentication of *H. sabdariffa* leaves was carried out. These leaves are checked for any impurities, kept for drying, grinded to coarse powder and finally subjected to successive solvent extraction by using Soxhlet apparatus. Soxhlet extraction was done by using following procedure: Defatting by of plant material was done by using petroleum ether (40-60°C) (PEHS), then extraction was carried out by using different chemicals as chloroform (60°C) (CEHS), ethyl acetate (50-60°C) (EAEHS), ethanol (40-60°C) (EEHS), and aqueous (60-70°C) (AEHS). Obtained extracts were left to air dry and semisolid form was obtained after few days. % Yield was calculated for all the obtained extracts by using following formula:

$$\%\text{Yield} = \left(\frac{\text{Weight of extract obtained in grams}}{\text{Total weight of raw material}} \right) \times 100$$

2.2.2 Phytochemical analysis of *H. sabdariffa* leaves

Preliminary phytochemical assessment was carried out to identify the presence of alkaloids, cardiac glycosides, tannins, phytosterols, proteins, amino acids and flavonoids by using standard procedures as described by Khandelwal (2007) (Shaji *et al*., 2019). The fourier transform infrared spectrophotometer (FTIR) is one of the most powerful techniques for identifying functional groups in substances. For FTIR analysis, dried aqueous extract was utilised. To make a translucent sample disc, 10 mg of dried extract powder was encapsulated in 100 mg of KBr pellet. Each plant specimen's powdered sample was placed into an FTIR spectroscope (Shimadzu, IR Affinity1, Japan), which has a scan range of 400 to 4000 cm^{-1} and a resolution of 4 cm^{-1}. Further phytochemical evaluation of *H. sabdariffa* was performed by HPLC (Shimadzu Japan) analysis, by using C18 column. Instrument was operated at room temperature with 1ml/min flow rate. Mobile phase consists of 2 solvents, *viz*., 0.1% trifluoroacetic acid in water (Sol-A) and 0.1% trifluoroacetic acid in acetonitrile (Sol-B). 100 mg of extract was dissolved for 24 h with 5 ml of methanol (5°C). This solution of extract was centrifuged at 2500 rpm for 15 min, followed by collection of supernatants. This was filtered by using 0.45 µm millipore membrane. This collected further diluted with double distilled water. About 100 µl of filtrate was injected and analysis was conducted at 521 nm. Elution program for analysis is as: for first 5 min 5-15% of Sol-B, then for next 10 min, 15-25% Sol-B was allowed, followed by 25-100% Sol-B for next 15min, and finally 100% of Sol-B for 30-40 min chlorogenic acid was used as an internal standard (Imad Uddin *et al*., 2020).

To carry out GC-MS analysis for 5 h, 10 g of AEHS was dissolved in 95% ethanol. To eliminate the sediments and traces of water in the filtrate, the extract was filtered using Whatmann filter paper No.41 with 2 gm sodium sulphate. The filter paper and sodium sulphate were wetted with 95% ethanol before filtering. Both polar and non-polar phytocomponents of the plant material were present in the extract. This was subjected to GC-MS analysis by using "Trace DSQ GC-MS" analyzer. This equipment contains fused silica capillary column of length 30 m, thickness 0.25 µm and diameter 0.25 mm. With a constant flow rate of 1ml/min helium gas was used as carrier. Injection volume was 2 µl. Temperature of injector and ion-source was maintained at 250°C and 280°C, respectively. In mass spectrometer, scan interval was about 0.5 sec and fragments from m/z 50 to 500 Da was programmed. Temperature of inlet and source was set to be 280°C and 250°C, respectively. By comparing the average peak area of each component to the total areas, the relative percentage quantity of each component was computed. The GC-MS was performed by Central Instrumentation Research Laboratory, University College of Technology, Osmania University, Hyderabad.

2.2.3 *In silico* analysis of *H. sabdariffa* leaves aqueous extract

The computational structure of the MAO-A was acquired from the Protein Databank website with PDB Id: 2z5y in order to explore *in silico* antidepressant effects of AEHS. The structure was improved by removing unattached water molecules that were more than 1Å. The energy of the entire structure was minimised by utilising the OPLS-2005 force field and the protein preparation wizard tool of the Schrodinger suite. Hydrogen atoms are added to satisfy the valences. Thereafter, amino acids are added to stabilize side chains. Using the Glide Xp docking methodology, structurally optimised protein was used to evaluate protein-ligand interactions of the dataset ligands. To begin, a 3D grid was created around the protein's binding site, into which all of the dataset ligands were docked. Glide score, which is a mix of hydrophilic, hydrophobic, metal binding groups, Vander Waals energy, frozen rotatable bonds, and polar interactions with receptor, was used to calculate binding interactions and efficiency (Abubucker, 2021).

To carry out post docking calculations, binding energies of the docked complexes were calculated using the Prime MM/GBSA (molecular mechanics based generalised born/surface area) module of the Schrodinger suite. This energy is the combination of OPLS molecular mechanics energies (EMM), an SGB solvation model for polar solvation (GSGB), and a non-polar solvation term (GNP) containing non-polar solvent accessible surface area and Vander Waals interactions. The findings of docking were rescored using an energy function that included a well-defined description of binding contributions. The total free energy of binding is then expressed as per below mentioned equation:

$\Delta G_{bind} = G_{complex} - (G_{protein} + G_{ligand})$ where ΔG_{bind} is binding energy of ligand.

3. Results

3.1 Extraction, characteristic features and phytochemical analysis of *H. sabdariffa* leaves

Successive solvent extraction process was used to extract *H. sabdariffa* leaves. Different solvents, *viz*., petroleum ether, chloroform, ethylacetate, ethanol and water are used for extraction process. EAEHS was brown in color, whereas PEHS was brownish black and all other extracts, *viz*., CEHS, EEHS, and AEHS are black in color. All these extracts are semi solid in nature. % Yield was found to 12.4% for AEHS (highest) (Table 1). In *H. sabdariffa* leaves, screening was done with different chemical extracts and according to the standard tests. Presence of alkaloids was detected by using Mayer's and Wagner's test. Alkaloids are found to present in all extracts. Molisch and benedict's test was carried out to check the presence of carbohydrates and was reported positive with all extracts. Both legal's and killer killani test showed positive results for the presence of cardiac glycosides in CEHS, EAEHS, and AEHS. Iodine and gelatin tests are positive for presence of tannins in CEHS and AEHS. Similarly, Salkowski's test and Libermann Bouchard test showed positive results for the presence of phytosterols in CEHS, EEHS, and AEHS. Proteins and amino acids showed presence in CEHS and EAEHS with positive results for both Ninhydrin and Millon's test. Whereas, alkaline reagent and lead acetate tests showed positive results for the presence of flavonoids in EAEHS, EEHS, and AEHS, EEHS and AEHS are positive only for anthocyanins (Table 2).

Table 1: Characteristics of different extracts of *H. sabdariffa* leaves

Extract	Color of the extract	Consistency of the extract	Wt. of the extract (gm)	Wt. of sample (gm)	% Yield
PEHS	Brownish black	Semi-solid	100	2.5	2.5
CEHS	Black	Semi-solid	100	3.5	3.5
EAEHS	Brown	Semi-solid	100	3.9	3.9
EEHS	Black	Semi-solid	100	7.8	7.8
AEHS	Black	Semi-solid	100	12.4	12.4

Table 2: Phytochemical screening of different extracts of *H. sabdariffa* leaves

S.No.	Phytochemical constituents	PEHS	CEHS	EAEHS	EEHS	AEHS
1	**Alkaloids**					
	1. Mayers test	++	+-	+-	+-	++
	2. Wagners test					
2	**Carbohydrates**					
	1. Molish test	++	++	+-	++	+-
	2. Benidicts test					
3	**Cardiac glycosides**					
	1. Legals test	+-	++	++	+-	++
	2. Killerkallani test					
4	**Tannins**					
	1. Iodine test	+-	+-	++	+-	++
	2. Gelatin test					
5	**Phytosterol**					
	1. Salkowiskis test	+-	++	+-	++	++
	2. Libermann buchard test					
6	**Proteins and aminoacids**					
	1. Ninhydrin test	--	++	++	+-	+-
	2. Millons test					
7	**Flavonoids**					
	1. Alkaline reagent test	+-	+-	++	++	++
	2. Lead acetate test					
8	**Anthocyanins**	-	-	-	+	+

Based on these characteristic and phytochemical screening results, AEHS was selected for further studies. FTIR analysis of AEHS was conducted to evaluate the presence of various functional groups. Peak at 3441 cm^{-1} corresponds to asymmetric stretching vibration of primary amine, *i.e.*, $–NH_2$ and peak at 3387 cm^{-1} is responsible for symmetric stretching vibration of the same $–NH_2$ group. Two peaks, *viz.*, 3404 cm^{-1} and 3421cm^{-1}corresponds to –OH stretching or –NH stretching vibrations. Peaks at 2362$cm^{-1}$and 2347 cm^{-1} are representing O=C=O stretching vibrations. 1629 cm^{-1} peak corresponds to vibration stretching of C=O group, 1394 cm^{-1} and 1383 cm^{-1} corresponds to –OH bending vibrations. Whereas, peaks at 1111 cm^{-1} and 1053 cm^{-1} corresponds to anthocyanins (Figure 1). HPLC analysis of AEHS showed the presence of two peaks, *viz.*, 22.81 min and 24.97 min which corresponds to delphinidin and cyanidin, respectively. 27.39 min peak represents chlorogenic acid (Figure 2). GCMS analysis revealed the presence of different constituents. 1st peak with retention time (RT) of 0.021 min indicates 2-heptanamine, 5-methyl with % area of 0.51. Next peaks with RT of 1.121 min and 2.029 min indicate 1,3-propanediol, 2-methyl-2-nitro-, dinitrate and 1,2,3-propanetriol, 1,3-dinitrate, respectively. P-cymene a triterpenoid was reported with RT of 5.124 min. It has 1.46% area and molecular formula as $C_{10}H_{14}$. Similarly, RT at 8.059 min indicates beta-pinene in our study with 1.98 of % area and 93.57 m/z value. Peak at RT value of 9.331 min indicates delphinidin, which is having a %area of 1.73 and 300 as the m/z value. Cyanidine is an another important phytoconstituent reported in GC-MS analysis with RT at 17.12 and 280.05 as m/z value. 9-Octadecenoic acid or oleic acid is reported at 20.308 with %area of peak as 7.15. Other constituents which are reported are 2-acetylbenzoic acid, hexadecanoic acid, methyl ester and methyl E-11-tetradecenoate at RT values of 14.43, 18.215, and 44.145, respectively (Figure 3 and Table 3).

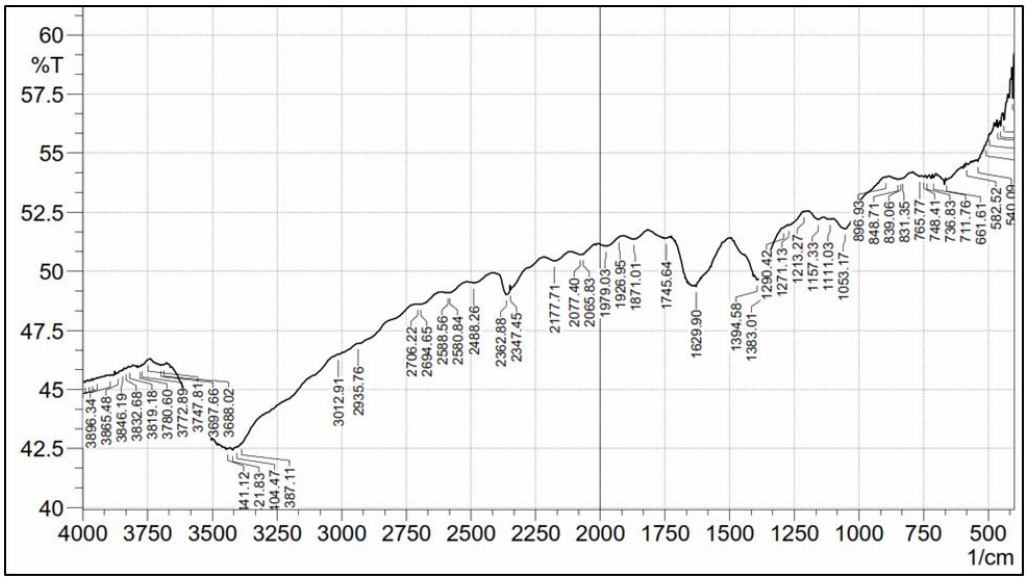

Figure 1: FTIR analysis of AEHS.

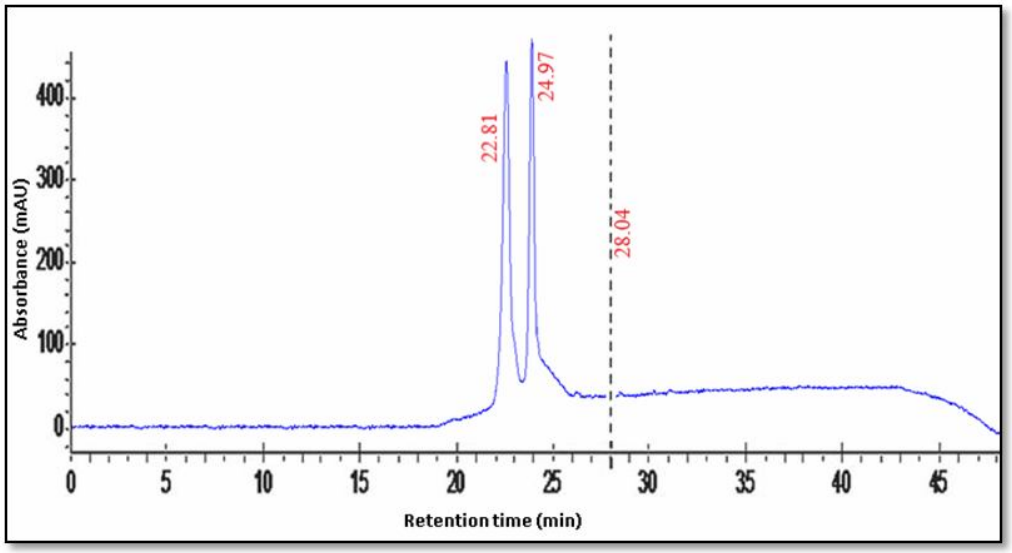

Figure 2: HPLC chromatogram of AEHS.

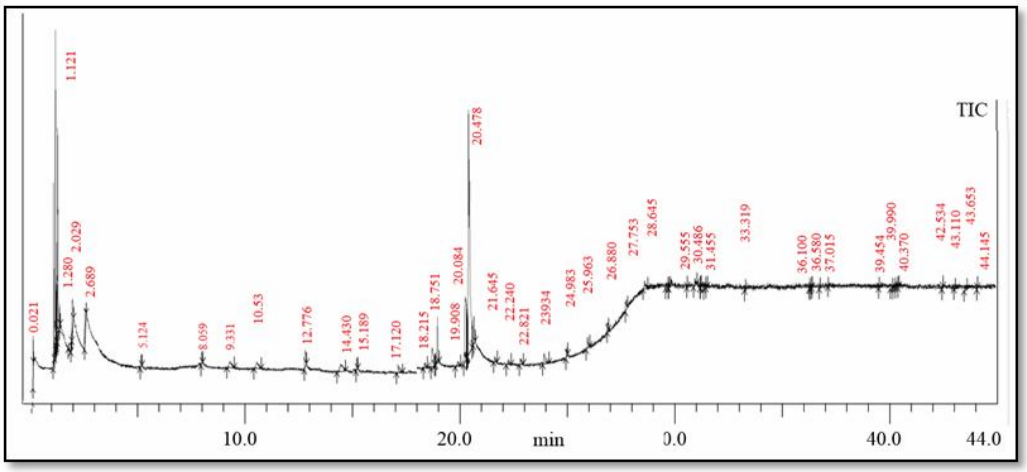

Figure 3: GC-MS analysis of AEHS.

Table 3: GC-MS analysis of AEHS

Peak	R. time	Name	Molecular formula	Molecular weight (g/mol)	Area %	Base m/z value
1	0.021	2-heptanamine, 5-methyl-	$C_8H_{19}N$	129.24	0.51	44.05
2	1.121	1,3-propanediol, 2-methyl-2-nitro-, dinitrate	$C_4H_7N_3O_8$	225.1	8.26	45.75
3	2.029	1,2,3-propanetriol, 1,3-dinitrate	$C_3H_6N_2O_7$	182.08	2.76	45.85
4	5.124	p-cymene	$C_{10}H_{14}$	134.2	1.46	85.16
5	8.059	Beta-pinene	$C_{10}H_{16}$	136.23	1.98	93.57
6	9.331	Delphinidin	$C_{15}H_{11}O_7$	303.2	1.73	300
7	10.533	Cyclohexasiloxane, dodecamethyl-	$C_{12}H_{36}O_6Si_6$	444.9	1.01	73.05
8	14.43	2-Acetylbenzoic acid	C_9H_8O	164.16	1.70	149.0
9	17.12	Cyanidine	$C_{15}H_{11}O_6$+	287.2	0.59	280.05
10	18.215	Hexadecanoic acid, methyl ester	$C_{17}H_{34}O_2$	270.45	0.33	74.00
11	20.308	9-Octadecenoic acid	$C_{18}H_{34}O_2$	282.47	7.15	55.05
12	44.145	Methyl E-11-tetradecenoate	$C_{15}H_{30}O_2$	242.40	0.13	252.85

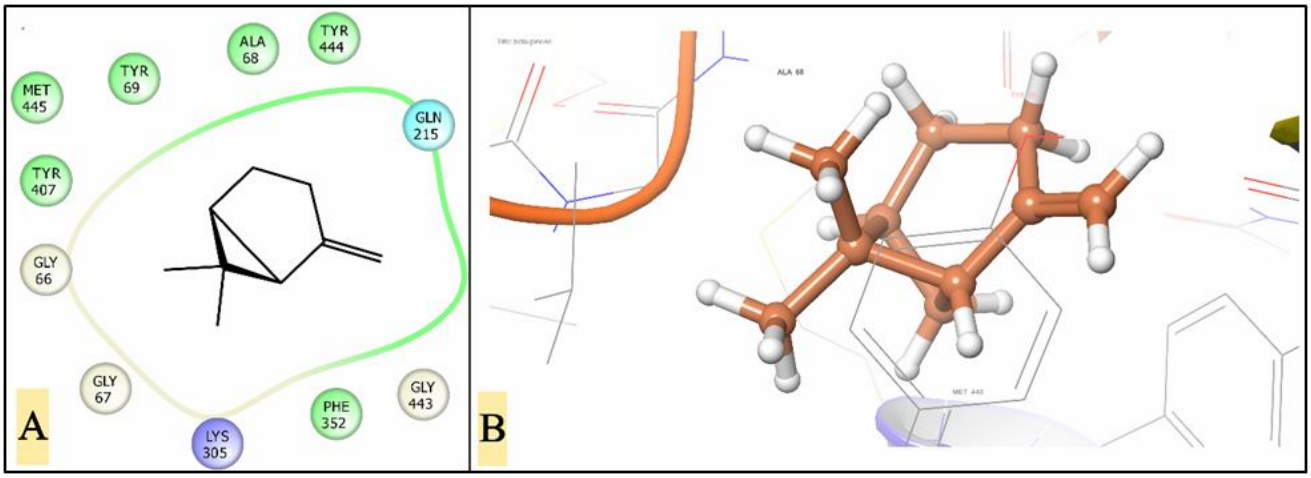

Figure 4: A-beta-pinenedoes not show binding interactions with amino acids of MAO-A, 2z5y protein. B-2D representation of beta-pinene, where it is not showing binding interactions with amino acids of MAO-A, 2z5y protein.

After successful characterization of AEHS by FTIR, HPLC and GC-MS analytical techniques, four constituents, *viz*., beta-pinene, cyanidin, delphinidin, and p-cymene are screened for *in silico* antidepressant efficacy by using MAO-A, 2z5y as a target protein. Glide XP module generates many protein ligand complexes for each protein ligand interaction but the complex having highest EModel energy is only represented in results. Table 4 explains about glide dock score, number of H-bonds, interacting amino acids, H-bond distance in Å, glide energy and EModel energy. Beta-pinene does not interact with any amino acid (Figure 4A and B), with very low dock score of –4.794 and a very low EModel energy of -11.61kcal/mol. Cyanidine have 3 hydrogen bonds and showed interaction with 3 amino acids, *viz*., GLN (215), MET (445), and TYR (69). Both dock score and EModel energy was found to be more than beta-pinene as –6.896 and –42.299 kcal/mol, respectively (Figure 5A and B). On the lead among four constituents, delphinidin have 5 hydrogen bonds and interacts with 4 different amino acids, *i.e*., GLY (443), GLN (215), MET (445), and TYR (69). It is also reported with highest dock score and EModel energy as –7.055 and –49.299 kcal/mol, respectively (Figure 6A and B). P-cymene with no hydrogen bond does not interact with any amino acids and have –5.43 as dock score and –26.454 kcal/mol as EModel energy (Figure 7A and B).

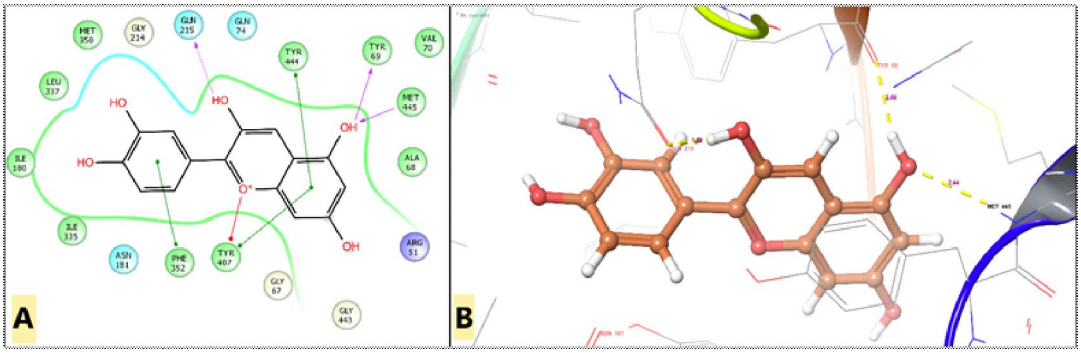

Figure 5: A-cyanidine shows binding interactions with GLN (215), MET (445), and TYR (69)amino acids of MAO-A, 2z5y protein. B-2D representation of cyanidine, where it is showing binding interactions with amino acids of MAO-A, 2z5y protein.

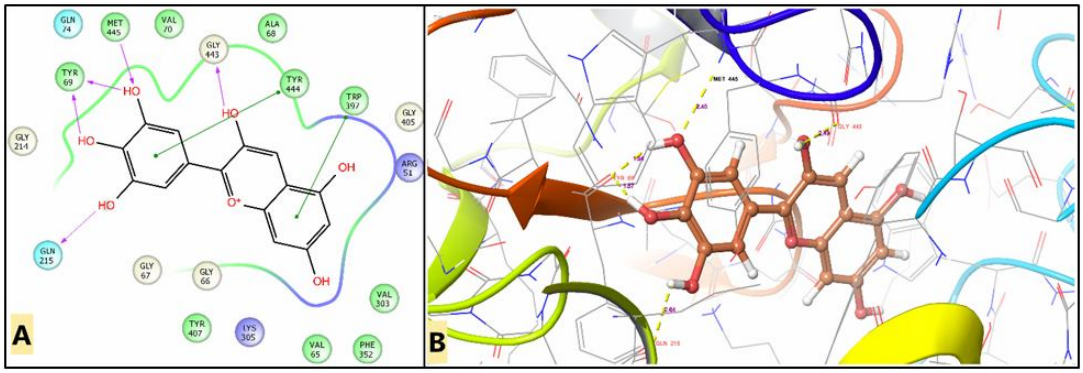

Figure 6: A-delphinidinshows binding interactions with GLY (443), GLN (215), MET (445), and TYR (69) amino acids of MAO-A, 2z5y protein. B-2D representation of delphinidin, where it is showing binding interactions with amino acids of MAO-A, 2z5y protein.

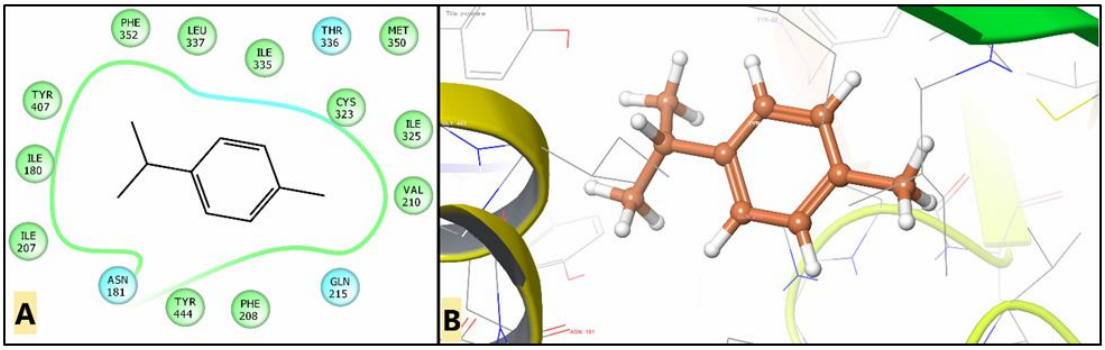

Figure 7: A-P-cymene does not show binding interactions with amino acids of MAO-A, 2z5y protein. B-2D representation of p-cymene, where it is not showing binding interactions with amino acids of MAO-A, 2z5y protein.

Table 4 : Docking results and protein-ligand binding interactions of different phytoconstituents of *H. sabdariffa* against MAO-A, 2z5y protein

Compound	Dock score	No. of H-bonds	Interacting amino acids	H-bond distance (Å)	Glide energy	EModel energy
Beta-pinene	–4.794	0	-	-	–17.421	–11.611
Cyanidin	–6.896	3	GLN 215	1.59		
			MET 445	2.44	–36.943	–42.299
			TYR 69	2.06		
Delphinidin	–7.055	5	GLY 443	2.49		
			GLN 215	2.64	–38.476	–49.492
			MET 445	2.45		
			TYR 69	1.94, 1.87		
P-cymene	–5.436	0	-	-	–21.878	–26.454

4. Discussion

The Malvaceae family includes *H. sabdariffa* (Roselle). In this plant 8-15 cm long, deeply three-to five-lobed leaves are placed alternately on the stalks. In English-speaking regions, rozelle, sorrel, red sorrel, and roselle are some of the common names for *H. sabdariffa*. Our study reported the good yield of ethanol and water extract as 7.8% and 12.4%, respectively. Different phytoconstituents are reported in various extracts of these leaves. Our study reported the presence of alkaloids which are in accordance with the results of Nkumah *et al.* (2015). Presence of phytosterols and glycosides reported in our study is also in accordance with the results of Aguirre-Garcia *et al.* (2019), where they reported the presence of many phytoconstituents like pigments (major pigment as cyanidine-3-glycoside), phytosterols, carbohydrates and glycosides in complete plant extract. Flavonoids a therapeutic potential phytoconstituent reported in our study is also reported in calyces by Alara *et al.* (2020).

FTIR spectroscopic analysis showed the presence of various functional groups in AEHS. In our study, two peaks 3441 cm^{-1} and 3387 cm^{-1} representing $–NH_2$ stretching was also reported by Fatoni *et al.* (2018). –OH, stretching peaks of amide reported in our study are 3404 cm^{-1} and 3421 cm^{-1}, similar peaks corresponding to –OH stretching like 3404 cm^{-1} and 3420 cm^{-1} are reported by Suresh *et al.* (2016). Similar 2362 cm^{-1} and 2347 cm^{-1} peaks representing O=C=O stretching are also reported by Alia *et al.* (1997). In the same way, 1111 cm^{-1} and 1053 cm^{-1} corresponding to anthocyanins is also reported by Paraiso *et al.* (2020).

After FTIR analysis, HPLC analysis of AEHS was conducted to evaluate the presence of various constituents. RT values of delphinidin and cyanidin reported in our study are in close accordance with the study conducted by Villalpando *et al.* (2013) as 27 min and 28.5 min fordelphinidin and cyanidin, respectively. gas chromatography is usually combined with mass spectroscopy to explore the presence of various constituents in plant extracts. P-cymene reported in our study is in accordance with the results of Pavlic *et al.* (2020) where p-cymene was reported with the RT of 5.283 min. Beta-pinene an important component of essential oil in many plants is also reported with a RT value of 8.059, this was also reported in the essential oil extracted from leaves and calyces of *H. sabdariffa* plant material collected from Iran by Amlashi *et al.* (2020). Delphinidin, is an important anthocyanidin pigment reported in GC-MS analysis, this was also reported Bochi *et al.* (2015) with a very near m/z value of 303. Another important anthocyanidin; reported in our study is cyanidine, our results are in accordance with the study conducted by Gouvea *et al.* (2012), where a very near m/z value of 287 was reported for cyanidine. Successfully, characterized AEHS revealed the use of four different phytoconstituents for *in silico* studies by using MAO-A, 2z5y protein. Among all four phytoconstituents screened, delphinidin showed maximum inhibition of MAO-A, 2z5y protein with highest dock score and EModel energy as –7.055 and –49.299 kcal/mol, respectively.

5. Conclusion

In conclusion, we report the importance of *H. sabdariffa* leaves as a rich source of various phytoconstituents. FTIR analysis of AEHS showed the presence of different functional groups and HPLC analysis reported the peaks of delphinidin and cyanidin. GC-MS analysis of AEHS showed the presence of more than 10 different phytoconstituents among which four were selected for evaluating *in silico* antidepressant effect. Delphinidin and cyanidin showed good docking score against MAO-A, 2z5y protein indicating their efficacy against depression. Authors are further exploring AEHS *in vivo* antidepressant activity. Further, bioactivity guided isolation can also be conducted to get highly effective compounds for the treatment of depression.

Conflict of interest

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

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Citation **Mohd Mohiuddin Shareef and E. Bhavya (2021). Extraction, phytochemical analysis and *in silico* antidepressant studies of aqueous extract of leaves of *Hibiscus sabdariffa* L. Ann. Phytomed., 10(2):448-455. http://dx.doi.org/10.21276/ap.2021.10.2.59**