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Antiglycation, Antioxidant and Anti Lipid Peroxidation Activities of *Microcephala lamelatta* With Low Cytotoxic Effects *In vitro*

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Abstract: Non-enzymatic protein glycation, oxidative stress and lipid peroxidation are the fundamental processes which are closely associated with various biological disorders particularly diabetes and late diabetic complications. Plant *Microcephala lamellata* (Bunge) Pobed, is the member of family Asteraceae which is widely used as folk medicine. To the best of our knowledge there are no previous reports regarding antiglycation, anti lipid peroxidation, antioxidant and cytotoxicity studies of *M. lamellata*. In this study we prepared various fractions of *M. lamellata* and among all five tested fractions, ethyl acetate fraction exhibited a significant antioxidant activity (IC_{50}= 106.314±1.021 µg/mL) while crude methanolic extract showed moderate antioxidant activity (IC_{50}=213.021±2.216 µg/mL) on 1,1-Diphenyl-2-picrylhydrazyl (DPPH) radical scavenging assay. Ethyl acetate fraction and crude methanolic extract exhibited significant antiglycation activity with IC_{50} values = 165.594±2.732 and 235.712 ±3.238 µg/mL, respectively while on thiobarbeturic acid reactive substance (TBARs) assay dichloro methane and crude methanolic extract showed 72.52% and 62.84% inhibition respectively. In cytotoxicity assay against mouse 3T3 fibroblast cells, dichloro methane fraction showed moderate toxicity with IC_{50} values = 12.367±0.56 µg/mL, while rest of the fractions were non-toxic on cytotoxicity test with IC_{50} values greater than 30 µg/mL. In conclusion, the crude methanolic extract and ethyl acetate fraction of *M. lamellata* are more active against protein glycation, free radicals and lipid peroxidation.

Key words: Protein glycation · Oxidative stress · *Microcephala lamellate* · Antioxidants · Lipid peroxidation · Cytotoxicity

INTRODUCTION

Free radicals derived from glycated proteins are one of the major causes of protein fragmentation and oxidation of lipids and nucleic acids [1, 2]. Advanced glycation endproducts (AGEs) induce lipid peroxidation by a direct reaction between glucose and amino groups on phospholipids [3]. Membrane lipids are mainly susceptible to oxidation due to their high concentration of polyunsaturated fatty acids and they are associated with enzymatic and non-enzymatic systems thus generate free radical species [4]. It is therefore established that oxidative stress, lipid per oxidation and non-enzymatic protein glycation are key processes in the development of diabetes mellitus and related complications [5, 6]. Despite the availability of the current therapies for prevention of the protein glycation and oxygen stress related diseases are still a major threat to human health. Antioxidants effectively protect against glycation derived free radicals and may have therapeutic potential for the inhibition of radical induced processes [7]. Moreover, plant samples with combined antioxidant, antiglycation and anti lipid per oxidation properties are highly desired because they can be more effective in treating various biological disorders [8].

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The plant *Microcephala lamellata* (Bunge) Pobed, belongs to family Asteraceae which is widely distributed in Ziarat, Baluchistan, Pakistan. It is erect or ascending, profusely branched above, up to 50 cm tall, glabrous herb with sulcate or obtusely angled internodes while flowering period is April to August. Traditionally the plant has been used to treat jaundice, long standing fever, colic pain and the dysentery of children [9], which also show its non-toxic effects. To the best of our knowledge plant *M. lamellata* was not previously evaluated for these bioactivities.

MATERIALS AND METHODS

Plant Extraction: The plant *Microcephala lamellata* was collected from Ziarat, Baluchistan, Pakistan. The Plant was identified and confirmed by Department of Botany, University of Baluchistan, Quetta, Pakistan. The whole plant of *M. lamellata* was dried in dark, chopped and grinded to coarse powder. The powdered plant (500 gm) was initially extracted with methanol for 24 hours at room temperature with occasional stirring. The process was repeated three times and the combined extracts were concentrated under reduced pressure in rotary evaporator to give the crude methanol extract as a gummy residue. A part of crude methanol extract was stored for further analysis while the remaining extract was dissolved and suspended in distilled water. The suspension was partitioned successively in various fractions though separating funnel using *n*-hexane, dichloromethane and ethyl acetate. The organic fractions were condensed down under vacuum while the aqueous fractions were concentrated in the freeze drier. All the fractions were stored at 4°C.

Reagents for DPPH and Superoxide Assays: 1,l-Diphenyl-2-picryIhydrazylradical (DPPH), naphthylethylenediamine, potassium dihydrogen phosphate, dipotassium hydrogen phosphate, 3- (2-pyridyl)-5,6-di(*p*-sulfophenyl)-1,2,4-triazine (disodium salt or ferrozine), reduced β-nicotinamide adenine dinucleotide (NADH), 5- methylphenazium methyl sulfate (PMS), nitro blue tetrazolium salt (NBT) and standard radical scavengers Propyl gallate (propyl 3,4,5-trihydroxybenzoate) were purchased mainly from Sigma Chemical Co.

Reagents for Antiglycation Assay: Bovine Serum Albumin (BSA) from Research Organics, anhydrous D-glucose from Fisher Scientific, Sodium azide and trichloro acetic acid (TCA) from Scharlau. Phosphate buffer (pH 7.4), phosphate buffer saline (pH 10) and rutin were purchased from Carl Roth.

Reagents for Lipid Peroxidation (TBARs) Assay: Phosphotidyl choline (substrate), Thiobarbituric Acid (TBA), Quercetin, Trichloro acetic acid (TCA), Butylated hydroxyanisole (BHA) were purchased from Sigma Aldrich while Ferrous Sulphate was purchased from Roth, Tris-HCL buffer pH.7.1 and Double Distilled Water (DDW) were also used in this assay.

Reagents for Cytotoxicity Assays: Reagents for cytotoxicity assay were purchased as mouse fibroblast (3T3) from European American Culture Collection (EACC), Dulbecco's Modified Eagle's Medium (DMEM) from Sigma-Aldrich, Fetal Bovine Serum (FBS) from GIBCO-BRL,MTT (3-[4,5-dimethylthiazole-2-yl]-2,5- diphenyl-tetrazolium bromide) from Amresco, Penicillin and Streptomycin from Sigma- Aldrich, Trypsin /EDTA(0.25%) from GIBCO.

DPPH Radical Scavenging Assay: Free radical scavenging activities of the test compounds were determined by measuring the change in absorbance of DPPH (l,l-Diphenyl-2-picrylhydrazyl radical) at 515 nm by the spectrophotometric method described by S.K. Lee [10]. In this assay, reaction mixture comprised of 95 µL (0.3mM) of ethanolic solution of DPPH' and 5 µL of the plant fraction (0.5mg/mL) dissolved in DMSO.

Superoxide Anion Assay: The reaction was performed in triplicate in a 96-well plate and the absorbance was measured on multiplate reader (Spectra Max 340). The reaction mixture contained 40 µL(0.2mM)of nicotinamide adenine dinucleotide (NADH), 40 µL(0.018mM) nitroblue tetrazolium (NBT), 90 µL of (100mM) phosphate buffer pH 7.4 and 10 µL (0.5mg/mL) of the test samples (Plant extracts) pre-read at 560nm. The reaction was initiated by the addition of 20 µL (0.008mM) of phenazine methosulphate (PMS) and then incubated at room temperature for 5 minutes. Formation of blue color formazan dye was measured at 560 nm. The control contained 10 µL of DMSO, instead of the test compound. The solutions of NBT, NADH and PMS were prepared in phosphate buffer, while the test fractions were dissolved in DMSO [11].

The radical scavenging activities (%) were calculated by using the following formula:

$$\% \text{ RSA} = [100-(AS/AC * 100)]$$

RSA is radical scavenging activity, AS is the absorbance of radicals and formazan dye in the presence of test sample and AC is the absorbance of formazan dye without sample (control).

Antiglycation Assay Protocol: BSA-fluorescent assay was used as described previously by Choudhary [12]. In this assay 0.5 mg/mL of unknown inhibitors (fractions) were dissolved in DMSO, along with standard inhibitor. The comparison of fluorescence intensity at 370 nm excitations and emission at 440 nm was obtained by using spectrofluorimeter [13-15]. Rutin, a standard inhibitor, showed IC_{50} value 98.01±2.03 µM.

Anti Lipid per Oxidation Assay: Lipid peroxidation, an indicator of tissue injury induced by reactive oxygen species is measured as thiobarbituric acid reactive substance (TBARS). The amount of tissue TBARS was measured by the thiobarbituric acid assay (TBA) as previously described by Buege and Aust [16].

20 µL of substrate (Phosphotidyl choline), 5 µL of Tris-HCL buffer (pH 7.4), 5 µL of Ferrous Sulphate (1mM) and 20 µL (0.3 mg/mL) of sample inhibitor were added in 96 well plate then 30 µL of double distilled water was added. It was then incubated at 37°C for 15 minutes. Then 50 µL of TCA (50%) and 100 µL of TBA (0.35g) were added to the reaction mixture. It was then incubated for 15 minutes at boiling water bath and pink colour chromogen appeared. Readings were taken at 532 nm (spectra Max-340). Percent radical scavenging activity by samples was determined in comparison with a DMSO treated control group. Following formula was used to calculate percent lipid per oxidation inhibition activity (% Inhibition). Quercetine and butylated hydroxyanisole (BHA) were used as standard inhibitors (0.5 mg/mL) in anti lipid peroxidation assay with 85.025% and 90.321% inhibition respectively.

$$\% \text{ Inhibition} = 100 - \{(\text{OD test compound} / \text{OD control}) \text{ X } 100\}$$

Cytotoxicity Assay: Cytotoxicity of the samples was evaluated in 96-well flat-bottom microplates using the standard MTT (3-[4,5-dimethylthiazole-2-yl]-2,5-diphenyltetrazolium bromide) colorimetric assay as described by Choudhary [12]. However, in this case 3T3 cells (mouse fibroblasts) were cultured in (MEM), supplemented with 5% (FBS), by using a 75 cm^2 flask in a 5% CO_2 incubator at 37°C. Cycloheximide was used as a standard (IC_{50} = 0.3 ± 0.089 µM).

Statistical Analysis: The results were expressed as mean ± SEM and the EZ-fit software (Perrella Scientific Inc., Amherst, U.S.A.) was used to calculate the IC_{50} values (µg/mL). IC_{50} values were measured by using different concentrations of the active samples.

RESULT AND DISCUSSION

Screening of traditionally used plants and discovery of their active components with combined antioxidant and antiglycation properties would be beneficial in the treatment of various disorders [17].

Ziarat a city of Baluchistan, Pakistan has enormous floral resources which are being used traditionally for the treatment of various ailments. The close relationship between free radicals generation, lipid peroxidation and non-enzymatic protein glycation actually motivated us to screen and evaluate different traditionally used plants which might have the potential to inhibit all these biological processes effectively.

Microcephala lamellata is an important plant which is used traditionally as a medicine. Various fractions of *M. lamellata* were prepared and tested for their antiglycation, antioxidant, anti lipid per oxidation and cytotoxicity effects.

In the DPPH radical scavenging assay ethyl acetate (EtOAc) fraction showed promising radical scavenging activity as 96.121% and crude methanolic extract exhibited antioxidant activity as 72.927% as shown in the Table 1. Then different concentrations of these active samples were used to calculate IC_{50} values which were found to be significant as 106.314±1.021 µg/mL and 213.021±2.216 µg/mL respectively, as compared to standard propyl gyllate with IC_{50} value = 34.537±1.311 μg/mL as shown in Figure 1. Similarly in same assay dichloro methane (DM) showed 50.036% antioxidant activity while other fractions were mainly inactive at 300 µg/mL. On super oxide anion assay aqueous and dichloro methane (DM) fractions showed mild antioxidant activity as 48.442%, 40.376% while others were less active in this assay.

These fractions were then evaluated for their inhibitory potential against protein glycation (one of the major causes of diabetic complications). On BSA-glucose fluorescent-based assay ethyl acetate (EtOAc) fraction and crude methanolic extract showed a moderate

Table 1: Results of various Bioactivities of *Microcephala lamellate* fractions

Extract/Fractions	DPPH Radical Scavenging % Inhibition	Super Oxide Anion % Inhibition	Antiglycation % Inhibition	Lipid peroxidation % Inhibition	Cytotoxocity IC_{50} (μg/mL)
Crude MeOH	71.927	19.580	60.169	62.843	>30
n-Hexane	15.718	38.660	35.149	2.453	>30
(DM)	50.036	40.376	40.654	72.526	12.367±0.56
EtOAc	96.121	30.731	68.257	30.490	24.80±0.18
Aqueous	32.179	48.442	22.565	21.295	>30

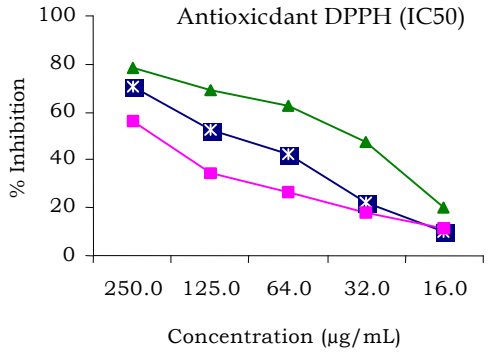

Fig. 1: Antioxidant activity of various fractions at different concentration and IC_{50} values calculation. Inhibition by propyl gyllate (standard antioxidant) ---▼---, Ethyl acetate fraction ---x---, Crude methanolic fraction ---●---

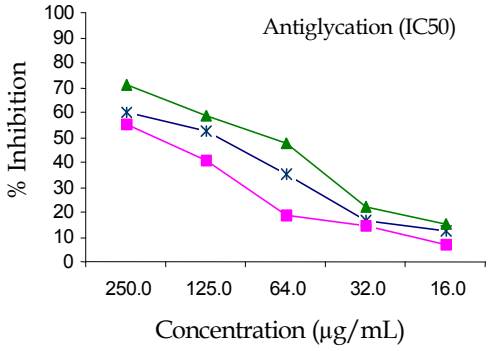

Fig. 2: Antiglycation activity of various fractions at different concentration and IC_{50} values calculation. Rutin (standard antiglycation agent) ---▼---, Ethyl acetate fraction ---x---, Crude Methanolic fraction ---●---

inhibitory potential with IC_{50} values 165.594±2.732 and 235.712±3.238 µg/mL respectively while dichloro methane (DM) fraction show 46.0542% inhibition against protein glycation *in vitro* while other fractions were found to be inactive. Rutin a standard antiglycation agent exhibited IC_{50} value 98.01±2.03 µM in this assay as shown in (Figure 2).

Since some of these fractions showed promising antioxidant activities so, in order to investigate their anti lipid per oxidation potential, TBARs assay was performed.

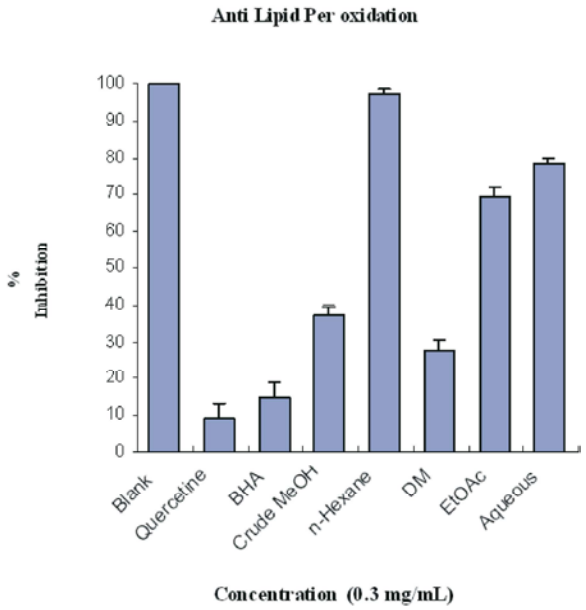

Fig. 3: Anti lipid peroxidation activity of various fractions of *Microcephala lamellate* as compared to standard inhibitors Quercetin and BHA. Blank sample is without any inhibitor sample.

Among these samples dichloro methane fraction and crude methanolic extract showed relatively good activity as 72.526% and 62.843% inhibition respectively at a concentration 0.3 mg/mL as shown in Fig. 3.

All these fractions were then subjected to cytotoxicity test on mouse fibroblast 3T3 cells. Dichloro methane (DM) fraction was found to be moderately toxic with IC_{50} value = 12.367±0.56 µg/mL while all other samples were non-toxic as shown in Table 1. Cycloheximide (IC_{50} = 0.3 ± 0.089 µM) was used as a standard in this assay.

CONCLUSION

These results suggest that plant *Microcephala lamellate* possesses inhibitory effects against various biological disorders and is medicinally important with non-toxic effects that support its traditional use against these problems. The current study shows that crude

methanolic (MeOH) extract and ethyl acetate (EtOAc) fraction have multiple inhibitory activities against protein glycation and oxidation process while other fractions such as dichloro methane (DM), n-hexane and aqueous have variable effects on these bioassays. In future, these active and non-toxic fractions can be subjected to animal studies and active ingredients responsible for different bioactivities can be isolated and characterized.

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