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Evaluation of antioxidant, DNA cleavage and α-amylase inhibitory activity of polyphenolics from the root bark of *Bauhinia racemosa*

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ABSTRACT

*Bauhinia racemosa Lamk. (Caesalpiniaceae) is a small tree where bark and leaves are used extensively for the treatment of inflammation, headache, fever, malaria, skin infections, dysentery and diarrhea. The primary objective of this study was to evaluate the antioxidant, DNA cleavage and α-amylase inhibitory effects of the phenolic compounds: racemosol (**BR-I**), de-O-methyl racemosol (**BR-II**), 1,7,8,12b-tetrahydro-2,2,4-trimethyl-2H-benzo[6,7]cyclohepta [1,2,3-de][1]benzopyran-5,10,11-triol (**BR-III**) and racemosolone (**BR-IV**), isolated from ethanolic extract of root bark of B. racemosa and their structures were determined by spectroscopic analysis. Further the total phenolic contents were also determined. The antioxidant activities were evaluated using the scavenging of 2-2-diphenyl-1-picrylhydrazyl radical (DPPH) and FRAP total reduction capability methods where **BR-II** exhibited most significant antioxidant effect at all concentrations while others were active at high concentrations only. In DNA cleavage activity measured using gel electrophoresis method, **BR-I**, **BR-II** and **BR-III** displayed partial cleavage of DNA at all concentrations while in **BR-IV** results were inconclusive as the compound was fluorescent under UV. The α-amylase inhibitory assay was performed using the chromogenic DNSA method where **BR-IV** showed 88.4% and 100% inhibition at 250 µg and 500 µg concentration respectively. The total phenolics were found to be 0.75 mg/ g catechol which could be correlated with the antioxidant capacity established by two different methods suggesting that these may be the active antioxidant ingredients of B. racemosa.*

Keywords: *Bauhinia racemosa* Lamk., polyphenolics, antioxidant activity, DNA cleavage activity, α-amylase inhibitory activity.

INTRODUCTION

Phenolic compounds are a class of low molecular weight secondary plant metabolites. Most of these compounds are able to scavenge free radicals such as those produced during cell metabolism (reactive oxygen species, ROS or free radicals such as hydrogen peroxide, hydroxyl radical and singlet oxygen) that can lead to oxidative stress. Oxidative stress is associated with major chronic health problems like cancer, inflammation, neurodegeneration diseases, heart diseases, aging and also food deterioration [1]. Until the endogenous mechanisms of protection are sufficient to contrast the various reactive oxidative injuries generated both, endogenously in metabolism [2] or exogenously by xenobiotics [3], severe damages occur to the biomolecules. When these defences are overwhelmed, an imbalance occurs, and it becomes necessary to provide, from external sources, the required protection to avoid oxidative stress and related irreversible alterations of biomolecules. *In vitro* and *in vivo* studies have shown the effective role of phenolics in the prevention or suppression of disorders such as oxidative damage to DNA, proteins and lipid, or many chronic diseases [4, 5].

Bauhinia racemosa Lamk. (Hindi : Kachnal) belonging to Family Caesalpinaeceae is a small deciduous tree widely distributed throughout the tropics. The bark and leaves are used extensively for the treatment of inflammation, headache, fever, malaria, skin infections, diarrhea and dysentery [6]. Methanolic extract of the stem bark exhibited strong free radical scavenging, antitumor, antimicrobial, analgesic, antipyretic and anti-inflammatory effects [7-9]. The alcoholic extract of the leaves showed different degrees of anti-inflammatory, analgesic, antipyretic as well as antispasmodic properties [10]. Fresh flower buds demonstrated inhibition of ulcer formation [11]. Cytotoxicity against CA-9 KB in cell culture, hypotensive and hypothermic activities are reported from the hydroalcoholic extract of this plant [12]. The antibacterial, antifungal and antiviral activities of the root bark extract and isolated compounds have been carried out [13]. In the present study, root bark of *B. racemosa* was extracted with ethanol keeping an aim of evaluating the total phenolic contents. Further, the compounds were isolated and antioxidant capacity (DPPH and FRAP assays), DNA cleavage and α-amylase inhibitory activities of the isolated polyphenolics were evaluated.

EXPERIMENTAL SECTION

2.1 Plant material

Plant material was collected from Jhalana area, Jaipur and authenticated from the Herbarium, Department of Botany, University of Rajasthan, Jaipur (RUBL 19765) where a voucher specimen is deposited.

2.2 Extraction and isolation

Air – dried and powdered root bark of *B. racemosa* (3.8 kg) was exhaustively extracted with EtOH (95%) on a steam bath for 8 h thrice. The extract was concentrated using rotary evaporator yielding dark brown semi-solid (120 g) which was extracted with hexane, $CHCl_3$ and EtOAc (500 mL each) to obtain n-hexane (10.55 g), $CHCl_3$ (22.87 g) and EtOAc (35.25 g) fractions when dried under vacuum. The hexane and $CHCl_3$ fractions exhibited nearly similar TLC profile and hence, were mixed together and chromatographed over silica gel column (46 x 4 cm) which afforded eight compounds. Elution was carried out with solvents of increasing polarity *viz.*, petroleum ether (60^0 - 80^0), CH_2Cl_2, EtOAc and MeOH. The eluates were collected in 250 ml portions using stepwise gradient to get thirteen fractions (A-M). A reddish brown solid obtained on the removal of solvent from fraction J (eluent CH_2Cl_2 : pet ether, 3 : 2) was crystallized as deep red prisms from $CHCl_3$ as **BR-I** (411 mg). Fraction M (eluent CH_2Cl_2) furnished **BR-II** as deep red crystals (610 mg) on crystallization from $CHCl_3$ / MeOH. Similarly, the EtOAc soluble fraction was applied over silica gel column (26 x 2.5 cm) using a solvent system of $CHCl_3$ and MeOH with increasing polarity as an eluent to give eleven fractions (N-Y). Fractions S and V (eluent MeOH : $CHCl_3$, 1: 49) furnished orange microcrystalline powder of **BR-III** (209 mg) and greenish grey crystalline needles of **BR-IV** (524 mg) on crystallization from $CHCl_3$ and $CHCl_3$ / MeOH respectively. Fractions O and T yielded compounds **BR-I** and **BR-II** respectively. However, compounds could not be crystallized from fractions Q, R, U, W, X and Y. Other compounds isolated have been reported earlier [14]. The phenolic compounds isolated from *B. racemosa* were determined by comparing the m.p. and spectral data (^1H, ^{13}C NMR and mass spectra) with those reported in literature [13, 14], and by direct comparison of TLC with authentic reference compounds.

2.3 Total phenolic contents

The total phenolic contents in the ethanolic extract of *B. racemosa* root bark were estimated using the Folin-Ciocalteau method [15]. Aliquot of sample was pipetted out in a test tube and volume was made up to 3 ml with distilled water. Folin-Ciocalteau reagent (0.5 ml) was added to the tube and incubated for 3 min at room temperature. Sodium carbonate (20%; 2 ml) solution was added, mixed thoroughly and the tube was incubated for 1 min in boling water bath. Absorbance was measured at 650 nm against a reagent blank. Standard curve using different concentrations of standard phenolic - catechol was prepared. From the standard curve, concentration of phenol in the test sample was determined and expressed as mg of catechol equivalent.

2.4 Antioxidant activity

2.4.1 DPPH free radical-scavenging effect

DPPH activity was carried out according to the method Khalaf et al [16]. A solution (2.5 ml) of 2×10^{-3} µg/ml of 1,1-diphenyl-2-picrylhydrazyl (DPPH) in methanol was mixed with equal volume of test compound/ascorbic acid (standard) solution in methanol and kept in dark for 30 min. The absorbance at 517 nm was monitored at different concentrations (10, 20, 40, 60, 80 µg/ml) using UV-Vis spectrophotometer. Blank was also carried out to determine the absorbance of DPPH, before interacting with the extract.

Radical scavenging activity [17] was calculated using the formula:

Radical scavenging activity = $[(Abs_0 – Abs_1) / Abs_0] \times 100$

Where Abs_0 is the absorbance of the blank and Abs_1 is the absorbance in the presence of test compound.

2.4.2 FRAP total reduction capability effect

Fe^{3+} - Fe^{2+} transformation assay was carried out following the method of Oyaizu [18]. To 1 ml of test compound/ascorbic acid (standard) at different concentrations (62.5, 125, 250, 500, 1000 µg/ml) in ethanol was added 1 ml of distilled water, 2.5 ml phosphate buffer (0.2 M, pH 6.6) and 2.5 ml potassium ferricyanide (1%). The mixture was incubated at 50°C for 20 min. Trichloroacetic acid (2.5 ml, 10%) was added to the mixture and mixed with distilled water (2.5 ml) and $FeCl_3$ (0.5 ml, 0.1%) and the absorbance was measured at 700 nm using UV-Vis spectrophotometer. Higher absorbance indicates greater reducing power.

2.5 DNA cleavage activity

2.5.1 Preparation of culture media

DNA cleavage experiments were done according to the literature method [19]. Nutrient broth [peptone, 10; yeast extract, 5; NaCl, 10 in g/l] was used for culturing of *Escherichia coli*. Fifty ml media was prepared and autoclaved for 15 min at 121^{o}C under 15 lbs pressure and inoculated for 24 h at 37^{o}C.

2.5.2 Isolation of DNA

The fresh bacterial culture (1.5 ml) was centrifuged to obtain the pellet which was dissolved in 0.5 ml of lysis buffer (100 mM Tris pH 8.0, 50 mM EDTA, 10% SDS). To this, 0.5 ml of saturated phenol was added and incubated at 55^{o}C for 10 min, then centrifuged at 10,000 rpm for 10 min and to the supernatant, equal volume of chloroform: isoamyl alcohol (24:1) and 1/20th volume of 3M sodium acetate (pH 4.8) were added. This was followed by centrifuging at 10,000 rpm for 10 min and to the supernatant, 3 volumes of chilled absolute alcohol were added. The precipitated DNA was separated by centrifugation, the pellet was dried and dissolved in TAE buffer (10 mM Tris pH 8.0, 1 mM EDTA) and stored in cold conditions.

2.5.3 Agarose gel electrophoresis

Cleavage products were analyzed by agarose gel electrophoresis method [19]. Test samples (1 mg/ml) were prepared in DMF. The samples (25 mg) were added to the isolated DNA of *E. coli* and incubated for 2 h at 37^{o}C and then 20 ml of DNA sample (mixed with bromophenol blue dye at 1:1 ratio) was loaded carefully into the electrophoresis chamber wells along with standard DNA marker containing TAE buffer (4.84 g tris base, pH 8.0, 0.5 M EDTA/l) and finally loaded on agarose gel and passed the constant 50 V of electricity for 30 min. After removing the gel and staining with 10 mg/ml ethidium bromide for 10-15 min, the bands were observed under Vilber Lourmat Gel documentation system and then photographed to determine the extent of DNA cleavage. The results were compared with standard DNA marker.

2.6 *α*-Amylase inhibitory activity

The inhibition assay was performed using the chromogenic DNSA method [20]. The total assay mixture composed of 1400 µl of 0.05 M sodium phosphate buffer (pH 6.9), 50 µl of amylase (Diastase procured from HiMedia, Mumbai, Cat No. RM 638) and compounds at concentration 100, 250 and 500 µg were incubated at 37°C for 10 min. After pre-incubation, 500 µl of 1% (w/v) starch solution in the above buffer was added to each tube and incubated at 37°C for 15 min. The reaction was terminated with 1.0 ml DNSA reagent, placed in boiling water bath for 5 min, cooled to room temperature and the absorbance measured at 540 nm. The control amylase represented 100% enzyme activity and did not contain any sample of analysis. To eliminate the absorbance produced by sample, appropriate extract controls with the extract in the reaction mixture in which the enzyme was added after adding DNS. The maltose liberated was determined by the help of standard maltose curve and activities were calculated according to the following formula

$$\text{Activity} = \frac{\text{Conc. of Maltose liberated x ml of enzyme used}}{\text{Mol. wt of maltose x incubation time (min)}} \text{ x dilution factor}$$

One unit of enzyme activity is defined as the amount of enzyme required to release one micromole of maltose from starch per min under the assay conditions. The inhibitory/induction property shown by the sample was compared

with that of control and expressed as % inhibition / induction. This was calculated according to the following formula

$$\% \text{ inhibition/induction} = \frac{\text{Activity in presence of compound}}{\text{Control activiy}} \times 100$$

RESULTS AND DISCUSSION

Three tetracyclic phenols racemosol (**BR-I**), de-O-methyl racemosol (**BR-II**), 1,7,8,12b-tetrahydro-2,2,4-trimethyl-2*H*-benzo[6,7]cyclohepta[1,2,3-*de*][1]benzopyran-5,10,11-triol (**BR-III**) and one pentacyclic phenol racemosolone (**BR-IV**) (Fig.1.) were isolated from ethanolic extract of root bark of *B. racemosa* and identified on the basis of spectral data (^{1}H, ^{13}C NMR and mass spectra) and melting point [13, 14].

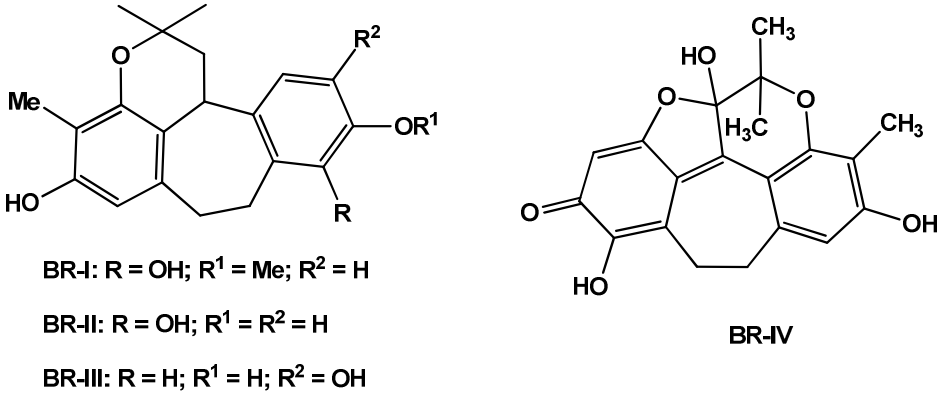


Fig.1. Phenolic compounds isolated from root bark of *B. racemosa*

3.1 Total phenolic contents

The samples investigated were analyzed for total phenolic contents as these, are largely responsible for the antioxidant activity of plant extracts [21, 22]. Total phenolic contents were quantified using the Folin-Ciocalteau reagent, which is a widely used method for estimating total phenolic compounds and catechol as the standard. The total phenolic content of the ethanolic extract of *B. racemosa* was determined to be 0.75 mg of catechol /g (dry weight).

3.2 Antioxidant activities

The % inhibition of DPPH indicated that all the tested compounds exhibited activity nearly equivalent to that of standard at high concentrations (Table 2). However, **BR-II** was active at all concentrations. It is also noteworthy that **BR-IV** was least active amongst the tested compounds at low concentrations although equally active at high concentration. The activity order observed in this system was **BR-II** ~ **BR-III** > **BR-I** > **BR-IV**. The main structural feature responsible for the antioxidative activity in these compounds is the phenolic groups. Phenols are able to donate the hydrogen atom of the phenolic -OH to the free radicals, thus these stop the propagation chain during the oxidation process. The behavior of this group depends markedly on two main factors: first, the electronic characteristics (the electron releasing or electron withdrawing character of the other substituents) and second, the steric hindrance of the molecules because the steric accessibility to the DPPH is determinant of the reaction, as reports indicate that small molecules which have better access to the radical site have relatively higher antioxidant capacity [23]. The lower activity of **BR-IV** which has a more voluminous group than compound **BR-I** can be ascribed to a greater steric hindrance of the former to react with DPPH radical. Compounds **BR-II** and **BR-III** presented similar and higher antioxidative activities than **BR-I**. In these, the presence of a second hydroxyl group at the ortho-position, yielding a catechol ring that also lowers the OH bond-dissociation enthalpy and increases the rate of H-atom transfer to peroxy radicals [24]. Inhibition of DPPH radicals was almost double for **BR-II** and **BR-III** containing a diortho phenolic motif than for **BR-I** where a methoxy group replaces the OH group at ortho position.

Table 1. Antioxidant activity of polyphenolics by DPPH method

Isolates	% Inhibition (concentration in µg/ml)				
	10	20	40	60	80
BR-I	78.85 ± 1.36	93.74 ± 0.36	94.39 ± 0.10	94.60 ± 0.18	94.80 ± 0.2
BR-II	90.85 ± 1.39	94.65 ± 0.40	94.70 ± 0.30	94.79 ± 0.2	94.88 ± 0.2
BR-III	84.44 ± 0.48	94.60 ± 0.42	94.67 ± 0.39	94.69 ± 0.24	94.79 ± 0.8
BR-IV	60.66 ±3.4	65.50 ± 3.5	77.6 ± 1.5	80.11 ± 1.4	93.54 ± 0.6
Ascorbic acid	93.7	94.3	96.25	97.02	97.2

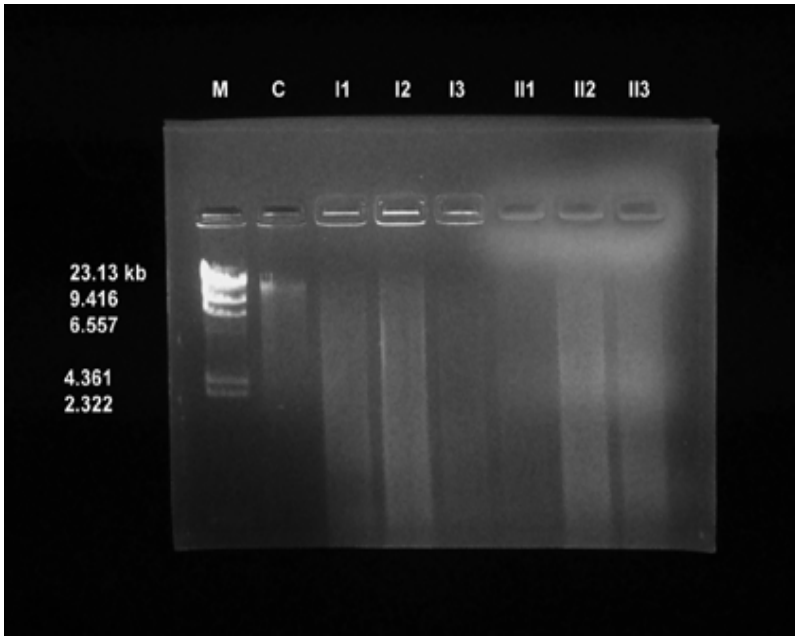
Compounds already confirmed as being good radical scavengers, also exhibited profound reducing capacity as can be concluded from the FRAP values (Table 2). The absorbance data indicated nearly same order of activity profile as by DPPH method with **BR-II** to be active at all concentrations.

Table 2. Antioxidant activity of polyphenolics by FRAP method

Isolates	Absorbance				
	62.5 µg/ml	125 µg/ml	250 µg/ml	500 µg/ml	1000 µg/ml
BR-I	0.322 ± 0.02	0.533 ± 0.41	0.786 ± 0.03	1.076 ± 0.07	1.182± 0.04
BR-II	0.660 ± 0.02	0.739 ± 0.02	0.942 ± 0.02	1.199 ± 0.14	1.273 ± 0.06
BR-III	0.362 ± 0.02	0.537 ± 0.01	0.827 ± 0.03	1.153 ± 0.06	1.246 ± 0.02
BR-IV	0.234 ± 0.02	0.382 ± 0.01	0.552 ± 0.03	1.080 ± 0.02	1.170 ± 0.01
Ascorbic acid	0.553	0.813	1.052	1.257	1.308

3.3 DNA cleavage activity

The DNA cleavage activity was determined using gel electrophoresis procedure [25]. The results indicated that the four phenolic compounds had promising effect. The inhibitory potency of the test compounds was assessed by comparing the cleavage of DNA by control and the title compounds. The relative efficacy of the drugs to stimulate DNA cleavage varied considerably from one congener to another. The gel after electrophoresis clearly revealed that all the tested compounds did act on the DNA, as little tailing in the bands can be observed in treated samples (Fig. 2). The difference was observed in bands of all the compounds compared to the control DNA. **BR-I**, **BR-II** and **BR-III** have displayed partial cleavage of DNA at all concentrations but the results of **BR-IV** are inconclusive due to the interference of the fluorescence of the compound under UV.



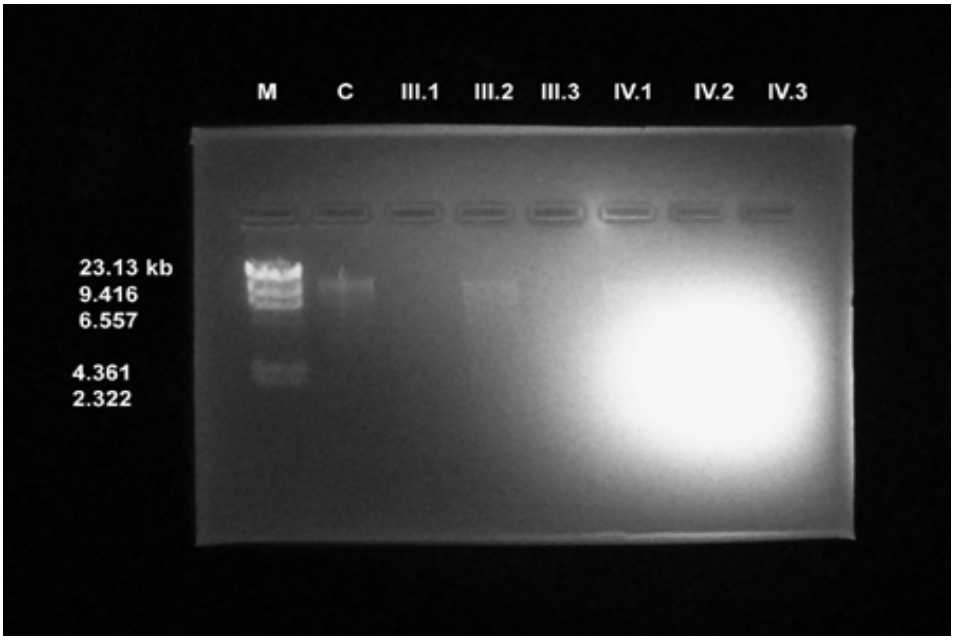


Fig.2. Gel pictures showing DNA cleavage analysis of BR-I (I1, I2, I3), BR-II (II1, II2, II3), BR-III (III.1, III.2, III.3) and BR-IV (IV.1, IV.2, IV.3) samples (where M is standard DNA molecular weight marker and C is control DNA (untreated sample) respectively)

Table 3. α-Amylase assay data of the polyphenolics

Sample	OD at 540 nm	Concentration of maltose liberated (µg)	Activity (µmoles/ml/min)	% Activity	% Inhibition
Control	1.72	138	0.0383	100.00	0.00
BR-I (100 µg)	1.59	128	0.0355	92.75	7.25
BR-I (250 µg)	1.33	108	0.0300	78.26	21.74
BR-I (500 µg)	1.29	104	0.0289	75.36	24.64
BR-II (100 µg)	1.29	104	0.0289	75.36	24.64
BR-II (250 µg)	0.98	80	0.0222	57.97	42.03
BR-II (500 µg)	0.88	72	0.0200	52.17	47.83
BR-III (100 µg)	1.27	112	0.0311	81.16	18.84
BR-III (250 µg)	0.94	76	0.0211	55.07	44.93
BR-III (500 µg)	0.93	75	0.0208	54.35	45.65
BR-IV(100 µg)	1.55	126	0.0350	91.31	8.69
BR-IV(250 µg)	0.19	16	0.0044	11.59	88.41
BR-IV(500 µg)	0	0	0.0000	0.00	100.00

3.4 α-Amylase inhibitory activity

The chromogenic DNSA method was used to evaluate this activity which might be extrapolated to detect a potential antidiabetic effect. α -Amylase catalyses the hydrolysis of α-1,4-glucosidic linkages of starch, glycogen and various oligosaccharides and α-glucosidase further breaks down the disaccharides into simpler sugars, readily available for the intestinal absorption. The inhibition of their activity in the digestive tract of humans is considered to be effective to control diabetes by diminishing the absorption of glucose decomposed from starch by these enzymes. The sample **BR-I** exhibited poor enzyme inhibitory activity at all concentrations. **BR-II** and **BR-III** have demonstrated almost similarly, though a better inhibition was displayed by **BR-II** at 100 µg concentration. **BR-IV**, though did not display significant inhibition at 100 µg, but showed 88.4% inhibition at 250 µg and 100% inhibition at 500 µg (Table 3, Fig. 3).

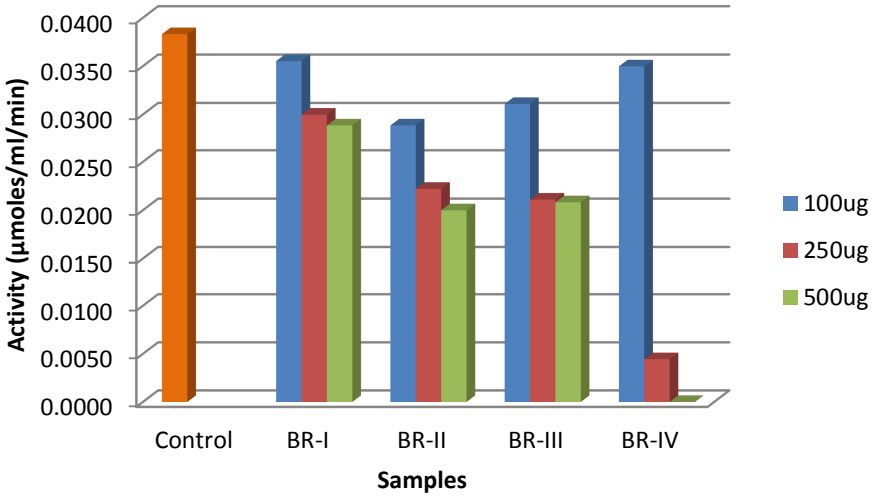


Fig.3. Comparative analysis of α-amylase inhibitory activity

CONCLUSION

The present study showed that the tetracyclic phenolic compounds , racemosol (**BR-I**), de-O-methyl racemosol (**BR-II**) and 1,7,8,12b-tetrahydro-2,2,4-trimethyl-2*H*-benzo[6,7]cyclohepta[1,2,3-*de*][1]benzopyran-5,10,11-triol (**BR-III**) as well as pentacyclic phenolic, racemosolone (**BR-IV**) possess a high antioxidant activity. Moreover, **BR-I, II and III** proved to be promising DNA cleavage agents while **BR-IV** exhibited a good α-amylase inhibition at higher concentration. It is thus evident that these four phenolic compounds can be used as prototypes to design new antioxidant and antidiabetic drugs.

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