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In vitro antioxidant activity and protective effect of *Hertia cheirifolia* L. n-butanol extract against liver and heart mitochondrial oxidative stress in rat

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Abstract: *The current study investigated the protective effect of Hertia cheirifolia L. n-butanol extract on oxidative stress in vitro by measuring lipid peroxidation (MDA) level and superoxide anion ($O_2^{\bullet-}$) production in liver and heart mitochondria of rat. In addition, the antioxidant potential of H. cheirifolia n-butanol extract was evaluated by using five methods: $ABTS^{\bullet+}$, $O_2^{\bullet-}$, Bleaching of β-carotene in linoleic acid, CUPRAC and Ferric reducing power. The results indicated that n-butanol extract contained large amounts of total phenolics (203.52±1.81 mg GAE/g) and flavonoids (104.86±0.57 mg QE/g), and had an interesting antioxidant activity and protective effect on mitochondrial oxidative stress. Therefore, H. cheirifolia n-butanol extract may serve as potential source of natural antioxidant for pharmaceutical applications.*

Keywords: *Hertia cheirifolia* L., phenolics, flavonoids, liver and heart mitochondria, antioxidant activity

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Introduction

Oxidative stress results from over production of reactive oxygen species (ROS), and often leading to peroxidation of membrane phospholipids and reactive aldehydes. Moreover, inner mitochondria membrane is considered as targets of apoptotic process, owing to alterations of potential membrane and to ROS production [1]. Flavonoids and phenolic compounds of plant origin have been reported as good scavengers of free radicals. Hence, nowadays search for natural antioxidant source is gaining much importance due to lesser side effects and multiple therapeutic advantages [2].

Hertia cheirifolia L. belongs to the Asteraceae family, it widely distributed in Northern Africa and it is endemic to both Tunisia and Algeria. This plant is traditionally used to treat spasm, inflammation, diarrhea and hemorrhoid [3]. Previous studies have shown that *H. cheirifolia* has important chemical and biological activities such as spasmolytic, anti-inflammatory [4] and acaricidal effects [5]. The antioxidant potency and the protective activity of *H. cheirifolia* methanol and aqueous extracts against biomolecule oxidative damages were also evaluated [6].

The present work was conducted to investigate the protective effect of *H. cheirifolia* n-butanol extract on oxidative stress *in vitro* for the first time by measuring malondialdehye (MDA) level as the end product of lipid peroxidation and superoxide anion (O^{2-}) production in liver and heart mitochondria of rat and, to determine its antioxidant potential by different methods: $ABTS^{\bullet+}$, $O_2^{\bullet-}$ scavenging, lipid peroxidation (β-Carotene-linoleic acid), ferric reducing power and CUPRAC.

Material and Methods

Plant material

The aerial parts of *H. cheirifolia* (HC) were collected from Oum El Bouaghi (East of Algeria). The plant was identified by Professor Zellagui Ammar and a voucher specimen under number ZA 122 was deposited in the Laboratory of Biomolecules and Plant Breeding, Oum EL Bouaghi University, Algeria.

Extraction

The extraction of n-butanolic extract of *H. cheirifolia* (BEHC) was prepared according to the extraction protocol described by Gordana et al. [20]. The powdered aerial parts of *H. cheirifolia* were macerated for three times in Ethanol 70% every 24 h by renewing the solvent each time. The resulting solution was filtered, concentrated, and then we proceeded to successive liquid-liquid extractions, by four organic solvents: n-hexane, chloroform, ethyl acetate, and n-butanol.

Total phenolics content

Total phenolics content in BEHC was determined according to the colorimetric method using Folin Ciocalteu as reagent (FC) [21]. A volume of 0.5 ml was introduced into test tubes with 2.5 ml of 10% FC

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reagent. After 10 minutes, 2 ml of 7.5% (w/v) sodium carbonate was added. The tubes were shaken and incubated in the dark for 90 min at room temperature before measuring the absorbance at 760 nm by a UV spectrophotometer. The concentration of phenolics was found using the equation of linear regression obtained from the standard calibration curve:

$Y = 0.017x + 0.080$ Gallic acid (µg), $R^2 = 0.996$.

Samples were analyzed in triplicates and the results were expressed as mg of gallic acid equivalent per gram of extract (GAE/g extract).

Total flavonoids content

Flavonoids content was quantified according to the method of aluminum trichloride [22] using quercetin (2–20 µg/ml) as standard. 1 ml of extract solution (1 µg/ml) was incubated in the presence of 1 ml of AlCl3 (2%) for 10 min at room temperature. The absorbance was measured at 430 nm. The concentration of flavonoids was calculated using the equation of linear regression obtained from quercetin curve:

$Y = 0.042x + 0.037$ quercetin (µg), $R^2 = 0.998$.

Samples were analyzed in triplicates and the results were expressed as mg quercetin equivalent per gram of extract (QE/g extract).

***In vitro* antioxidant activity**

ABTS Cation Radical Scavenging Assay

The ABTS scavenging activity was assessed according to the method of Re et al. [23]. Briefly, 2.45 mM potassium persulfate was added to 7 mM ABTS solution in water, and then stored in the obscurity at room temperature for 12 h. The absorbance of this solution was adjusted with H2O to 0.700±0.020 at 734 nm. 160 µl of this solution was added to 40 µl of sample and standards in triplicate. Methanol was used as negative control while BHT and BHA were used as standard antioxidants. After 10 min of incubation at room temperature the absorbance was read at 734 nm and the results were established as 50% inhibition concentration (IC_{50} = µg/ml) that means sample concentration providing 50% activity.

β-Carotene-linoleic acid assay

Estimation of lipid peroxidation *in vitro* was performed using the method of β-carotene-linoleic acid [24]. Briefly, β-carotene and linoleic acid solution was prepared with 0.5 mg of β-carotene in 1 ml of chloroform, 25 µl of linoleic acid and 200 µl of Tween 40. After evaporation of chloroform under vacuum at 40°C, the residue was recuperated with 100 ml of distilled water saturated with oxygen.

Absorbance was adjusted to (0.800-0.900) at 470 nm with oxygenated water. 40 µl of sample at concentrations (6.25-200) µg/ml, BHT and BHA which were used as positive controls were added to 160 µl of this solution. After incubation of two hours at 50°C, absorbance was measured at 470 nm and the bleaching ratio of β-carotene was determined as follows:

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$R = (ln\ a_1/a_2)/t$,

where: a_1 and a_2 were respectively absorbance at start of reaction (t_0) and after 120 min (t_{120})

Whereas, antioxidant activity (A%) of the samples was calculated using the following equation:

$A\% = [(R_{control} - R_{sample})/ R_{control}] \times 100$

Superoxide anion radical scavenging assay

Measurement of $O_2^{\bullet-}$ scavenging activity was based on the colorimetric method showed by Elizabeth and Rao [25] with slight modification. Briefly, Alkaline DMSO was prepared with dissolving 20 mg of NaOH in 100 ml of DMSO to generate $O_2^{\bullet-}$, 130 µl of this solution was mixed with 40 µL of extract at different concentrations (6.25-200) µg/ml, and then add 30 µL of NBT (1µg/ml in distilled water).

Ascorbic acid and methanol was used as positive and negative control respectively. After 10 min of incubation at room temperature Absorbance was read at 560 nm and percentage of $O_2^{\bullet-}$ inhibition was measured using the equation:

$I\% = [(A_1 - A_2)/A_1] \times 100$,

where A_1 and A_2 are respectively absorbance of negative control and sample.

Reducing power assay

Ferric reducing power of the extract was evaluated using the method described by Oyaizu [26]. 40 µL of phosphate buffer (pH=6.6) and 50 µL of potassium ferricyanide (1%) (1 g of $K_3Fe(CN)_6$ in 100 ml H_2O) was mixed with 10 µL of sample (1.562-50) µg/ml. After incubation at 50°C for 20 min, 50 µL of trichloroacetic acid (10%), 40 µl H_2O and 10 µl ferricchloride $FeCl_3$ (0.1%) were added to the mixture. Then absorbance was measured at 700 against a blank (methanol). BHT and BHA were used as positive controls. Results were assessed as absorbance $A_{0.5}$ which corresponding the concentration indicating 0.5 absorbance intensity.

Cupric reducing capacity (CUPRAC)

The cupric reducing antioxidant capacity was performed according to the method of Apak et al. [27]. 60 µL ammonium acetate buffer (1M, pH=7.0) was mixed with 50 µL of 7.5 mM neocuproin and 50 µL 10 mM ($CuCl_2$, $2H_2O$) and then 40 µL of sample solution at concentrations (6.25-200) µg/ml was added to this mixture. The absorbance at 450 nm was read against a blank reagent after 60 min of incubation at room temperature. Results were given as absorbance and $A_{0.5}$. BHT and BHA were used as antioxidant standards for comparison of the activities.

Evaluation of mitochondrial oxidative stress

Isolation of mitochondria

Mitochondria were isolated by differential centrifugation technic from liver and heart of the adult rat using the method of Rustin et al. [28]. 5 g of liver and 1 g of heart were cut into small pieces and

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homogenized with three volumes of ice cold liver isolation medium (TSE) [Tris 50 mM, sucrose 250 mM and EGTA 5 mM; pH=7,2 at 4ºC] and heart isolation medium Mannitol 225 mM, sucrose 75 mM, Hebes 5 mM, EGTA 1mM, and Albumin µg/ml; pH=7,2 at 37ºC) using a manual poter. Homogenates were centrifuged at 1770 rpm for 10 min at 4ºC to remove cell debris and nuclei and then supernatants were collected and centrifuged again at 9600 rpm for 10 min at 4ºC to separate mitochondria. The pellet mitochondrial was washed and suspended in TSE and then centrifuged at 9600 rpm for 10 min 4ºC. The new pellet was resuspended in TS buffer (Tris 50 mM, sucrose 250 mM; pH=7,2 at 25°C) and KSK (KH_2PO_4 1 mM, sucrose 50 mM, KCl 100 mM; pH=7,4 at 37ºC) for liver and heart mitochondria respectively. This mitochondrial suspension was centrifuged at 9600 rpm for 10 min 4ºC for a last once and the finally pellet was resuspended in respiratory buffer (KH2PO4 10 mM and sucrose 200 mM; pH=7,2 at 25ºC) and KSK for liver and heart mitochondria respectively for measuring the lipid peroxidation (MDA) level and superoxide anion (O^{2-}) production. Protein concentration of mitochondrial suspension was assayed by the Bradford's method [29].

Mitochondrial lipid peroxidation assessment

Lipid peroxidation was assessed according to the method of Okhawa et al. [30]. A mixture containing 800 µl of suspended mitochondria in 0.9% NaCl and 100 µl of BEHC in deferent concentration was incubated for 10 min at 37°C and then 100 µl of the oxidizing solution Fe^{2+} /Fe^{3+} (50µM/50µM) was added and the mixture was incubated for 30 min at 37°C, this reaction was stopped by the addition of 1ml of 3% trichloracetic acid (TCA). After a centrifugation at 3000 rpm for 15 min 4ºC, 1 ml of thiobarbituric acid (1% TBA) reagent was added to the supernatant and the mixture was heated at 100°C for 30 min and then tubes were recooled in ice for 5 min. The absorbance was read at 530 nm.

Mitochondrial superoxide anion assay

Generation of superoxide anion was achieved as described by Zini et al. [31]. Briefly 16.5 µl mitochondrial suspension (1 µg/ml), 12 µl nitrobluetetrazolium (NBT+2 100 µM), 1,2 µl cyclosporine (1 µM) and 12 µl of BEHC in different concentrations were mixed in 1.2 ml of respirant buffer (250 mM sucrose, 5 mM KH_2PO_4, pH=7.2 at 25°C) supplemented with 2 µM rotenone at 25°C. The addition of 7,2 µl succinate (6 mM) initiates the superoxide production and the NBT reduction to monoformazan was measured at 560 nm for 3 min.

Statistical analysis

All data and results were performed in triplicate and expressed as mean ± standard deviation (S.D). The one-way analysis of variance (ANOVA) was applied for the calculation of differences among various groups, followed by Tukey's post hoc test using Graph-Pad Prism 5 software: ns: indicates a non-significant effect ($P>0.05$); *: indicates a significant effect ($P<0.05$); **: indicates a very significant effect ($P<0.01$).

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Results and Discussion

Medicinal plants are known as an important source of natural drugs. These natural resources appear interesting to develop alternative treatments. Moreover, plants are rich in antioxidants and can be used against oxidative stress related disorders and tissue damage [7]. Mitochondria are one of the main sources of ROS in the cell, which renders mitochondrial function particularly sensitive to oxidative damage [8].

Lot of researches were performed concerning chemical composition; antioxidant and biological activities of *H. cheirifolia* extracts, but a few studies were focused on n-butanolic extract of *H. cheirifolia* (BEHC). This study evaluated the antioxidant activity and the protective effect of *H. cheirifolia* n-butanol extract on oxidative stress *in vitro* by measuring lipid peroxidation (MDA) level and superoxide anion (O^{2-}) production in liver and heart mitochondria of rat.

Total phenolics and flavonoids contents

Total phenolics and flavonoids contents of BEHC are presented in Table 1. The results showed that BEHC has a high amount of total phenolics (203.52±1.81 mg GAE/g) and flavonoids (104.86±0.57 mg QE/g). The extraction yield was evaluated to be 0.98% in dry weight of plant. These results were not in agreement with those of [9] which mentioned that the lowest amounts of polyphenols were detected in the butanolic extract of *H. cheirifolia* flowers (11.18±2.00 mg GAE/g). Moreover, BEHC had higher polyphenolics content than the other extracts of *H. cheirifolia* found in previous studies [6, 9, 10]. Our results were probably related to the highly polyphenolic content of plant aerial part, furthermore the extraction of polar compounds with n-butanol was more efficient than ethyl acetate [11].

Table 1. Total phenolics and flavonoids contents in n-butanolic extract of *H. cheirifolia*

Extraction yield %	Total phenolics (mg GAE/g)[a]	Flavonoids (mg QE/g) [b]
0.98	203.52 ± 1.81	104.86 ± 0.57

Values are expressed as mean ± SD of triplicate.

[a] Microgram of Gallic Acid Equivalent per milligram of extract.

[b] Microgram of Quercetin Equivalent per milligram of extract.

***In vitro* antioxidant activity**

The antioxidant activities of plant extracts could not be evaluated using a single method [12]. However, in the present study, a variety of *in vitro* antioxidant assays were performed to determine the antioxidant status of BEHC and the results were given in Figure 1, as a function of inhibition percentage and of concentration. Values of IC50 and $A_{0.5}$ are present in Table 2. The BEHC exhibited an interesting

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antioxidant activity when compared with those of standard antioxidants in all tests, but the highest activity was recorded in the ß-carotene-linoleic acid assay (IC_{50}=9,99±0,53 µg/ml) which was stronger than those of the other fraction extracts of *H. cheirifolia* reported in previous studies [6, 9, 10, 13]. It's well known that β-carotene bleaching method is a commonly used as model to analyze the antioxidant activity of the plant extracts because of the extremely sensitivity of β-carotene to mediate free radical oxidation of linoleic acid [14]. Furthermore, in CUPRAC and Ferric reducing power tests, BEHC showed a good activity with $A_{0.5}$= 29,25±1,55 µg/ml and $A_{0.5}$ = 47,20 ±1,05 µg/ml, respectively.

Evaluation of mitochondrial Lipid peroxidation

Data in Figure 2 shows that BEHC revealed a good significant effect by decreasing MDA levels of liver and heart mitochondria (p<0,001) compared to the control (Fe^{2+}/Fe^{3+}). But, BEHC at the concentration of 2 µg/ml significantly reduced MDA production rate in both liver (38.26±0.4 nmol/g) and heart mitochondria (40.19±0.1 nmol/g; P<0.001), and exhibits better effect than the other two used concentrations (1 µg/ml and 0,5 µg/ml). Lipid peroxidation is the process of oxidative degradation of polyunsaturated fatty acids. Its occurrence in mitochondrial membranes causes impaired membrane function, impaired structural integrity, decrease in fluidity, and inactivation of a number of membrane bound enzymes and protein receptors [15]. Ferrous ions are the most powerful pro-oxidants among the various species of metal ions [16], and the transition of these metal ions can stimulate lipid peroxidation via Fenton reaction, and accelerate lipid peroxidation by decomposing lipid hydro peroxides into peroxyl and alkoxyl radicals that can propagate the chain of lipid peroxidation [17]. In this system, BEHC exhibited potent lipid peroxidation inhibition by reducing significantly MDA production rates in both liver and heart mitochondria. BEHC may serves as secondary antioxidant, as it reduces the redox potential and thereby.

Table 2. Values of IC_{50} and $A_{0,5}$ for antioxidant activities of BEHC

Antioxidant activities	**Samples**			
	BEHC	**BHT**	**BHA**	**Ascorbic acid**
ABTS-+ assay IC_{50} (µg/ml)	27.54±2.87	1.29±0.30	1.81±0.10	nt
β-carotene-linoleic acid assay IC_{50} (µg/ml)	9.99 ±0.53	1.05±0.01	0.90±0.02	nt
$O_2^{\bullet-}$ scavenging assay IC_{50} (µg/ml)	>200	nt	nt	90.73±2.28
CUPRAC assay $A_{0,5}$ (µg/ml)	29.25±1.55	9.62±0.87	3.64±0.19	nt
Reducing power assay $A_{0,5}$ (µg/ml)	47. 20 ±1.05	>50	8.41±0.67	9.01±1.46

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Values expressed are means ± S.D. of three parallel measurements, nt: not tested, BHA: Butylated-hydroxyl anisole, BHT: Butylated-hydroxyl toluene

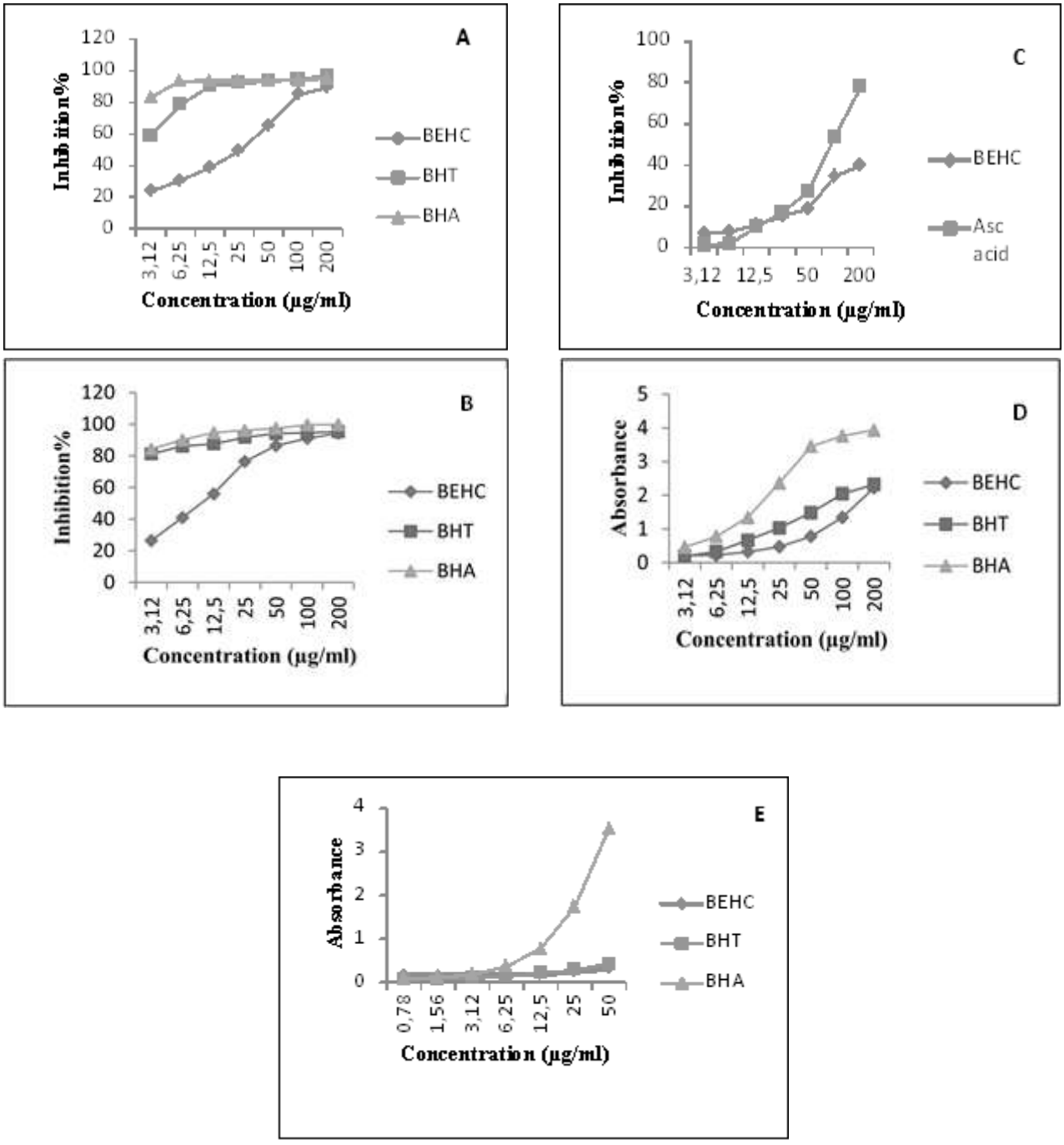


Figure 1. Antioxidant activity of *H. cheirifolia* L. butanolic extract; $ABTS^{\bullet+}$ scavenging **A**, Bleaching of β-carotene in linoleic acid **B**, $O^{2\bullet-}$ scavenging **C**, CUPRAC **D** and Ferric reducing power **E**.
•BHA: Butylated-hydroxyl anisole, BHT: Butylated-hydroxyl toluene

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The ability of BEHC to scavenge radicals and then to inhibit lipid peroxidation may be attributed, as cited before, to its constituents that are electron donors, which can react with ROS to convert them to more stable products.

Evaluation of mitochondrial anion superoxide production

The measurement of mitochondrial superoxide anion production, especially by respiratory chain complex III, has a special importance in the study of oxidative stress because this phenomenon constitutes the primary event in the oxidative stress induction [18]. The effect of BEHC on mitochondrial superoxide anion production in both liver and heart is presented in Figure 3. When compared with standard (Vit C), BEHC exhibited a good significant effect ($p<0,001$) at the concentration of 2 µg/ml where the percentage of inhibited mitochondrial superoxide anion production was 45,54% and 42,24% in liver and heart respectively. However, at the concentration of 1µg/ml and 0,5 µg/ml of BEHC, the percentage of this inhibition were (37,62% and 35,64%) and (26,73% and 22,77%) in liver and heart respectively, *H. cheirifolia* appears to be rich in polyphenolic compounds which could explain the strong antioxidant effect, as these polyphenolic compounds exhibited multiple biological activities including adsorbing and neutralizing of free radicals [15-19].

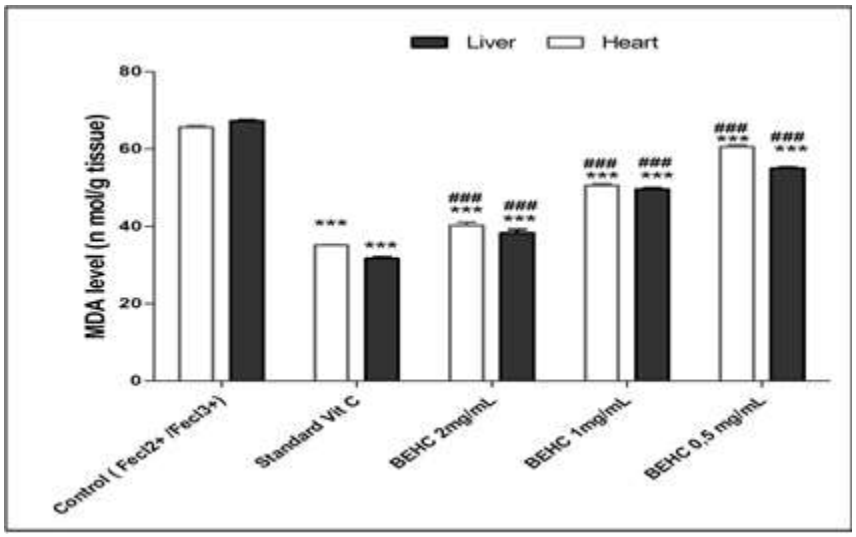


Figure 2. Effect of BEHC on liver and heart mitochondrial MDA level *in vitro*

Values are expressed as means ± SD (n=3). ***p<0.001 compare to control

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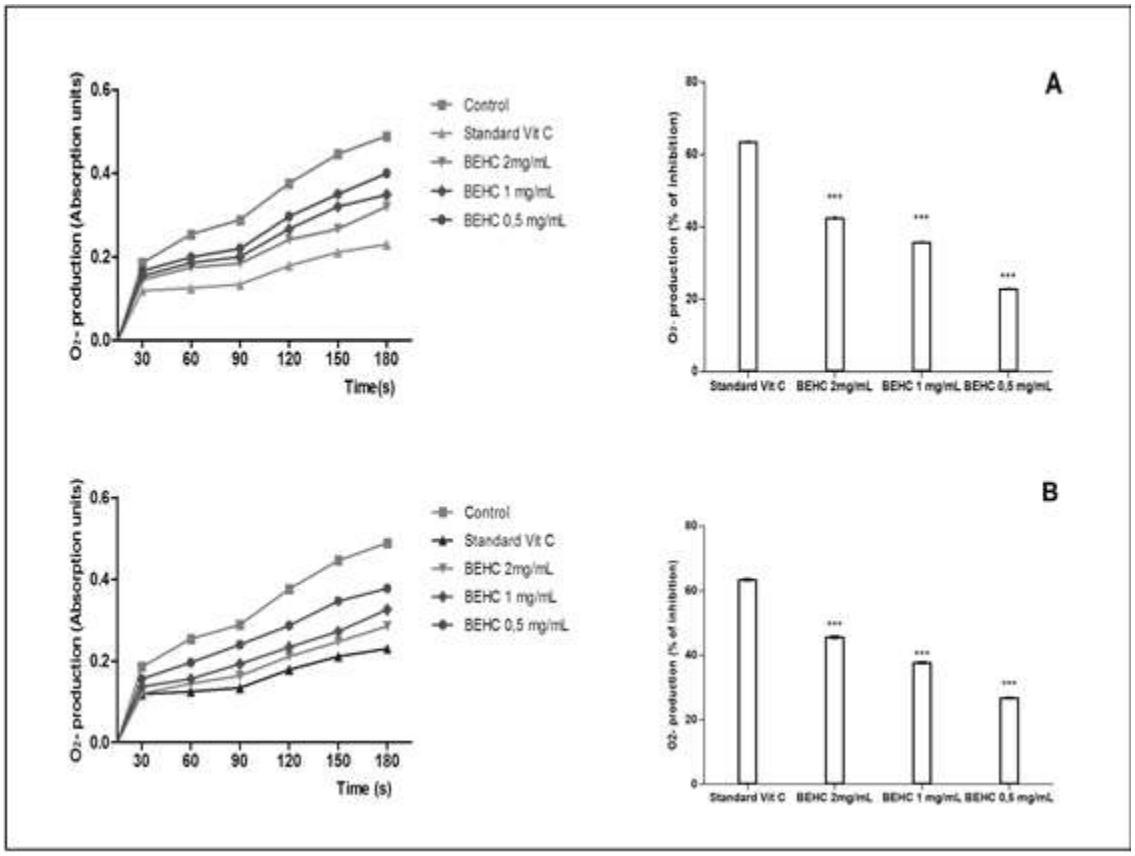


Figure 3. Effect of BEHC on heart (A) and liver (B) mitochondrial O^{2-} production *in vitro*

Values are expressed as means ± SD (n=3), *** $p<0.001$ compared to standard Vit C (as positive control)

Conclusion

It can be concluded that *H. cheirifolia* n-butanol extract possesses potential antioxidant activity and protective effect on oxidative stress *in vitro*. Consequently, it may serve as potential source of natural antioxidants for pharmaceutical applications and food preservation. However, further investigations are required to isolate and purify the active compounds of the plant extract, and to study their biological activities *in vivo*.

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