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Original Research Article

PHYTOCHEMICAL SCREENING, ANTIBACTERIAL AND ANTIOXIDANT STUDIES OF *HYOSCYAMUS AUREUS* L. LEAF EXTRACT

AIREMWEN COLLINS OVENSERI[1,4,*], HALILU MSHELIA EMMANUEL[1,2], OMOBOYEDE OJO[4], OBARISIAGBON AIWAGUORE JOHNBULL[3]

1. Department of Pharmaceutics and Pharmaceutical Technology, Faculty of Pharmacy, University of Benin, Benin City, Nigeria.
2. Department of Pharmacognosy and Ethnomedicine, Faculty of Pharmaceutical Sciences, Usmanu Danfodiyo University, Sokoto, Nigeria.
3. Department of Pharmaceutics and Pharmaceutical Technology, College of Pharmacy, Igbinedion University, Okada, Edo State, Nigeria.
4. Faculty of Pharmacy, Cyprus International University, Nicosia, Cyprus.

ABSTRACT

Hyoscyamus aureus L belongs to the Family, Solanaceae and it has been used both in ancient times and in modern medical practice for the treatment of certain disease conditions such as the relief of pain, inflammation, constipation, palpitations of the heart, cough, treatment of rheumatism, and rejuvenation of the body tissues and cells. The purpose of this study was to evaluate the phytochemicals, antioxidant and antimicrobial activities of the leaf extract. The leaves were equally subjected to microscopic, macroscopic, and physicochemical examinations. The antioxidant assay was done using the DPPH, hydrogen peroxide and β-carotene methods. The macroscopic examination of the plant showed a brownish-green powder with a characteristic smell and taste, and a gritty texture. The microscopic evaluation revealed the presence of microscopic structures such as stomata and trichomes. The antimicrobial evaluation was done using the agar disk diffusion method. The results of the physicochemical analyses showed a moisture content of 5.5%, ash content of 6.0%, while the alcohol-soluble and petroleum ether soluble values were 5.0% and 8.0%, respectively. The results of the phytochemical tests revealed the presence of flavonoids, tropane alkaloids and quinones. The antioxidant assay results revealed that the leaf extract demonstrated significant free radical scavenging activity compared to the standard, with IC50 values of 3.25 and 2.18 mg/mL for the extract and ascorbic acid, respectively. The antimicrobial studies showed zones of inhibition ranging from 5.94-9.38 mm, which indicated significant antibacterial activity against both the gram-positive and negative bacteria used in the research. The findings of this research showed that leaf extract possessed significant antibacterial and antioxidant activities.

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INTRODUCTION

Medicinal herbs play a vital role in medical and pharmaceutical sciences as they are a natural source of pharmaceutical excipients and drugs because they are very rich in secondary metabolites and essential oils [1]. Medicinal plants have been widely explored for their diverse chemical constituents, known as phytochemicals, which possess

***Corresponding author:** collins.airemwen@uniben.edu, +234 813 373 7933

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various bioactive properties. *H. aureus,* a plant that belongs to the Family Solanaceae, has a variety of about 100 genera and approximately 2,800 species [2]. They include many important medicinal plants that have been used over the years by humans as medicines, food and ornamental plants [3]. The Solanaceae Family contains several important medicinal plants rich in alkaloids with potent antimuscarinic effects such as atropine, hyoscyamine, hyoscine and scopolamine, which have been used medically in the treatment of several diseases [4].

H. aureus, commonly known as golden henbane, is known for its distinctive yellow flowers and deeply lobed leaves. It is a perennial plant that completes its cycle in two years. It is commonly found in the Mediterranean, Aegean region and some parts of Asia. *H. aureus* has also been reported to contain several classes of phytochemicals, ranging from flavonoids, terpenoids, alkaloids, and phenolic compounds, that are mostly responsible for its pharmacological activities [5]. It is active pharmacologically because of its high content of scopolamine and hyoscyamine. Previous studies have reported that the plant possesses significant antioxidant, anti-diabetic, antisecretory, anti-asthmatic, Ca^{2+} channel-blocking, anticancer, antiallergic, anti-diarrhoeal, hypotensive, hepatoprotective, cardioprotective, antidepressant, and anticonvulsant activities [6,7]. Previous studies have also reported that the plant possesses significant anticholinergic effects due to its rich tropane alkaloid content; hence, it is also used to treat several diseases such as gastric ulcers, nausea and vomiting associated with motion sickness and Parkinson's disease [8]. It is also used as a cycloplegic, spasmolytic, mydriatic, and an antidote for intoxications of several poisons [9]. However, consuming the roots and leaves of the *H. aureus* in excessive amounts has been reported to induce poisoning by paralysing the parasympathetic system nerve endings [10].

Several disease conditions, such as cancer, diabetes mellitus and Parkinson's disease, have been linked to damage to cellular structure due to an increase in the release of free radicals such as reactive oxygen species (ROS) and oxidative stress [11]. Antioxidants have been reported to protect cells from the harmful effects of ROS, which normally stimulate oxidative stress, and the best approach to reduce these free radicals in the body is to scavenge them using antioxidants. Previous studies have reported that the methanol extract of *H. niger* possessed significant antibacterial activity [12,13]. However, limited studies have been done on the species *H. aureus;* hence, this research aimed to investigate the antioxidant and antibacterial activities of the leaf extract.

MATERIALS AND METHODS

Reagents

Ethanol, acetone, chloroform, DPPH, petroleum ether, ascorbic acid, hydrochloric acid and Dragendorff's reagent were all obtained from Sigma Aldrich (Germany). The reagents used were of analytical grade. The Nutrient agar, Lysogeny broth (LB) and Mueller-Hinton agar were purchased from Merck KGaA (United Kingdom).

Collection of the plant material

Fresh leaves of *H. aureus* were collected in April 2022, at Girne, Cyprus (Latitude: 35.1856° N; Longitude: 33.3823 E). The leaves were identified by a Botanist, Dr M.E Halilu and dried for fourteen (14) days in the shade. The specimen number, CIU/PHARM/TLEF/002, was assigned to the voucher sample and kept for future reference. The dried leaves were powdered, sieved and stored in an airtight container.

Macroscopic evaluation of the powder plant sample.

This was done by examining the form, size, colour, surface traits, texture and odour of the plant [14].

Microscopic evaluation

Microscopic evaluation was done to investigate structures such as fibers, starch, grains, phloem, steroids, calcium oxalate crystals and cork cells. 5 mL of chloral hydrate was added to the powdered leaves (2 g) in a glass tube and heated on a hot water bath for 5 min. The microscopic slides were prepared by adding a drop of the plant extract and diluted glycerin. The slides were then viewed under the microscope (Leica, Turkiye).

Determination of physicochemical constituents

The alcohol soluble, water soluble, acid insoluble and total ash values were determined by methods previously described by Lih *et al.,* [15].

Extraction of *H. aureus* leaves

The maceration method was used to extract 20 g of the powdered leaves using 200 mL of ethanol in a beaker, stirred for 6 h and then kept for 24 h. The plant mixture was filtered twice into another conical flask to ensure that there were no particles left in the extract, and kept in a tight jar for further studies.

Phytochemical tests

The test samples were analysed qualitatively for the presence or absence of some secondary metabolites such as saponins (frothing test), alkaloids (Mayer's and Dragendorff's test), tannins (lead acetate test), phenolics (Ferric chloride test), and flavonoids (sodium hydroxide test) [15].

Thin Layer Chromatography (TLC)

This method was used to demonstrate the separation of the plant compounds. An aluminium plate coated with silica gel, which is the stationary phase, was cut, a baseline was drawn, and a point was marked on it; a drop of the plant extract was spotted on that point. This was done for several TLC sheets. The mobile phase was prepared by combining different non-polar, semi-polar and polar solvents in varying ratios. Petroleum ether, chloroform and ethanol (6:3:1) were the solvents used and the spotted TLC sheets were developed

for each fraction in a beaker of different mobile phases. Since there was no physical separation, the sheets were viewed under UV light and some spots were seen. Then the sheets were soaked with 10% H_2SO_4 and dried with a hand dryer, and then observed under an ultraviolet light. The retention factor (Rf) value of the spots was calculated using equation 1.

$$Rf = \frac{distance\ moved\ by\ the\ compound}{distance\ moved\ by\ solvent\ front} \quad - \quad -$$

- Equation 1

Gas chromatography-mass spectrometry (GC–MS) analysis

The qualitative chemical composition of the extract was determined via GC-MS analysis, as described by Shibula and Velavan [16], using an author injection GC-MS-QP2010 Plus System (Shimadzu, Japan). A comparison of the mass spectra of the compounds against the Wiley library 7 was used to identify the compounds.

Antioxidant assay

DPPH radical scavenging assay

The concentrated plant extract (0.2 g) was weighed into a beaker, 10 mL of absolute ethanol and 5 mL of DPPH (4 mM) solution were added and mixed. The samples were analyzed with a UV spectrophotometer (UV-2450, Shimazdu, Japan) at a wavelength of 480 nm [17].

Hydrogen peroxide (H_2O_2) scavenging assay

The ability of *Hyoscyamus aureus* extract to scavenge H_2O_2 was quantified as previously described by Ghasemi et *al.,* [18]. Hydrogen peroxide solution was prepared using a phosphate buffer. Varying concentrations of the plant extract were prepared and 6 mL of hydrogen peroxide was added into each glass tubes and incubated for 20 min. The absorbance (Abs) was read at a maximum wavelength of 480 nm. The antioxidant activity was determined using equation 2.

$$\frac{Abs\ control - Abs\ sample}{Abs\ control} \times 100 \quad - \quad - \quad -$$

Equation 2

β-carotene and linoleic acid bleaching assay

A mixture of β-carotene linoleate was prepared by dissolving 0.5 mg of β-carotene in 300 µL linoleic acid and 2 mL of chloroform. Tween 80 (200 mg) was added and mixed [19]. The chloroform was then evaporated, 50 mL of purified water was added to the residue, and mixed to form an emulsion which was then transferred into different test tubes. The absorbance of the samples was read spectrophotometrically at 470 nm at time (t=0) and after incubation for 1 h at 40°C. Butylated hydroxyanisole was used as a positive control. The β-carotene bleaching inhibitory activity of a sample was calculated using equation 3.

$$\% \text{ Activity} = \frac{At}{A0} x100 \quad - \quad - \quad \text{-equation 3}$$

where A_0 is the absorbance a time zero and A_t is the absorbance after 1 h.

Antibacterial activity

Two (2) microbial isolates, namely, *Staphylococcus aureus* and *Salmonella typhi,* were used for the experiment. The agar disk diffusion method was used for the antimicrobial study as reported by Hossain *et al.* [20]. The zones of inhibition were calculated.

Statistical analysis

The experiments were done in triplicate, and the results were documented as mean ± standard deviation. Data was analysed using Microsoft Excel (2019 version).

RESULTS

Macroscopic evaluation

The macroscopic evaluation of the powdered leaves of *H. aureus* revealed a brownish green colour, gritty texture, bitter taste and a strong characteristic smell, which may be because of the presence of volatile substances such as flavonoids and tannins (Table 1, Figure 1). Previous studies by Dulger *et al.* [21] also reported a similar observation.

Microscopic evaluation

The results of microscopic evaluation revealed the presence of trichomes and stomata in the powdered leaves of *H. aureus* (Figure 2). Collak *et al.,* [22] also reported the presence of trichomes, stomata and calcium oxalate crystals in the powdered leaves.

Physicochemical values

The results of the physicochemical evaluation revealed a moisture content value of 5.5%, total ash value of 6%. The water and alcohol-soluble extractive values were 2.0% and 5.0% respectively suggesting that the plant materials were more soluble in alcohol than in water, while the percentage solubility in petroleum ether was 8% (Table 2).

Phytochemical analysis

Phytochemical screening was done to evaluate the chemical compounds responsible for the biological and antioxidant activities of the plant. The characteristic colour change when reagents were mixed with the leaf extract indicated the presence of phytochemical constituents. The findings confirmed the presence of saponins, terpenoids, quinones, phlobatannins and alkaloids in the leaf extract (Table 3). Lih *et al.,* [15] also reported similar findings.

TLC

This is a technique used in the analysis of crude extract by separating the compounds in the mixture. The Rf value, which is the distance travelled by a compound, was calculated, and the values obtained indicated the presence of flavonoids, tannins, alkaloids, and some phenolic compounds (Table 4). This chromatographic technique also resulted in the separation of other phytochemicals such as saponins and quinones. The Vitali Morin test gave a purple colour, confirming the presence

Table 1: Macroscopic evaluation

Parameters	Result
Smell	Characteristic smell
Taste	Bitter
Colour	Brownish green
Texture	Gritty

Figure 1: Powdered leaves of *Hyoscyamus aureus*

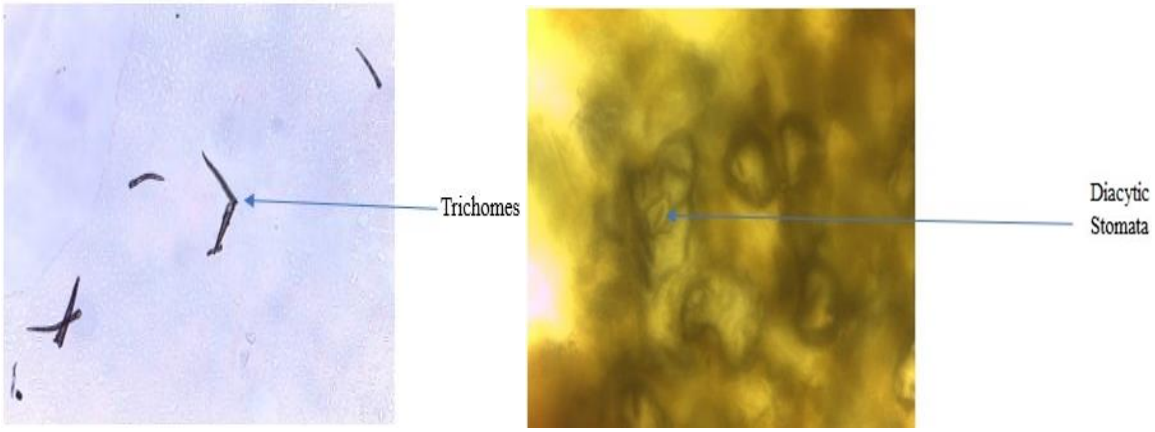

Figure 2: Microscopic features of *H. aureus* showing trichomes and stomata

Table 2: Physicochemical values

S/N	Experiments	Results (%)
1	Total ash	6.0
2	Moisture content	5.5
3	Acid insoluble ash value	1.0
4	Water-soluble extractive value	2.0
5	Alcohol soluble extractive value	5.0
6	Petroleum ether extractive value	8.0

Table 3: Results of phytochemical screening

Test	Observations	Results
Frothing test	Formation of foam	Presence of saponins
Salkowski test	Reddish brown colouration	Presence of terpenoids
Dragendorff test	Orange red precipitate	Presence of alkaloids
Alkaline reagent test	Colourless	Presence of flavonoids
Sulphuric acid test	Formation of red colour	Presence of quinones
Bontrager's test	No colour change	No presence of Anthraquinones
HCl test	Formation of red precipitate	Presence of phlobatannins
Test for Anthocyanins	No visible colour change	Negative
Guignard sodium picrate test	Presence of pink solution	Presence of cyanogenic lycosides

Table 4: Rf values of tropane alkaloids from preparative TLC.

Solvent Ratio (Acetone + Toluene)	SD (cm)	Spot (cm)	Rf values
9:1	7.4	1.0	0.13
8:2	7.6	0.9	0.11
7:3	6.9	1.0	0.14
6:4	6.4	2.0	0.31

SD = Solvent Distance

Table 5: Identification and qualitative analysis of the tropane alkaloid

Test	Observation	Results
Dragendorff's test	Orange colour formed	Positive
Hager's test	Yellow ppt formed	Positive
Dragendorff's spray test	Formation of atropine rings	Presence of tropane alkaloid
Vitali Morin Test	Colour change to purple	Presence of tropane alkaloids

Table 6: GC/MS data and relative composition of the alkaloids in the extract of *H. aureus*

S/N	Compound Name	Formula
1	4-Fluoro-N,N-dimethylaniline	C_2H_6FN
2	N-Nitroso-N-methylurea	$C_2H_5N_3O_2$
3	1-Chloro-2-nitroethane	$C_2H_4C_lNO_2$
4	Panthenyl ethyl ether	$C_{11}H_{23}NO_4$
5	1-Nitroso-1Trideutriomethyl urea	$C_2H_5N_3O_2$
6	1-chloro-1-nitrosoethane	C_2H_4CINO
7	Cyanogen Chloride	CNCI
8	N, N-Tetramethylputrescine	$(CH2)_4(NH2)_2$
9	2,4-N-Methylpyrrolidinylhygrine	C_5H_9NO
10	Hexamethylenetetramine	$C_6H_{12}N_4$
11	3-Phenylacetoxy-6-hydroxytropane	$C_6H_{21}NO_3$
12	6-Hydroxylhyoscyamine	$C_{17}H_{23}NO_4$
13	1-Methyl-2-pyrrolidinone	C_5H_9NO

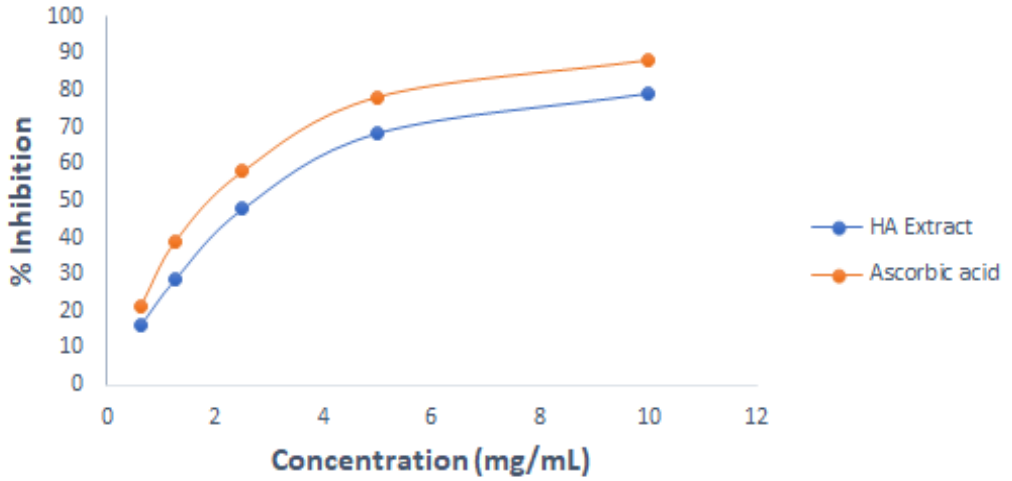

Figure 3: Percentage inhibition of *H. aureus* extract and ascorbic acid using DPPH assay

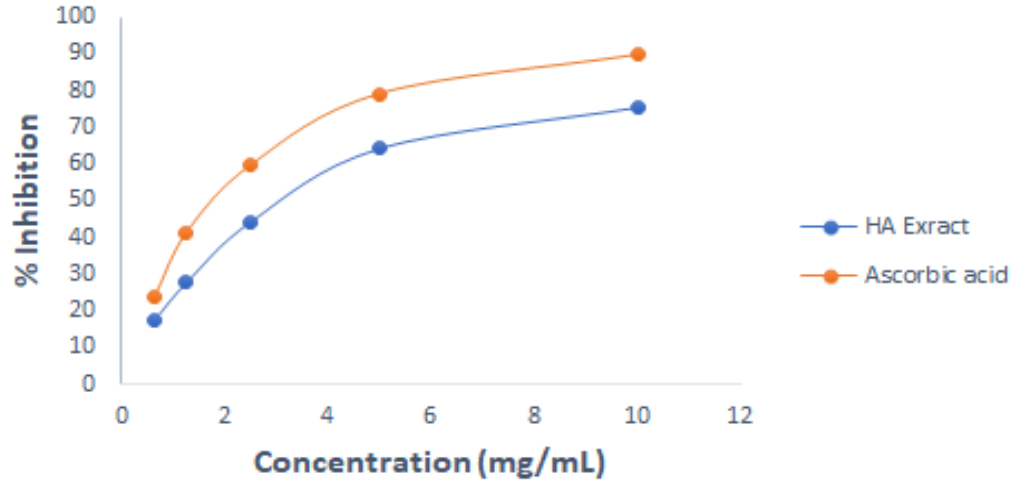

Figure 4: Percentage inhibition of *H. aureus* extract and ascorbic acid using hydrogen peroxide assay.

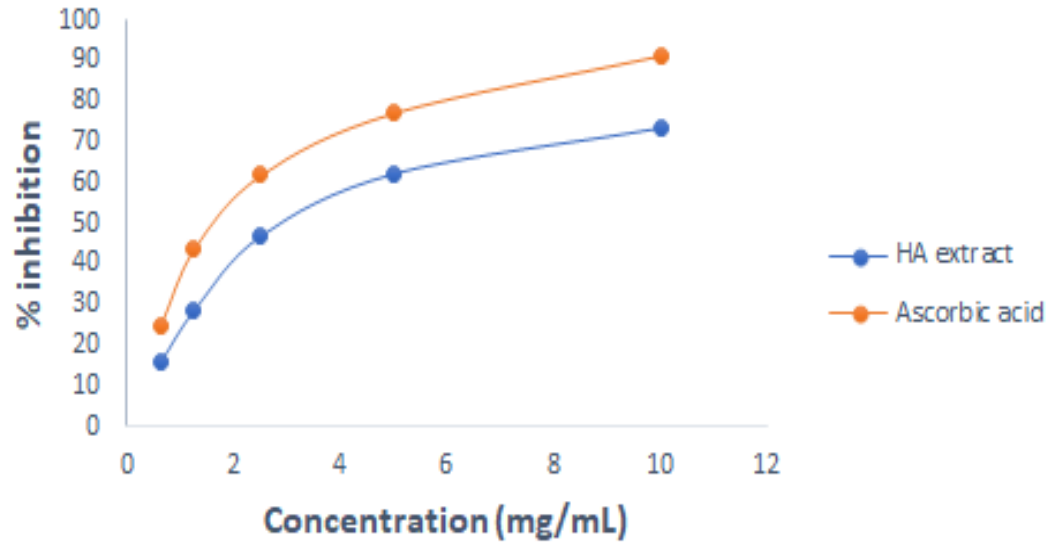

Figure 5: Percentage inhibition of *H. aureus* extract and ascorbic acid using β-carotene assay

Table 7: Results of antibacterial activity of *H. aureus* leaf extract

Organisms	*H. aureus* extract (mm)	Gentamicin (mm)	Distilled water
S. aureus	9.38±0.01	13.16±0.01	0
S. typhi	5.94±0.01	11.39±0.02	0

of atropine, and Dragendorff's spray test also confirmed the presence of tropane alkaloids (Table 5). The total percentage of the tropane alkaloid was calculated to be 2.0 %.

GC-MS

The GC-MS results revealed the presence of more than ninety (90) compounds present in the leaf extract of *H. aureus*, but only the alkaloidal compounds detected were documented in this study (Table 6). Previous studies also reported similar findings [23].

Antioxidant assay

The results of the antioxidant assay were reported as half-maximal inhibitory concentration (IC50) and percentage inhibition. Based on the plot of the percentage inhibition versus the concentration using DPPH assay, the IC50 of the leaf extract and ascorbic acid were 3.25 mg/mL and 2.18 mg/mL, respectively (Figure 3). The results of the hydrogen peroxide scavenging assay revealed that the leaf extract demonstrated good H_2O_2 scavenging ability when compared to ascorbic acid. The IC50 of the leaf extract and ascorbic acid were 4.05 mg/mL and 2.25 mg/mL, respectively (Figure 4). From the results of the β-carotene/linoleic acid bleaching assay, the IC50 of the leaf extract and ascorbic acid were 4.19 mg/mL and 2.15 mg/mL, respectively (Figure 5). The leaf extract inhibited the bleaching of β-carotene in comparison with the standard (Ascorbic acid). Previous studies by Kero *et al.* [24] also reported similar findings.

Antibacterial activity

The results of the antibacterial study showed an inhibition of zone diameter of 9.38±0.01 mm and 5.94±0.01 mm for *Staphylococcus aureus* and *Salmonella typhi* respectively implying that the test sample at a concentration of 5 mg/mL possessed significant antibacterial effect against the bacteria used in the study, with the highest activity seen against the Gram-positive bacteria (*S. aureus*) (Table 7). From the zone of inhibition obtained, the antibacterial activity of the plant extract showed some promising results against the bacterial strains employed

The microscopic and macroscopic evaluations of powder plant samples are primarily done to establish some diagnostic features for the identification and authentication of crude vegetable drugs, as stated by the WHO [25]. These features help to differentiate one species from the other species with well-known characteristics. The brownish green colour, bitter taste, characteristic odour and gritty texture of the leaves have been reported to be due to the presence of tannins, flavonoids and phenolic compounds [26]. Microscopy of plants helps in the authentication of herbal drugs and the determination of impurities or contamination in the plant material. The moisture content represents the amount of moisture still present in the plant after collection, air drying and powdering. High moisture content can lead to the spoilage of the drug via fungal and bacterial growth and hydrolytic degradation of the active chemical compounds [27].

The results obtained in this study were similar to previous studies done by Omeche *et al*. [28]. Total ash value was 6.0%, and this represents the amount of the residual substance not volatized on ignition at 550 ^{O}C when crude drugs were ignited [24]. The acid insoluble ash indicates the amount of sample that is not hydrolysed by acid, and it is not subsequently volatilized upon the incineration of the residue, and the value obtained was 1.0 %. The alcohol soluble value (5%) was higher than the water-soluble value (2%), implying more solubility in alcohol than in water. Halilu *et al*. [2] reported that polar solvents tend to extract higher amounts of phytochemicals than nonpolar solvents. All these evaluations provided more information, which is vital for the proper identification of the plant.

The findings of the study revealed that the alkaloids present in the leaf extract were atropine, scopolamine, and hyoscyamine. Previous studies have reported that *H. aureus* belongs to the family, Solanaceae and hyoscyamine, scopolamine, and atropine, which are referred to as tropane alkaloids, are dominant alkaloids present in plant [6]. Different separation was done on the tropane alkaloid extract, and the Rf values of the solvents (toluene and acetone) gave the optimised separation results, which also confirmed the presence of tropane alkaloids. The Vitali Morin test confirmed the presence of atropine, while Dragendorff's spray test also confirmed the presence of tropane alkaloids [26].

DPPH is a relatively unreactive free radical that is usually used to evaluate the scavenging activity of plant extracts. Antioxidants have been reported to have the ability to scavenge DPPH radicals by donating electrons or a hydrogen atom and transforming them into a colourless product [27]. The antioxidant activities of the *H. aureus* plant extract have been reported to be due to secondary metabolites such as flavonoids, alkaloids and the phenolic compounds that can absorb, neutralise and scavenge oxygen molecules [28]. The antioxidant effects demonstrated by all the batches were concentration dependent. The findings of the study revealed that leaf extract possessed a significant antioxidant effect and DPPH scavenging activity compared to the reference ($P<0.05$). Previous studies by Baliyan *et al.* [30] also reported that *H. aureus* leaf extract possessed significant antioxidant activity. Rakmai *et al.* [12] reported that the tendency of a compound to scavenge H2O2 is an indicator of its antioxidant activity. From the results of the antioxidant assay, the

leaf extract scavenged H_2O_2 in a concentration-dependent manner, and ascorbic acid had a significantly higher antioxidant activity ($P<0.05$). The hydrogen peroxide scavenging activity by the sample could be a result of the presence of phenolic compounds that can react with H_2O_2 and neutralise it to water [30]. The β-carotene bleaching assay evaluates the ability of antioxidants to interrupt lipid peroxidation in the initiation and propagation phases. Generally, β-carotene/linoleic acid undergoes a quick discolouration when antioxidants are not present in the body system. The linoleic acid free radicals formed during the production of a hydrogen atom from one of its methylene

diallylic groups rapidly attacked the unsaturated β-carotene. This leads to the β-carotene getting oxidised and broken up, partially leading to the loss of its orange colouration [19,30]. Although the β-carotene goes through a quick colour change in an oxidative state, however, antioxidants reduce the oxidation reaction. Linoleic acid bleaching assay determines the ability of the plant to inhibit the hydroperoxide formation coupled during the linoleic oxidation of the linoleic acid. The leaf extract demonstrated significant antioxidant activity depending on the concentration.

The findings of the antibacterial studies showed that *H. aureus* leaf extract demonstrated significant antimicrobial activity against *Staphylococcus aureus* and *Salmonella typhi,* while the positive control (gentamicin) showed the highest activity ($P<0.05$). The antibacterial activity against the gram- positive bacteria (*S. aureus*) was significantly higher than that of the gram-negative bacteria (*Salmonella typhi*) ($P<0.05$). This result is similar to that of a previous study done by Hossain *et al.* [20] who reported that *H. aureus* leaves possessed significant antibacterial activities which could be attributed to tannins, flavonoids and other compounds present in the extract.

CONCLUSION

This study evaluated the phytochemical screening and biological studies of the H. aureus leaf extract, which showed that it contains several phytochemicals that possess beneficial therapeutic effects in the treatment of diseases such as muscular spasm, motion sickness, etc. H. aureus leaf extract also demonstrated significant antibacterial and antioxidant activities. Further research should be done to better understand the underlying mechanism of the antioxidant effect.

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AUTHORS' CONTRIBUTION

HME was involved in the conceptualisation and supervision of the study; OO performed the laboratory experiments; ACO designed the study and drafted the manuscript, and OAJ analysed the data and reviewed of manuscript.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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