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Research Article

COMPARATIVE STUDY OF PHYTOCHEMICAL, ANTIMICROBIAL, CYTOTOXIC AND ANTIOXIDANT ACTIVITIES IN BLEPHARIS GENUS PLANT SEEDS

Kiran Kumar Mundla, B.Sitaram.Ph.D Associate Professor

PVP SIDDHARTHA INSTITUTE OF TECHNOLOGY, VIJAYAWADA

ABSTRACT

Background:

India is a vast repository of medicinal plants that are used in traditional medical treatments. India gave 'Ayurveda' the oldest golden book in herbal medicine system. Medicinal plants play a vital role for the development of new drugs. Natural products from plant, animal and minerals have been the basis of the treatment of human disease but plants stood above all. Traditional knowledge can serve as powerful search engine, which will greatly facilitate intentional, focused and safe natural product drug discovery. These traditions have relatively organized database, and more exhaustive description of botanical material[1,2]. People of small village are directly in touch with herbs and plant for treatment purpose, so we have to work together for better result. Although the herbal medicine doesn't have side effect but we should follow the strict quality control process. Globalization of herbal medicine system comes with the better advancement of many oxidative stress related diseases are as a result of accumulation of free radicals in the body. A lot of researches are going on worldwide directed towards finding natural antioxidants of plants origins. The aims of this study were to evaluate *in vitro* phytochemical, antimicrobial, cytotoxic and antioxidant activities and to screen for phytochemical constituents of ***Blepharis genus*.** [Family Acanthaceae] Methonolic crude extract.

Methods:

I assessed the antioxidant potential and phytochemical constituents of crude methanolic extract of *Blepharis genus (B.molluginifolia, B.meyeri, B.glomerans, B. capensis*) using tests involving inhibition of superoxide anions, DPPH. The flavonoid, proanthocyanidin and phenolic contents of the extract were also determined using standard phytochemical reaction methods. The cytotoxic activity was determined by MTT test. General antimicrobial analysis was also done for the crude extracts of all the four seed extracts of *Blepharis genus.[3,4,5]*

Results:

Phytochemical analyses revealed the presence of tannins, flavonoids, steroids and saponins. The percentage inhibition of superoxide at the initial stage of oxidation showed antioxidant activity of 87% compared to those of BHT (84.6%) and gallic acid (96%). Also, the percentage inhibition of malondialdehyde by the extract showed percentage inhibition of 78% comparable to those of BHT (72.24%) and Gallic (94.82%).

Conclusions:

Our findings provide evidence that the crude aqueous extract of *B.molluginifolia, B.meyeri, B.glomerans, B. capensis* are the potential source of natural antioxidants, and this justified its uses in folkloric medicines.

Key words:

Herbal medicine, *Acanthaceae*, *B.molluginifolia, B.meyeri, B.glomerans, B.capensis, Phytochemical screening, Antioxidant activity, Cytotoxicity, MTT test etc.*

INTRODUCTION

The art of herbal treatment has very deep roots in Indian culture. Even today in most of the rural areas people are depending on herbal drug systems for primary health care. The present paper deals with the first hand information of the traditional medicinal claims referring to cytotoxic activity of medicinal plants in Andhra Pradesh[6].

So far no systematic medical ethno survey has been made in this area. The goal of this ethno medico botanical survey is to gather information on Biological source, family, parts of the plant used, regional name, local use and therapeutic uses (traditional use) along with chemical constituents and cytotoxic actions[7,8].

Figure1: Blepharis molluginifolia

Regnum: Plantae
Cladus: Angiosperms
Cladus: Eudicots
Cladus: Core eudicots
Cladus: Asterids
Cladus: Euasterids I
Ordo: Lamiales
Familia: Acanthaceae
Subfamilia: Acanthoideae
Tribus: Acantheae
Genus: *Blepharis*

Sample collection:

The seed samples were randomly collected from the nurseries. Representative sampling should be done of specific seeds at the growth stage that is most closely associated with critical values as provided by research data. Sampling criteria and procedures for individual samples are similar to those of soil testing in that the sample should be representative of the field. A predetermined, representative number of plants from a homogenous sampling unit contribute to the composition of bulk sample [9, 10, and 11].

Extraction:

1. 2 kg dried powder of seeds of each plant was extracted by percolation with methanol.
2. The alcoholic filtrate was concentrated under reduced pressure to yield a 200 g gummy residue.

PHYSIOCHEMICAL ANALYSIS:

Total ash:

2 g powder of seed of each plant was taken in a silica crucible and ignited it by gradually increasing the heat to 500°C until it was white, indicating the absence of carbon. Ash was cooled in a desiccators and weighed without delay. Total ash value was calculated in mg per g of air-dried material.

Acid insoluble ash:

To the crucible containing total ash, 25 ml of hydrochloric acid (~70g/l) is added; and is covered with a watch-glass and boiled gently for 5 minutes. The watch-glass is rinsed with 5 ml of hot water and this liquid was added to the crucible. The insoluble matter was collected on an ash less filter paper and it was washed with hot water until the filtrate was neutral. The filter paper containing the insoluble matter was transferred to the original crucible; it was dried on a hot plate and ignited to constant weight. The residue was allowed to cool in desiccators for 30 minutes and then weighed without delay. Acid insoluble ash was calculated in mg per g of air dried material.

Water soluble ash:

To the crucible containing the total ash, 25 ml of water was added and boiled for 5 minutes. The insoluble matter was collected on an ash less filter paper. It was washed with hot water and ignited in a crucible for 15 minutes. Weight of insoluble matter was subtracted from the weight of total ash. The content of water soluble ash was calculated in mg per g of air dried material.

Determination of alcohol soluble extractive:

Four grams of crude powder of seed was macerated with 100 ml of alcohol in a closed flask and was kept on a rotary shaker for 24 h. Thereafter, it was filtered and the filtrate was evaporated to dryness at 105ºC till constant weight was obtained. The percentage of extractable matter was calculated with reference to the sample taken initially.

Determination of water soluble extractive:

Four grams of crude powder of seed was macerated with 100 ml of water in a closed flask and was kept on a

rotary shaker for 24 h. Thereafter, it was filtered and the filtrate was evaporated to dryness at 105ºC till constant weight was obtained. The percentage of extractable matter was calculated with reference to the sample taken initially

Loss on drying:

2 g of crude powder of seed of seed was taken in an evaporating dish and then dried in an oven at 105ºC till constant weight was obtained. The weight after drying was noted and loss on drying was calculated. The percentage was calculated on the basis of sample taken initially.

PHYTOCHEMICAL ANALYSIS

A phytochemical is a natural bioactive compound found in seed foods that works with nutrients and dietary fiber to protect against disease. Research suggests that phyto chemicals, working together with nutrients found in fruits, vegetables and nuts, may help slow the aging process and reduce the risk of many diseases, including cancer, heart disease, stroke, high blood pressure, cataracts, and osteoporosis and urinary tract infections.

Phytochemical Analysis:

Tannins:

A portion of the extract was dissolved in water and clarified by filtration. 10% Ferric chloride solution was then added to the resulting filtrate. The bluish color indicates presence of tannins.

Alkaloids:

0.5g of the extract was stirred in 5.0ml of 1% HCl on steam bath and filtered while hot. Few drops of distilled water were added and 1.0ml of the filtrate was treated with few drops of Wagner's reagent. A reddish brown precipitate indicates presence of alkaloids.

Cardiac Glycosides:

0.5g of the extract was dissolved in 2.0ml glacial acetic acid containing a drop of Ferric chloride solution followed by 2ml of Conc. H_2SO_4. A brown ring formation at interphase indicates presence of deoxy sugars.

Flavonoids:

2.0mls of dil NaOH was added to 2.0ml of the extract. The appearance of a yellow color indicates presence of flavonoids.

Saponins:

1.0ml distilled was added to 1.0ml extract and shaken vigorously. A stable persistent froth indicates the presence of saponins.

Phenols:

Equal volumes of extracts and $FeCl_3$ were mixed. A deep bluish green solution indicates presence of phenol.

Anthraquinones :

0.5g of extract was shaken with 10ml of benzene and filtered, 10% of ammonia solution was added to filtrate and the mixture shaken. The formation of a pink, red or violet color on the ammonical phase indicates anthraquinones.

Reducing Sugars:

3.0ml of extracts was dissolved in 5ml of distilled water followed by Fehling's A and B solution, it was then boiled. A red precipitate indicates a reducing compound.

Protein:

0.5g of the extract was added to 10ml distilled water and the mixture was left to stand for 3hrs and filtered. The 2ml portion of the filtrate was added to 0.1ml Million's reagent. A yellow precipitate indicates presence of protein.

Carbohydrate:

0.5g of the extract was shaken vigorously with water and then filtered. To the aqueous filtrate, few drops of Molisch reagent were added, then 1.0ml Con H_2SO_4 to form a layer of aqueous layer. A brown ring at interphase indicates carbohydrates.

Volatile Oils:

The extract was dissolved in 90% ethanol and few drops of $FeCl_3$ were added. A green color formed indicates presence of volatile oils.

Steroids:

0.5g of extract was dissolved in 3ml of chloroform and filtered Conc. H_2SO_4 was carefully added to the filtrate. A reddish brown color at interphase indicates a steroid ring.

Amino Acids:

Few drops of ninhydrin reagent were added to 1.0ml of extract. Appearance of purple color shows the presence of amino acids.

ANTI MICROBIAL ANALYSIS:

Assay for antimicrobial testing:

Isolated test bacteria were grown overnight on nutrient agar plates and fungi were grown on sabouraud dextrose agar plates. Bacterial inoculums were prepared from overnight grown cultures (24 h) in peptone water (HiMedia, Mumbai, India), and the turbidity was adjusted equivalent to 0.5 McFarland units (approximately 108CFU/ml for bacteria and fungi inoculums turbidity was equivalent at 105 or 106 CFU/ml). The micro organisms were inoculated into peptone water and incubated at 35 ± 2°C for 4 h. The positive control was taken streptomycin (10 µg/ml) for antibacterial activity and keto canozole (10 µg/ml) for antifungal activity[12-18].

The DMSO added disc was taken as negative control to determine possible inhibitory activity of the diluents of extract. The anti microbial activities were determined by the modified Kirby-Bauer disc diffusion method with Muller Hinton agar plates. Aliquots of inoculums were spread over the surface of agar plates with a sterile glass spreader. To test the antimicrobial activity all extracts were dissolved in DMSO to make a final concentration of 200 mg/ml. 20 µl of each extract was soaked separately into sterile discs (Hi Media, Mumbai, India), and the discs were dried in oven for 4 hours at 35°C. These discs were placed on Muller Hinton agar plates, previously swabbed with the bacterial and fungal inoculum. These plates were incubated for a period of 24 h at 37°C in incubator for bacteria and at 30°C for 24-48 h in B.O.D incubator for fungi. Each experiment was done in triplicate and mean values were taken. Antimicrobial activity was measured in the diameter (mm) of the clear inhibitory zone formed around the disc [19-21].

Minimum inhibitory concentration (MIC) and Minimum fungicidal concentration (MFC):

The MIC and MFC values of extracts were determined based on a micro broth dilution method in 96 multi-well microtitre plates with slight modifications. The crude seed extracts were first diluted to the highest concentration, 40000 to 625 µg/ml), to be tested, and 50µl of normal saline was distributed from the second to the ninth well. A volume of 50 µl from each extracts was pipetted into the first test well of each microtitre line which acts as sterility control, and then 50 µl of scalar dilution of seed extract was transferred from the second to the ninth well. To each well was added 10 µl of resazurin indicator solution (prepared by dissolving a 270 mg tablet in 40 ml of sterile distilled water) is added[22]. Using a pipette 30 µl of Muller Hinton broth was added to each well to ensure that

the final volume was of single strength of the normal saline. Finally, 10 µl of the bacterial suspensions were added to each well. In each plate, a column with a broad-spectrum antibiotic was used as the positive control (streptomycin in serial dilution 40000 to 625 µg/ml).

The plates were wrapped loosely with cling film to ensure that bacterium did not become dehydrated, and were prepared in triplicate. Subsequently, they were placed in an incubator at 37°C for 24 h. Any color change from purple to pink or to colorless was recorded as positive.

The lowest concentration at which the color change occurred was taken as the MIC and MFC value. The average of three values was calculated to determine the MIC and MFC of the test material.

ANTIOXIDANT ACTIVITY:

2, 2-Diphenyl-1-Picrylhydrazyl (DPPH) assay:

The method of Liyana-Pathiana and Shahidi was used for the determination of scavenging activity of DPPH free radical. One ml of 0.135 mM DPPH prepared in methanol was mixed with 1.0 ml of aqueous extract ranging from 0.2-0.8 mg/ml. The reaction mixture was vortexed thoroughly and left in dark at room temperature for 30 min. The absorbance was measured spectrophotometrically at 517 nm. The scavenging ability of the seedextract was calculated using this equation;

$$\text{DPPH scavenging activity}(\%) = \frac{(\text{Abs}_{\text{control}} - \text{Abs}_{\text{sample}})}{(\text{Abs}_{\text{control}})} \times 100$$

Where $\text{Abs}_{\text{control}}$ is the absorbance of DPPH + methanol; $\text{Abs}_{\text{sample}}$ is the absorbance of DPPH radical + sample (i.e. extract or standard)[23].

Scavenging activity of superoxide anion:

The scavenging activity of superoxide anion was determined by the method of Yen and Chen. The reaction mixture consists of 1 ml of seedextract (1 mg/ml), 1 ml of PMS (60 µM) prepared in phosphate buffer (0.1 M pH 7.4) and 1 ml of NADH (phosphate buffer) was incubated at 25°C for 5 min, the absorbance was read at 560 nm against blank samples.

RESULTS AND DISCUSSION

Phytochemical Screening:

The phytochemical analysis conducted on *Blepharis seed extract* revealed the presence of tannins, flavonoids, steroids and saponins. The total phenol content of the aqueous seed extract was 0.499 mg gallic acid equivalent/g of extract power. The total flavonoid and proanthocyanidin contents of the seedwere 0.705 and 0.005 mg gallic equivalent/g of extract powder respectively with reference to standard curve ($Y = 0.0067x + 0.0132$, $r^2 = 0.999$). These phytochemical compounds are known to support bioactive activities in medicinal plants and thus responsible for the antioxidant activities of this seed extract used in this study.

Physicochemical characters of seeds of Blepharis molluginifolia, meyeri, glomerans and capensis:

WHO Parameters	Average values (%w/w)	Average values (%w/w)	Average values (%w/w)	Average values (%w/w)
Total ash	6.91	9.1	8.56	8.7
Acid insoluble ash	0.78	3.6	2.4	2.23
Water soluble ash	6.11	3.89	8.56	5.67
Sulphated ash	5	14.06	5.4	3.47
Alcohol extractive value	23.14	7.5	21.07	28.51
Water extractive value	21.97	10.97	17.84	20.09
Loss on drying	3.09	71	7.5	6.7

Phytochemical screening is of paramount importance in identifying new source of therapeutically and industrially valuable compound having medicinal significance, to make the best and judicious use of available natural wealth. A number of medicinal plants have been chemically investigated .The screening of Blepharis molluginifolia for medicinal value has been carried out by some of the workers but there is no conclusion by them that's why I am using that for the research.

A general screening conducted to characterize chemical composition of Blepharis genus (B. mollugenifolia, B.meyeri, B.glomerans, B. capensis) seed samples and these are compared to the results of seed samples that were done by other workers in the past decades. The screening covered mainly nitrogenous compounds, isoprenoids, acetogenins, which are reported to have dramatic physiological activities mainly on central nervous system. All the seed samples showed positive test with 2 different alkaloids on the basis of their Rf values in TLC. 0.15% and 0.21% per gm of sample appeared in the samples respectively. Acetogenin screening included tannins, flavanoids, coumarins, emodins, anthocyanidins, anthocyanins, anthroquinones, anthracene derivatives, phenolics and fatty acid. All seed samples gave a positive test for tannins flavanoid, and phenolics. On the basis of different Rf values, TLC showed abundant occurrence of few of these compounds, phenolics while flavanoids. Rest of the acetogenic compounds were not found either of the sample. 3mg/gm, 3.2mg/gm and 2.2mg/gm total phenolics content appeared in seed samples respectively.

Total flavanoids in seed samples of four plants were found to be 1.1mg, 1.5mg, 1.25mg and 1.0mg /gm of sample respectively. Tannins decrease the bacterial proliferation by blocking key enzymes at microbial metabolism. Tannins play important role such as potent antioxidant. The screening for isoprenoids was confined to steroids, iridoids, triterpenoids, saponins, cardiac glycosides and carotenoids. Saponins are widely well known to have expectorant and anti tussive activity. Total 2 saponins were found to be present, 1 of which were observed in alcoholic extract and other which are observed in all extracts they are found to have RF value 0.5 and 0.30. Total Saponins content in seed samples were found to be 123mg, 101mg, 96mg and 111mg per gm of samples respectively. Saponins are found in many plants and animals. Several workers carried out an extensive phytochemical analysis of plants for the presence of saponin. Steroids and cardiac glycosides were found to be present in all samples that are in four plant seeds, whereas Fatty acid and lipids, volatile oil was found in some seed samples also.

ZONE OF INHIBITIONS :

ORGANISM	ZONE OF INHIBITION B.molluginifolia	ZONE OF INHIBITION B.meyeri	ZONE OF INHIBITION B.glomerams	ZONE OF INHIBITION B.capensis
Enterobacter aerogens	8.5 mm	12mm	15mm	10mm
Escherichia coli	8mm	11.6mm	12mm	8mm
Klebsiella pneumonia	6mm	10mm	12mm	9.8mm
Proteus mirabilis	9.6mm	6mm	12.1mm	11mm
Proteus vulgaris	9.5mm	5.9mm	12mm	11mm
Pseudomonas aeruginosa	7.9mm	8.7mm	9.8mm	13mm
Salmonella typhi	7mm	6.5mm	7.8mm	12.1mm
Shigella dysenteriae	5mm	6.7mm	7.9mm	12.5mm
Staphylococcus aureus	10mm	6mm	8.9mm	11mm
Bacillus subtilis	11.9mm	8.8mm	10.9mm	13mm
Aspergillus niger	10.6mm	8.3mm	11mm	13mm
Candida albicans	6.1mm	9.9mm	13mm	12.9mm
Candida glabrata	6mm	10mm	13.1mm	12mm
Pencilium notatum	12.4mm	11.9mm	12mm	15mm

The use of higher plants and preparations made from them to treat infections is a longstanding practice in a large part of the population, especially in the developing countries, where there is dependence on traditional medicine for a variety of ailments [23-26]. Interest in plants with antimicrobial properties increased because of current problems associated with the antibiotics. Recently, the antimicrobial effects of various seedextracts against certain pathogens have been reported by a number of researchers. Disc diffusion method is the most widely used procedure for testing antimicrobial susceptibility. The disc diffusion procedure (Kirby-Bauer method) has been accepted by the Food and Drug Administration (FDA) and as a standard by the National Committee for Clinical Laboratory Standards.

The extracts of higher seedcan be very good source of antibiotics against various bacterial pathogen. Seedbased antimicrobial compounds have enormous therapeutics potential as they can serve the purpose without any side effects that are often associated with synthetic antibacterial compounds. The study was made against six pathogenic bacteria and four fungal strains using the standard disc diffusion method. Antimicrobial activity of the extracts of Blepharis molluginifolia was first time investigated against *Enterobacter aerogens, Escherichia coli*, *Salmonella typhi*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa, Bacillus subtilis*, *Staphyllococcus aureus, shigella dysentryahea, proteus sps, Aspergillus niger* (moult), *Penicillium notatum* (moult) and *Candida albicans and candida galbrata.*

All the extracts were inhibited growth of almost all the selected bacteria in the range of 5-15 mm and selected fungi in the range 0f 6-15 mm. Among them Ethanol extract showed great activity against *Bacillus subtilis* (11.9 mm) and moderated activity were reported against *Enterobacter* (8.5 mm) *Pseudomonas aeruginosa* (7.9 mm), *Salmonella typhi* (7 mm) and *Staphyllococcus aureus* (10mm) followed by *Klebsiella pneumonia* (6 mm). Antifungal activity of Blepharis molluginifolia Ethanol seed extract showed great activity against *Penicillium notatum (12.6* mm) and moderated activity were reported against Aspergillus (10.6mm) and *Candida sps; (6.1 mm)*. Results of minimum inhibitory concentration (MIC) is shown in Table . The result showed that *shigella sps show greatest activity that is (4.5 mg/ml)* while the lowest MIC of 1 mg/ml was shown by *Enterobacter sps;*.

Cytotoxic assays of methanol extracts of all the four seeds:

% of inhibition of cell viability:

Extracts	10µg/ml	100µg/ml	250µg/ml	500µg/ml
B.molluginifolia	5.3%	11.3%	32.6%	46.2%
B. meyeri	4.6	15.3	20.61	25.5
B.glomerans	3.5	6.5	14.2	21
B.capensis	2.4	5.6	8.9	12.21

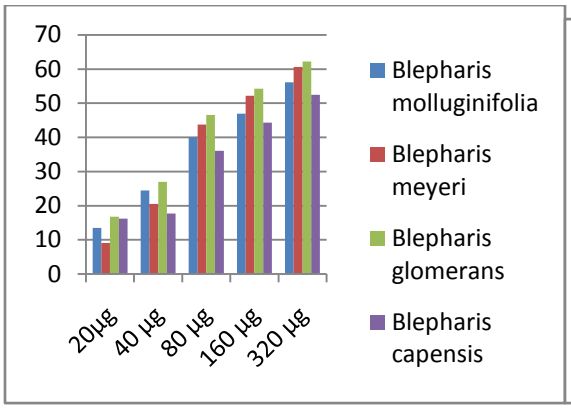
ANTIOXIDANT ASSAYS:

Concentration dependent % inhibition of DPPH radical of different seed extracts:

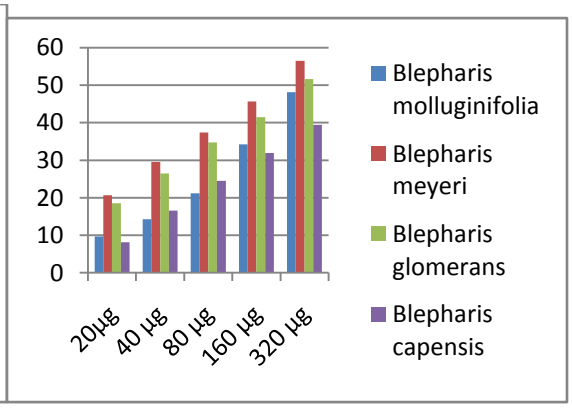
Extracts	20µg	40 µg	80 µg	160 µg	320 µg
Blepharis molluginifolia	13.52 ± 0.3	24.45 ± 0.26	40.00 ± 0.4	46.97 ± 0.2	56.16 ± 0.6
Blepharis meyeri	9.12 ± 0.3	20.51 ± 0.9	43.74 ± 0.52	52.16 ± 0.54	60.66 ± 0.41
Blepharis glomerans	16.81 ± 0.29	26.99 ± 0.65	46.56 ± 0.24	54.27 ± 0.23	62.23 ± 0.62
Blepharis capensis	16.25 ± 0.65	17.75 ± 0.4	36.06 ± 0.6	44.37 ± 0.4	52.46 ± 0.7 6

Concentration dependent % inhibition of superoxide radical of different extracts:

	Blepharis molluginifolia	Blepharis meyeri	Blepharis glomerans	Blepharis capensis
20µg	9.63 ± 0.4	20.69 ± 0.63	18.51 ± 0.35	8.12 ± 0.5
40 µg	14.26 ± 0.6	29.54 ± 0.47	26.45 ± 0.72	16.55 ± 0.3
80 µg	21.14 ± 0.5	37.36 ± 0.26	34.74 ± 0.24	24.46 ± 0.8
160 µg	34.24 ± 0.7	45.66 ± 0.39	41.48 ± 0.73	31.88 ± 0.4
320 µg	48.16 ± 0.5	56.49 ± 0.66	51.62 ± 0.73	39.44 ± 0.2



% inhibition of DPPH radical



% inhibition of superoxide radical

The result of DPPH scavenging activity assay in this study indicates that the seed was potently active. This suggests that the seed extract contain compounds that are capable of donating hydrogen to a free radical in order to remove odd electron which is responsible for radical's reactivity. The ability of this seed extract to scavenge DPPH could also reflect its ability to inhibit the formation of ABTS+. The scavenging activity of ABTS+ radical by the seed extract was found to be appreciable; this implies that the seed extract may be useful for treating radical-related pathological damage especially at higher concentration [27].

Superoxide anion radical is one of the strongest reactive oxygen species among the free radicals that are generated. The scavenging activity of this radical by the seed extract compared favorably with the standard reagents such as Gallic acid suggesting that the seed is also a potent scavenger of superoxide radical [28].

Plants with antioxidant activities have been reported to possess free radical scavenging activity. Free radicals are known as major contributors to several clinical disorders such as diabetes mellitus, cancer, liver diseases, renal failure and degenerative diseases as a result of deficient natural antioxidant defense mechanism [29-31].

CONCLUSION

This study affirms the pytochemical, antimicrobial, *in vitro cytotoxic and* antioxidant potential of crude extract of the seeds of *Blepharis genus* plants, with results comparable to those of the standard compounds such as gallic acid and butylated hydroxyl toluene (BHT). Further studies are needed to clarify the *in vivo* potential of this plant in the management of human diseases resulting from oxidative stress and this is a subject of investigation in our group.

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