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Geno-Protective, Free Radical Scavenging and Antimicrobial Potential of *Hyptis suaveolens* Methanolic Fraction: An *In-vitro* Study

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Authors' contributions

This work was carried out in collaboration among all authors. Author DI designed the study, performed the statistical analysis, wrote the protocol, and wrote the first draft of the manuscript. Authors ABD and MSK managed the analyses of the study. Author MSK managed the literature searches. All authors read and approved the final manuscript

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ABSTRACT

Aims: *Hyptis suaveolens* (L.) Poit, is one of the natural herbs with several medicinal properties. However, many medicinal aspects of this herb still need to be explored. Therefore, our aim was to examine the antioxidant, antimicrobial properties and genoprotective effect of *H. suaveolens* methanolic extracts (HSME) of seed, stem, and root.
Study design: extraction and therapeutic aspects of *H. suaveolens*.
Place and Duration of Study: 1) Department of Medical Laboratory Sciences, College of Applied Medical Sciences, Majmaah University, Al-Majmaah and 2) Clinical Biochemistry & Natural Product research laboratory, Department of Biosciences, Integral University, lucknow between 2018-2020.
Methodology: HSME were extracted through soxhlet extractor and further analyzed for TPC, antioxidant activity through DPPH and FRAP assay followed by antimicrobial potential through

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zone of inhibition and MIC/MBC assay. We also examined the genoprotective properties of HSME on oxidative DNA damage.
Results: Our results showed that TPC (180±5 mg GAE/g dw), DPPH scavenging activity (IC_{50} value = 72±0.45 µg/ml) and FRAP value (1.443±0.02 µM ferrous ion/mg extract) was highest in HSME seeds followed by root and stem. The results also illustrated that the antimicrobial activity of HSME (seed and stem) against five bacterial strain were found very effective than root part. Moreover, genoprotective effect of HSME seeds (80±3 % retention) was better than stem (41±2 %) and root (32±2 %) extract.
Conclusion: The study revealed that HSME seed extract showed potential bioactivities might be due to presence of high TPC and can be used to treat diseases related with oxidative stress or microbial infections.

Keywords: DNA damage; hyptis suaveolens; methanolic fraction; microbicidal; oxidative stress.

1. INTRODUCTION

Oxidative stress is responsible for the development of cell injury, aging, cardiovascular diseases, gastrointestinal infection, neurodegenerative diseases, kidney disorders and cancer. Overproduction of free radicals which can also induce oxidative stress leads to the damage to lipids, nucleic acid, and other biomolecules [1,2]. Microbial infections can also damage biomolecules, induce oxidative stress, and are involved in pathogenesis of various diseases [3], such as periodontal diseases, inflammation, Parkinson's disease [4,5].

Microbes develop the resistance mechanism due to overuse of antibiotics. Therefore, it has become a global concern which required novel findings to treat microbial infection [6,7]. Certain large number of synthetic drugs have been developed against microbial infections and oxidative stress having various side effects, toxicity and uneconomical in long term use [7,8]. Therefore, it is necessary to develop novel drugs from natural resources because of its high availability, cost affectivity, low toxicity and of having least side effects in prolonged use [9].

Previously, it has been reported that plants are the good source of natural bioactive compounds against vast variety of diseases [10]. Medicinal plants or their bioactive fractions were also reported as potent antioxidant, antimicrobial, geno-protective, hypolipidemic, antidiabetic, neuroprotective agents [11-23]. Studies also revealed the antioxidant and antimicrobial effects of several plant species, such as *Nandina domestica* [24]. Previously, plant species of *Lamiaceae* family have been reported for their medicinal properties, such as *Hyptis suaveolens*, *Ocimum tenuiflorum* L. and *Ocimum basilicum* L [25,26,27].

Hyptis suaveolens (L.) Poit (*Lamiaceae*), is a rigid sweetly aromatic herb well known for its insecticidal activity, against *Anopheles gambiae* [28], *Drosophila melanogaster* and *Artemia salina* [29]. *H. Suaveolens* (Fig. 1) have also been reported for its potent antimicrobial, antiplasmodial and antioxidant properties [27,28,30,31]. Various bioactive compounds were isolated from essential oils of *H. suaveolens* [29]. Moreover, bioactive fractions of *H. suaveolens* have shown decreased neurotoxicity [32], hypoglycemic [33] and anticancer [34] properties. Seeds and leaves extract of *H. suaveolens* were reported to control Trogoderma granarium Everts in stored groundnut [35].

Fig. 1. *H. suaveolens*
Source: https://flora.indianbiodiversity.org/flora/angiosperm/lamiales/lamiaceae/hyptis/hyptis-suaveolens

However, there is no report on *H. suaveolens* methanolic extracts (HSME) of seed, stem, and root for their antioxidant, antimicrobial and

genoprotective properties. Specified the fact of traditional knowledge and the recent pharmacological studies for *Hyptis suaveolens*, the aim of the present study was to evaluate the phytochemical, DPPH free radical scavenging activity, FRAP value, Geno-protective potential, and antimicrobial properties against gram positive (*Staphylococcus epidermidis*, *Bacillus subtilis*, *Bacillus cereus*) and gram negative (*Enterobacter aerogenes* and *Klebsiella pneumoniae*) microbes of HSME (seed, stem, and root).

2. MATERIALS AND METHODS

2.1 Chemicals

All reagents and chemicals were of analytical reagent grade and procured from Sigma Aldrich. Culture media was procured from Himedia Laboratory Pvt. Ltd.

2.2 Extract Preparation

Different parts (seeds, stem, and root) of *H. suaveolens* were collected from neighboring area of Lucknow near Integral University, washed with distilled water and shade dried for 15 days. Plant materials then pulverized using an electric grinder. The extractions of 20 gm powder with methanol (200 ml) were done using soxhlet extractor for about 8-10 hours [36]. The extracts were then concentrated using rotatory evaporator and placed at -20^{o}C till use.

2.3 Phytochemical Analysis

The freshly prepared crude extracts were qualitatively analyzed for the presence of phytochemicals as described by Harborne [37].

2.4 Estimation of Total Phenolic Content

Total phenolic content (TPC) of HSME root, stem and seed was determined by using Folin-Ciocalteu (F-C) reagent and is expressed as gallic acid equivalent (GAE) in µg/mg dry weight of extract [38].

2.5 DPPH Radical Scavenging Property Assay

The DPPH assay was performed according to the method described by Brand-Williams [39], with some modifications [20]. The scavenging property was calculated as IC_{50} value based on the percentage of DPPH radical scavenged using the following equation:

> % scavenging effect = [(control absorbance - sample absorbance)/ control absorbance] x 100

IC_{50} value is the efficient concentration that could scavenge 50% of the DPPH radicals. Ascorbic acid standard was used as positive control.

2.6 Ferric Reducing Antioxidant Power (FRAP) Assay

FRAP assay of HSME (seeds, stem, and root) was done by following the procedure of Benzie and Strain [40], with a few modification [21]. The standard curve was plotted using ferrous sulphate solution, and results were expressed as μM Fe(II) /mg dry weight of extract.

2.7 DNA Protection Assay

Oxidative DNA damage by Fenton's reagent was measured on pUC18 plasmid DNA, according to the procedure described by Iqbal et al. [21]. Briefly, reaction mixture (RM) was prepared by adding 10 µl of HSME of seed, stem, and root at different concentrations (50 & 100 µg) and 1 µl (100 ng) plasmid DNA were incubated for 10 min at 27ºC temperature followed by the adding up of 10 µl Fenton's reagent (30 mM H_2O_2, 50 µM Ascorbic Acid & 80 µM $FeCl_3$) in 0.5 ml micro centrifuge tubes. This reaction mixture was then run through electrophoresis (1% agarose) and protection of DNA damage by plant extracts were analyzed followed by ethidium bromide (EtBr) staining with Gel Doc XR system (Bio-Rad, USA). Mannitol (10 µg/RM) was used as standard in this assay.

2.8 Antimicrobial Activity Assay

Bacterial strains [*Staphylococcus epidermidis* (NCIM 2493), *Bacillus subtilis* (NCIM 2920), *Bacillus cereus* (NCIM 2156), *Enterobacter aerogenes* (NCIM 5139) and *Klebsiella pneumoniae* (NCIM 2957)] were obtained from NCL (National Chemical Laboratory) Pune, India. For antibacterial testing fresh inoculums was prepared for each bacterial strain and incubated at 37°C for 24 h. To obtain turbidity comparable to that of McFarland 0.5 standard (1.5×108 cells/ml) the cells suspension was adjusted with nutrient broth according to Jorgensen HJ [41]. The antibacterial assays of the HSME of different parts (seeds, stem, and root) were carried out by means of agar well diffusion method [42].

Hundred micro liters (µl) of diluted inoculums (1.5 ×10^8 CFU/ml) of bacteria strain was swabbed over Nutrient agar (pH 7.2) plates. Wells of 4 mm diameter were punched into agar plate and filled with 40 µl of extract prepared in DMSO at different concentrations (10-50 µg/ml). The plates were left for 30 min at 27ºC temperature to allow the diffusion of the extract and then incubated at 37°C for 18 h. The diameter of inhibition zone was measured in millimeters including the size of the wells. DMSO without extract was used as a control and antibiotics such as Penicillin and Tetracycline having potency of 10 µg per disc were used as standard.

2.9 Analysis of Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC)

Minimum inhibitory concentration (MIC) was analyzed for each plant extract showing antimicrobial activity against test pathogens [43]. Plant extracts were re-suspended in DMSO to prepare 5 mg/ml final concentration as stock and then 5-fold serial dilutions were done; added to broth media of 96-well microtiter plate. Thereafter, 100 µl inoculums (1×10^8 CFU/ml) were added to each well. This microtiter plate was incubated at 37°C for 24 h. Each plant extract was assayed in triplicate and each time two sets of microtiter plate were prepared; one was kept for incubation while another set was kept at 4°C for comparing the turbidity in the wells of microtiter plate. The MIC was defined as the lowest concentration preventing visible growth. The MBC of the HSME was determined as described by Mishra et al. [44]. The plates of MIC, which showed no visible growth, were cultured on fresh nutrient agar plates. The lowest concentration of antimicrobial agent/plant extract, from which bacteria do not recover on fresh medium, was treated as MBC.

2.10 Statistical Analysis

For all protocols, samples were analyzed in triplicate and the results were expressed as mean ± SD. Fifty percent inhibitory concentration (IC_{50}) were calculated by Origin version 6.0 Professional software (Origin Lab Corporation, Northampton, MA, USA), and the results were evaluated using one-way analysis of variance (ANOVA) and two tailed Student's t-test. Statistical significance was expressed as *p<0.05, **p<0.01.

3. RESULTS

3.1 Phytochemical Analysis

Qualitative phytochemical analysis represented in Table 1, reveals that HSME of stem is rich in flavonoids, terpenoids and proteins. Meanwhile, HSME of seeds is abundant only with phenol and scarcity of terpenoid was reported. However, HSME of root is only abundant with flavonoids.

3.2 Total Phenolic Content

Our results show that total phenolic content (TPC) of various HSME fractions was found to be in the following decreasing order: seeds > root > stem. The result presented in Table 2, clearly demonstrated that HSME of seed have better phenolic content (180 ± 3.72 μg GAE/mg of dry plant extract) than other plant parts like roots and stem (100 ± 2.96 and 82 ± 2.64 μg GAE/mg of dry plant extract, respectively).

3.3 In-vitro Antioxidant Activity

HSME (seeds, stem, and root) were screened for their *in-vitro* antioxidant properties by DPPH and FRAP analysis method. We found that HSME (seed) showed better DPPH radical scavenging activity (IC_{50} value = 72±0.45 µg/ml) than HSME of stem (>250±5.46 µg/ml) and root (143±2.15 µg/ml), respectively Table 2 and Fig. 2. Whereas, our results also showed in Table 2, that seeds of HSME have higher FRAP value (1.443±0.02 µM ferrous ion/mg extract) than the stem (0.367±0.004 µM ferrous ion/mg extract) and root (0.513±0.01 µM ferrous ion/mg extract) of HSME, respectively.

3.4 Geno-protective properties of HSME

Our results (Table 3) depicted that seed extract of *H. suaveolens* showed better DNA protection activity as represented in terms of retention percentage (35% ±2 at 50 µg/RM and 80% ±3 at 100 µg/RM) than stem (21% ±1 at 50 µg/RM and 41% ±2 at 100µg/RM) and roots (15% ±2 at 50 µg/RM and 32% ±2 at 100 µg/RM) of HSME, which is in accordance with free radical scavenging activity of HSME (seed).

3.5 Antimicrobial Activity

The *H. suaveolens* extract showed significant antimicrobial activity against different bacterial strains. The antimicrobial activity of methanolic extracts of *H. suaveolens* (seed, stem, and root)

and standard drugs against five bacterial strain *B. cereus, E. aerogens, K. pneumoniae, B. substilus, S. epidermidus* at different concentration (0.1-5 mg/ml) were evaluated. Our results elaborated Fig. 3,4,5 that, HSME of seeds showed better activity against gram negative bacteria (*E. aerogens, K. pneumoniae*), than stem and root parts. Whereas HSME of stem showed better potency against gram positive bacteria (*B. substilus* and *B. cereus)* than seeds and root part. HSME of root part was found to be more effective against *S. epidermidus* (gram positive).

Among standard drugs, penicillin showed lower activity than tetracycline against all tested microbial pathogens Fig. 6. MIC and MBC results Table 4 were found to be in correlation with zone of inhibition assay for antimicrobial activity of plant extract. MIC and MBC for HSME of seeds against *E. aerogens* and *K. pneumoniae* were reported better than root and seeds extract. Whereas stem extract showed higher potency of MIC and MBC against *B. substilus* and *B. cereus* than seeds and root part whereas root part is more effective against *S. epidermidus.*

Table 1. Phytochemical screening of HSME of seeds, stem, and root

Plant part	Flavonoids	Glycosides	Reducing sugar	Tannins	Terpenoids	Phenol	Proteins
HSME (seed)	+	+	+	+	–	++	+
HSME (stem)	++	+	+	—	++	—	++
HSME (root)	++	+	+	+	+	+	+

Table 2. TPC, FRAP value and DPPH radical scavenging activity of HSME seeds, stem, and root. Each value in the table is represented as mean ± SD (n = 3)

Plant extracts	TPC (µg GAE/mg of dry plant extract)	FRAP µM ferrous ion/mg extract	DPPH assay (IC_{50} value in µg/ml)
HSME (seed)	182±3.72	1.443±0.02	72±0.45
HSME (stem)	82±2.64	0.367±0.004	>250±5.46
HSME (root)	100±2.96	0.513±0.01	143±2.15
Ascorbic acid	-----	----	15±0.89

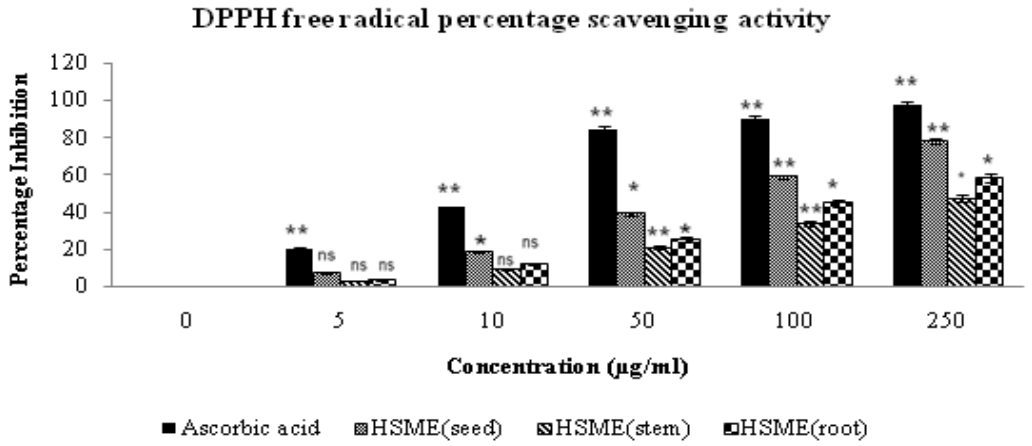

Fig. 2. DPPH free radical percentage scavenging activity of methanol extract of *Hyptis suaveolens* [HSME] seed, stem, root extract at various concentrations. Each value in the figure is represented as mean ± SD (n = 3). Non-significant (ns), significantly different *P<0.05, **P<0.01 vs 0 µg/ml

Table 3. Retention percentage of supercoiled pUC18 plasmid DNA in hydroxyl radical-mediated in-vitro systems with extracts of *H. Suaveolens*.

Concentration/Sample	00 µg/RM	10 µg/RM	50 µg/RM	100 µg/RM
Control	10±1			
HSME (seed)			35±2	80±3
HSME (stem)			21±1	41±2
HSME (root)			15±2	32±2
Mannitol		90±3		

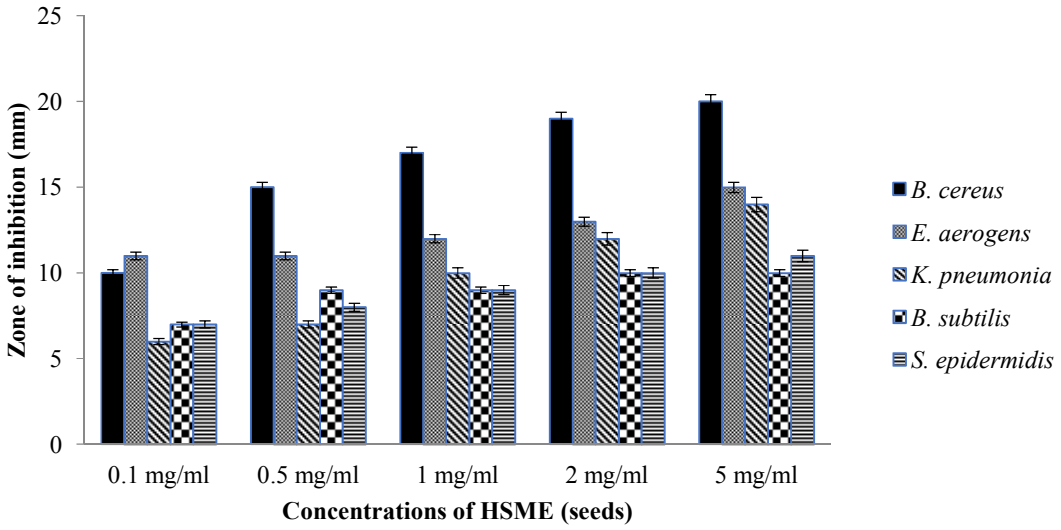

Fig. 3. Zone of inhibition (mm) at various concentrations (mg/ml) for HSME of seed against bacterial strains. Each value in the figure is represented as mean ± SD (n = 3)

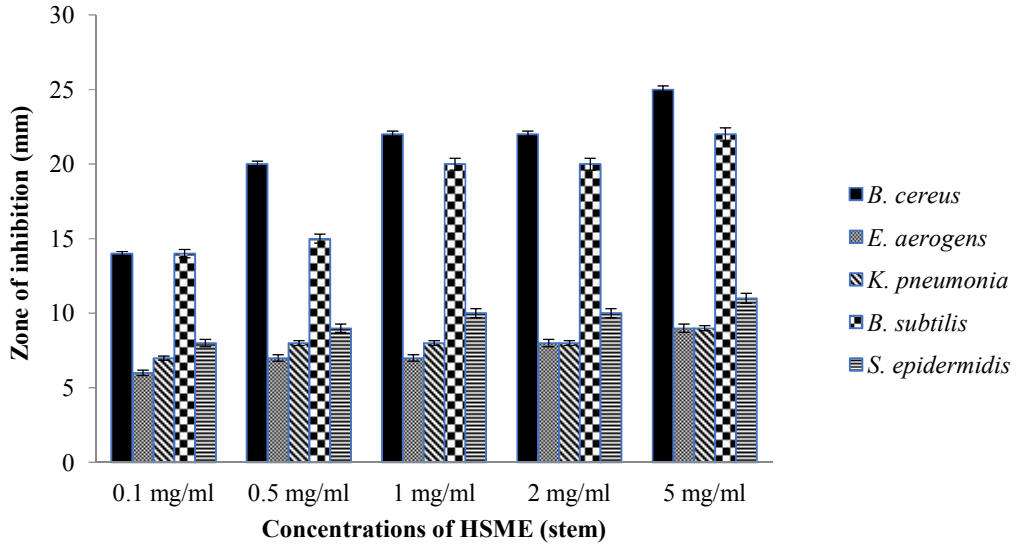

Fig. 4. Zone of inhibition (mm) at various concentrations (mg/ml) for HSME of stem against bacterial strains. Each value in the figure is represented as mean ± SD (n = 3)

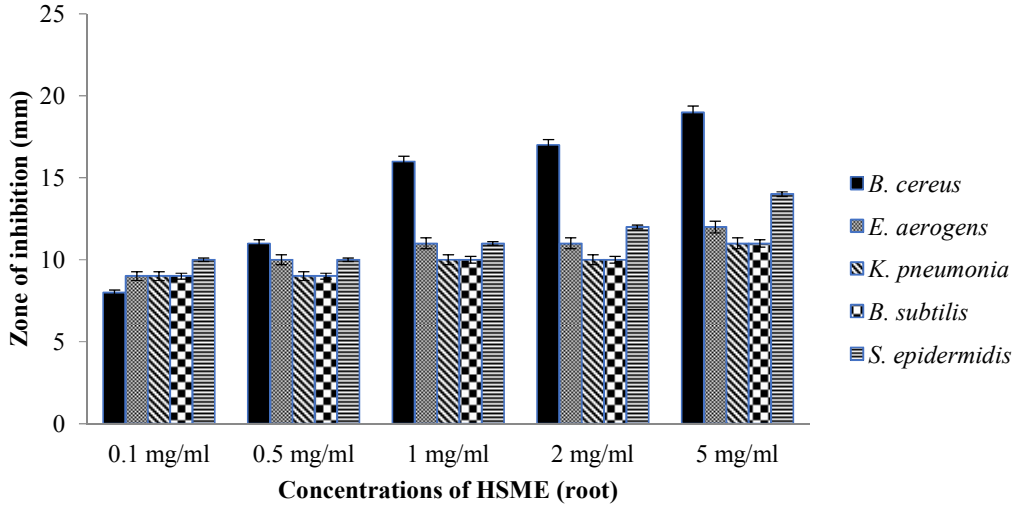

Fig. 5. Zone of inhibition (mm) at various concentrations (mg/ml) for HSME of root against bacterial strains. Each value in the figure is represented as mean ± SD (n = 3)

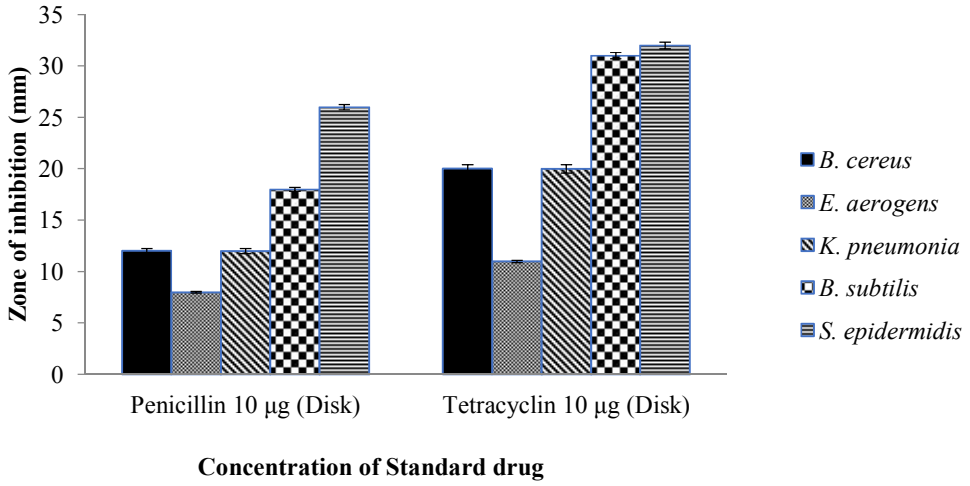

Fig. 6. Zone of inhibition (mm) at 10 µg per disk for standard antibiotic against bacterial strains. Each value in the figure is represented as mean ± SD (n = 3)

4. DISCUSSION

4.1 Phytochemical Analysis

Phytochemicals are the rich source of plant derived medicinal drugs having antioxidant, antimicrobial properties and can be used to cure various diseases [13,19,24,45]. Previous studies revealed that aerial parts, roots, and essential oils of *H. suaveolens* possess medicinal properties might be due to prodigious number of phytochemicals [29,31,34]. Our qualitative study (Table 1) elaborated the presence of phytochemicals in seed, stem, and root of HSME which exhibited the presence of flavonoids, glycoside, reducing sugar, tannins, and proteins with abundance of phenol, however lower terpenoids were found in HSME seeds. HSME root was high in flavonoids although scarcity of tannins and phenols. HSME stem contains most of the phytochemicals with high in flavonoids content.

Table 4. The MIC and MBC values (mg/ml) of HSME (seed, stem, and root) against bacterial strains. The results are shown as average values from three separate experiments.

Sample/Strains	*B. cereus*		*E. aerogens*		*K. pneumonia*		*B. subtilis*		*S. epidermidis*	
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
HSME (seed)	0.07±0.004	1±0.07	0.06±0.004	5±0.14	0.07±0.006	5±0.11	0.09±0.005	>5	0.09±0.006	>5
HSME (stem)	0.04±0.002	0.5±0.08	0.4±0.07	>5	0.19±0.009	>5	0.05±0.002	2±0.1	0.09±0.005	>5
HSME (root)	0.09±0.007	3±0.09	0.08±0.007	>5	0.08±0.006	>5	0.08±0.004	>5	0.08±0.004	4±0.012

4.2 Total Phenolic Content

The Folin–Ciocalteu assay, recognized as one of the standard antioxidant testing procedures, measures the level of total phenolic content in natural products. Phenolic compounds are the major plant secondary metabolites with antioxidant activity [46]. In the present study, as part of analysis of chemical composition, total phenolic contents of seeds, stem, and root of HSME were determined. Our results illustrate the total phenolic content (TPC) of various fractions of HSME which were found to be in the following decreasing order: seeds > root > stem. The result presented in Table 2, clearly demonstrated that HSME of seed have better phenolic content (180 ± 3.72 μg GAE/mg of dry plant extract) than other plant parts like roots and stem (100 ± 2.96 and 82 ± 2.64 μg GAE/mg of dry plant extract, respectively). Furthermore, the existence of considerable amount of TPC in seeds of HSME is in agreement with prior phytochemical information on different parts of *H. suaveolens* which have phenolic compounds as major components [32].

4.3 In-vitro Antioxidant Activity

Excessive free radical generation leads to the oxidative stress which results in macromolecule damage and progression of various diseases [1,2]. Therefore, it is necessary to scavenge these free radicals to balance the homeostasis. Most common protocol to evaluate the free radical scavenging properties of plant extracts uses DPPH as free radical and FRAP assay for antioxidant potential [20,21,32,47]. HSME (seeds, stem, and root) were screened for their *in-vitro* antioxidant properties by DPPH method. We found that HSME (seed) showed better scavenging activity (IC_{50} value = 72±0.45 µg/ml) than HSME of stem (>250±5.46 µg/ml) and root (143±2.15 µg/ml), respectively Table 2 and Fig. 2). Whereas, at low pH of about 3.6, reduction of ferric tripyridyl triazine (Fe3+ -TPTZ) complex to blue colored Fe2+ -TPTZ takes place, which has absorbance at 593 nm. Our results showed in Table 2, illustrated that seeds of HSME have higher FRAP value (1.443±0.02 µM ferrous ion/mg extract) than the stem (0.367±0.004 µM ferrous ion/mg extract) and root (0.513±0.01 µM ferrous ion/mg extract) of HSME, respectively.

The higher DPPH scavenging activity of HSME (seeds) might be due to its high phenolic content as TPC is directly related with antioxidant properties. Our results are in correlation with previous studies where the antioxidant potential of aerial parts and essential oils of *H. suaveolens* were illustrated [31,32]. Biological properties of *H. suaveolens* have been investigated in numerous studies and the results indicate that the reported therapeutic properties are mainly due to the presence of antioxidants compound [32,33]. Our findings are similar with the previous reports on other plants [20,21,48], where it is represented that antioxidant profile of plant extracts are significantly correlated with their total phenol content.

4.4 Geno-Protective Properties of HSME

DNA is a genetic material which controls all the metabolic process of living system. Oxidative stress induced highly reactive free radical species OH· through Fenton's reagent, are prone to cause damage of plasmid supercoiled DNA to single stranded or nicked circular form [49]. It is apparent from our results Table 3 that the mixing of *H. suaveolens* extract at the concentration of 50 µg/10µl, 100 µg/10µl to reaction mixture protects DNA of pUC18 may be due to scavenging of the OH· radicals generated through Fenton's reaction. HSME of seeds showed better reversal (80%) of oxidative DNA damage to its supercoiled form at 100 µg/RM

Table 2. Previously, it has been reported that scavenging of hydroxyl free radical have its impact on genoprotective properties, where it was observed that *Ficus virens*, *Aegle marmelos* and *Ficus palmata* extracts were reported to restrain the OH· dependent break of plasmid DNA [20,21,50].

4.5 Antimicrobial Activity

Appearance of multi-drug resistance in pathogenic microbes as well as adverse side effects of certain antibiotics has triggered enormous interest in the search for novel antimicrobial drugs especially from the natural resources [6,9]. Various plant species were analyzed for antimicrobial efficacy to compensate the resistant drugs for microbial pathogens [11,24,48]. *Lamiaceae* family contains good biological activity, such as *H. suaveolens* [31,32].

Therefore, antimicrobial activity of the *H. suaveolens* extracts and their effectiveness was quantitatively assessed by the presence or absence of zone of inhibition and scaling their diameter. The antimicrobial activity in this study was determined by using agar well diffusion assay, MIC, and MBC. The *H. suaveolens* extract showed significant antimicrobial activity against different bacterial strains. HSME of Seeds showed better activity against gram negative bacteria (*E. aerogens,* and *K. pneumoniae*), than stem and root parts.

We found the antimicrobial activity of HSME of seeds in the following decreasing order: *B. cereus> E. aerogens> K. pneumoniae> S. epidermidus> B. subtilis.* Whereas activity of HSME of stem in the following decreasing order: *B. cereus> B. subtilis> S. epidermidus> K. pneumoniae> E. aerogens* and HSME root is in the following decreasing order: *B. cereus> S. epidermidus> E. aerogens> K. pneumoniae> B. subtilis.* MIC and MBC results Table 4 were found to be in correlation with zone of inhibition assay for antimicrobial activity of plant extract. Similar results were found in previous studies where essential oils of *H. suaveolens* leaves, seeds showed antibacterial activity against gram-positive or gram-negative bacteria [26,27].

The study exposed that HSME of seeds exhibited potential bioactivities which might be due to presence of high TPC and can be used to treat ailments related with oxidative stress or microbial infections. Our study has some limitations such as only one microbial strain from each genus was analyzed for antimicrobial assays, and purification of bioactive compounds was not done. Therefore, further research can be done to check the potency of pure compounds of HSME of seeds on different microbial strains.

5. CONCLUSION

This prelude screening is an interesting assessment of the potential antimicrobial, DNA protective and antioxidant activity of *H. suaveolens*. On the light of these experiments, it could be concluded that the methanolic extracts of different parts of *H. suaveolens* exhibited a fascinating antibacterial activity against most of the strains tested. Study showed that methanolic extract of the *H. suaveolens* investigated are rich in flavonoids, tannins, phenols, reducing sugar, terpenoids and showed presence of glycosides. The methanol extract of *H. suaveolens* seeds showed maximum phenolic content, which in turn revealed maximum scavenging potential and hence also showed better genoprotective potential in comparison to stem and root extracts. The seed methanolic extract of *H. suaveolens* possess strong anti-oxidative activity. The occurrence of antioxidants is a desirable aspect which may have helpful health effect on anticipation of many diseases. Further studies require to be carried out to describe active principle(s) of fractions and to study the relation among chemical structure and antioxidant activity *in vitro* and *in vivo* and the mechanism by which *H. suaveolens* extract exhibit pharmacological actions.

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.

CONSENT

It is not applicable

ETHICAL APPROVAL

It is not applicable

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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