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Biomedical Activity of Egyptian *Hibiscus sabdariffa* L. as Modulator of Alzheimer's Beta-Amyloid$_{1-42}$ Secretion in a Human Neuroglioma Model

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Abstract

β-Amyloid$_{1-42}$, the major constituent of amyloid plaques, was believed to play a central role in the neuropathology of AD according to the ''amyloid cascade hypothesis''. In our study, we evaluated the in vitro profiles of four Roselle extracts against Aβ_{42} production. The in-vitro Aβ_{42} inhibiting activity was evaluated in a human neuroglioma cell line (H4) carrying the double Swedish mutation (K670N/M671L) of the human amyloid precursor protein (APPsw) under the transcriptional control of the hamster prion protein promoter. The in-vitro anti-COX activity was also investigated using human recombinant enzymes isolated from transfected Sf-9 cells. In addition, the cytotoxicity study by standard MTT assay and the antioxidant activity of Roselle extracts using DPPH Free Radical Scavenging Assay was also investigated. All Extracts concentrations showed a good inhibitory activity against DPPH free radical. The antioxidant activity of our extracts as IC_{50} was in the range of (45.5 - 88.5 µg/ml) among the four Roselle extracts within the two solvent system compared to 47.5 µg/ml of Gallic acid. No toxicity was detected by standard MTT assay against H4 cells treated with Roselle extracts by concentrations up to 600 ng/ml. A dose dependent inhibition of Aβ_{42} secretion was observed. Our Roselle extracts were found to be 1.5- and 3-fold more potent than *R*-flurbiprofen in inhibiting Aβ_{42} secretion and able to modulate the in vitro Aβ_{42} secretion without any cytotoxic effect. No inhibition activity was also observed against cyclooxygenase-1 (COX-1) and cyclooxygenase-1 (COX-2) up to 600 ng/ml concentration indicating that the reduction in Aβ_{42} levels may be independent of COX activity.

Keywords: Alzheimer's disease, Amyloid-*β* peptide (A*β*), *Hibiscus sabdariffa* L., Roselle.

1. Introduction

Alzheimer's disease (AD) is one of the most prevalent severe neurological disorders affecting our aged population and clinically characterized by a progressive and gradual decline in cognitive function and neuropathologically by the presence of neuropil threads, specific neuron loss, and synapse loss additionally to the hallmark findings of neurofibriallary tangles and senile plaques [1]. The plaques are composed of a tangle of regularly well-ordered fibrillar aggregates called amyloid fibers, a protein fold shared by other peptides such as the prions associated with protein misfolding diseases surrounded by dystrophic neurites, astrocytic processes and microglial cells [2]. Recent studies suggest that the soluble oligomeric varieties of the peptides could also be the causative agent within the development of Alzheimer's disease [3] and is believed to play a vital role in the neuropathology of AD consistent with the ''amyloid cascade hypothesis''.

A*β* is formed after sequential cleavage of amyloid precursor protein (APP), a transmembrane glycoprotein of undetermined function. APP can be processed by α-, β- and γ-secretase; A*β* protein is

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generated by successive action of the β and γ-secretase. The γ-secretase, which produces the C-terminal end of the Aβ peptide, cleaves within the transmembrane region of APP and may be generate a number of isoforms of 36-43 amino acid residues in length. The most common isoforms are Aβ_{40} and Aβ_{42}; the shorter form is often produced by cleavage that happens within the endoplasmic reticulum while the longer form is produced by cleavage within the trans-Golgi network. The Aβ_{40} form is that the more common of the two, but Aβ_{42} is the more fibrillogenic and more prone to aggregation and is thus associated with disease states [4]. Mutations in APP associated with early-onset Alzheimer's have been noted to intensify the relative production of Aβ_{42}, and thus one suggested avenue of Alzheimer's therapy involves modulating the activity of β and γ secretases to provide mainly Aβ_{40} and inhibition of Aβ_{42} production [5].

The improvement of disease-modifying drugs is a major challenge and would be a sizeable asset for future of AD treatments. Plants are a well-known source of new bioactive compounds with unique structures that have served throughout history and still function as models enriching our therapeutic armamentarium. Many natural molecules have been isolated, acting on several targets in AD [6]. Roselle (*Hibiscus sabdariffa* L.) belongs to the genus *Hibiscus* L. and *Malvaceae* family and usually thought to be safe (GRAS) as foodstuff. Many medicinal applications of this herb have been developed around the world. In China, it is used to treat hypertension, pyrexia, and liver damage and recently its sepal extract has been used as an efficient treatment against leukemia, due to its high content in polyphenols, particularly protocatechic acid [7]. We propose that Roselle extracts have significant antioxidant activity and protective effects against inflammation and suspect the extract could be useful in preventing AD through modulation of Aβ_{42} production.

2. Experimental

2.1. Phytochemica1 study

a. Plant material

The dark red calyces of *H. sabdariffa* cultivated in the applied research center for medicinal plants at central administration of drug control (CADC) were collected. These sepals were kindly authenticated by botanist specialist agricultural engineer. The voucher specimens have been deposited and dried in the herbarium of the applied research center for medicinal plants at (CADC).

b. Preparation of plant extracts

A weighed quantity of dark red (DR) calyces were dried and grounded. An aliquot of 50 gram of grounded sepals of *H. sabdariffa* L. were separately homogenized at ratio 1:5 (w/v) for cold extraction and 1:10 (w/v) for hot extraction with two solvent systems (30% aqueous ethanol and ultrapure distilled water). The hot aqueous ethanol extraction was performed under condensation in water bath while the hot water extraction was performed on hot magnetic stirrer and both of them at 60 °C for one hour [8]. These gave 4 samples to be biologically analyzed. Serial extracts of all sepals with their respective solvents were presented at table (1). The plant homogenates were, filtered through Whatman No. 1 filter paper. Extracts were evaporated under reduced pressure to remove ethanol and water. Then, the extracts were freeze-dried using a freeze drier and saved at −20°C for further analyses.

Table 1. Full description of the abbreviations for the serial extracts.

	Description
DR_1	Dark red Roselle sepals (DR) extracted with 30% aqueous ethanol at hot temperature.
DR_2	Dark red Roselle sepals (DR) extracted with 30% aqueous ethanol at cold temperature.
DR_3	Dark red Roselle sepals (DR) extracted with distilled water at hot temperature.
DR_4	Dark red Roselle sepals (DR) extracted with distilled water at cold temperature.

c. Spectrophotometric determination of total flavonoids of Hibiscus sabdariffa **L.**

Principle: The method adopted was based on measuring the intensity of the color developed when flavonoids are complexed with aluminum chloride [9] with some modification. According to a pre-established standard calibration curve, the concentration of flavonoids was calculated as rutin equivalent (QE).

The calibration curve was prepared using rutin as a standard flavonoid. A stock solution (1 mg/ml) was prepared in methanol and aliquots (2.5, 5, 8, 15, 20, 30.25 µl/ml) were transferred to test tubes and evaporated to dryness at 40 ºC. Residue of each concentration was mixed with 5 ml 2% aluminum chloride (w/v) in methanol. The content was incubated for 30 min at room temperature and the

absorbance was measured at 415 nm against a blank prepared in the same way but replacing rutin solution by methanol, using the spectrophotometer. For each concentration, triplicate determinations were carried out and the average of the obtained absorbance was plotted versus the concentrations.

Procedure: 125 mg of hibiscus dry extracts (DR_1, DR_2, DR_3 and DR_4) were accurately weighed. 20 ml of methanol was added and sonication was performed for 15 min then the solution was filtrated and the volume was completed to 25 ml using methanol.

Each of the prepared extracts (1 ml) was transferred to a separate test tube and evaporated to dryness in water bath at 40 ºC, and the residue was mixed with 5 ml 2% aluminum chloride (w/v). The content was incubated for 30 min at room temperature and the absorbance was measured at 415 nm against blank. Total content of flavonoids in the extracts was calculated as rutin equivalent from the calibration (X). The flavonoid (%) calculated as rutin and was deduced from the established standard calibration curve.

Flavonoid (%) calculated as rutin in the extracts:
Flavonoid (%) = X/5000 *100

X: amount of rutin in µg obtained from standard curve for each extract.

d. In-vitro Determination of antioxidant activity

Principle: The DPPH antioxidant assay is based on the ability of DPPH, a stable free radical, to decolorize in the presence of antioxidants. The DPPH radical contains an odd electron that is responsible for the absorbance at 517 nm and also for the visible deep purple color. When DPPH accepts an electron donated by an antioxidant compound, the DPPH is decolorized, which can be quantitatively analyzed from the changes in absorbance.

Procedure: quantitative measurement of radical scavenging properties of dark red calyces extracts from *H. sabdariffa* was carried out according to the method mentioned in the literature with some modification [10]. 0.1 mM solution of 2, 2-diphenyl-1-picryl-hydrazyl (DPPH.) in methanol was prepared and 1 ml of this solution was added to 3 ml of the aqueous extract at different concentrations (2 mg/ml – 10 µg/ml). The mixture was shaken vigorously and allowed to stand for 30 min in the dark (until stable absorption values were obtained). The extent of reduction of DPPH free radical and discoloration was determined by measuring the absorption at 517 nm. Methanol (1.0 mL) plus plant extract solution (3 mL) was used as a blank. DPPH solution (1.0 mL of 0.1 mM) plus ultrapure water (3 mL) was used as a negative control (Abs control). The experiment was performed in triplicate, and the average absorbance for each concentration (Abs sample) was taken. Gallic acid was used as a reference standard.

The capacity to scavenge the DPPH* radical was calculated and elucidated as inhibition percentage by the following equation:

I%= 100- {(Absorbance of test – Absorbance of blank/ Absorbance of control) ×100}

The IC_{50} values (concentration of sample required to scavenge 50% of free radicals) were calculated by regression analysis of plot where the abscissa represented the concentration of tested plant extracts and the ordinate represented the average percent of antioxidant activity from three separated tests.

2.2. In-vitro biological Assay

H4 Cells, a human neuroglioma cell line, expressing the human APP gene with the Swedish double mutation (K670N/M671L) under the transcriptional control of the hamster prion protein promoter [11] were employed in our research for the in-vitro study at Egypt cell bank unit in the Military Veterinary Hospital of the Armed Force, Egypt.

a. Preparations for Cell Culture of H4 Cells expressing the double Swedish mutation (K595N/M596L) of human APP (APPsw)

H4 Cells expressing the double Swedish mutation (K595N/M596L) of human APP (APPsw) were cultured in DMEM (high glucose) medium supplemented with 10 % heat-inactivated fetal bovine serum (FBS), antibiotics (100U/mL penicillin and 100U/mL streptomycin) and 2mM L-glutamine. and finally supplemented with 200 µg/mL G418 [12]. The cells were maintained in monolayer culture and allowed to grow to approximately 75 - 80% confluence at 37°C in a humidified atmosphere of 5%CO2/95% air. The medium was changed every three days.

The cultured cells were removed from the incubator and processed in the laminar air flow hood. Culture medium was sucked using a sterile, disposable plastic pipette, and few milliliters of phosphate buffered saline (PBS) were added to wash

the cells. PBS was sucked and the cells were sub-cultured by trypsinization (2ml; 0.25% trypsin and 0.04% EDTA; Biowest) as a thin layer over the cultured cells. Cells were incubated at 37 °C for 5-10 minutes and were then checked under the inverted phase contrast microscope to assess for proper detachment of the cultured cells (the cells round up, float and move freely in the trypsin solution). The action of trypsin was stopped by addition of 5ml of serum-containing medium to prevent over-digestion of cell membranes. Cells suspension was collected into 15 ml sterile Falcon tubes and centrifuged at 2000 rpm for 5-10 minutes. Supernatant fluid was discarded and the cell pellets were dissolved in an appropriate amount of medium according to the requirements of the experiment. Cells were counted using a hemocytometer.

b. Exposure of H4 cells to Roselle extracts

For experiments, H4 cells were seeded onto 24 well culture plates (2 x 105cell/well) in fresh media and allowed to grow to confluence for 24 h, in 5% CO2/95% air in humidified atmosphere. After 24h, the media were removed and a fresh serum-free media (450 µL/well) were added together with 50 µL of a series of different concentrations from each Roselle extracts (0 - 600 ng/ml) overnight in a final volume of 0.5 ml. *R*-flurbiprofen was used as a reference drug (3 - 1000 µM). The different extracts concentrations were prepared in fresh media without serum as in cells to obtain the required concentration. At the end of the incubation, 100 µl of each supernatant were removed for measuring β-amyloid peptides [12]. Each experiment was repeated on three different days and conducted in triplicate. The probit analysis using the SPSS software program (version 20, SPSS Inc., Chicago, IL, USA) was used to determine each of IC_{50}.

c. Detection of $A\beta_{42}$ Peptides

Each supernatant was treated with a biotinylated mouse monoclonal antibody (4G8, Signet Laboratories Inc., Dedham, MA, USA), specifically recognizing the 17-24 amino acid region of Aβ and a rabbit polyclonal antibody (C-term 42, BioSource International, Camarillo, CA, USA), specifically recognizing the C-terminus of $A\beta_{42}$. Antigen–antibodies complexes were identified by TAG-donkey anti-rabbit IgG (Jackson Immuno Research Laboratories, Soham, UK). The detected complexes were captured by streptavidin coated magnetic beads and the signals were investigated by an electrochemiluminescence instrument (Origen M8 Analyzer, BioVeris Corporation, Gaithersburg, MD, USA). Aβ levels in cell supernatants were evaluated in comparison to an Aβ peptide standard [12].

d. Inhibition of COX-1 and COX-2 assay

The inhibition of the cyclooxygenase activity was estimated by measuring prostaglandin E2 (PGE2) production from arachidonic acid according to a modified version of a method previously mentioned in literature [13]. Recombinant human prostaglandin H2 synthase-1 (PGHS-1) and synthase-2 (PGHS-2) were expressed in transfected spodoptera frugiperda (Sf-9) cells (Invitrogen, San Diego, CA, USA). The microsomal fractions were prepared from the transfected cells and used to assay the enzymatic activities. Briefly, the recombinant enzymes (2 g) reconstituted in a buffer (100 mM Tris-HCl, pH 8.0 at 37°C) containing 2 mM phenol. The enzyme preparations were preincubated with vehicle (media) or the different *H. sabdariffa* extracts (0 - 600 ng/ml) in media for 20 min at 22ºC. The reaction mixture was completed with 1M hematin 1 min prior to initiation of reaction. The reaction was initiated adding arachidonic acid (4 and 2 µM for COX-1 and COX-2, respectively) and the mixture was incubated for 5 min at 22ºC for COX-1 assay and 10 min at 25ºC for COX-2 assay. For reference control measurements, arachidonic acid not present in the reaction mixture. The sequential additional of 1 M HCl and 1 M Tris-HCl (pH 8.0), followed by cooling to 4ºC were used to stop the reactions. The amount of PGE2 present in the reaction mixture was quantified using an enzyme-immunoassay detection kit (Cayman Chemical, Ann Arbor, MI, USA) and the measurements were made with a microplate reader.

e. Notch-Mediated Hes1-Luciferase Reporter Assay

The Notch-Hes1 reporter assay was modified from procedures as described previously in literature [14]. HEK293 cells were plated in a six-well dish (≈ 5 × 105 cells) and transiently co-transfected with the following plasmids: luciferase reporter construct Hes1-Luc (0.3 µg), Notch construct pCS2-ΔEMV-6MT (also called Notch ΔE) (3.0 µg), which includes the transmembrane and intracellular domains of Notch1 or control pCS2 vector (3.0 µg), and pCH110

vector (0.1 µg), which expresses *β*-galactosidase (Amersham Pharmacia) for normalization of the transfection efficiency and luciferase activity. The cells were transfected with these plasmids for 24 h and then fresh media and tested samples were added to the cells. The cells were cultured after 24 h and lysed for measuring luciferase activity using the Luciferase Reporter Gene Assay kit (Roche). The luciferase activity was standardized based on *β*-galactosidase activity.

f. MTT Cytotoxicity Assay

The anti-proliferative activity of various extracts of *H. sabdariffa* against H4 human cell line was estimated using the 3-[4,5-dimethyl-2-thiazolyl)-2,5diphenyl-2H-tetrazolium bromide (MTT) assay, which is based on the cleavage of the tetrazolium salt by mitochondrial dehydrogenases yielding a blue formazan product in viable cells [15], but not in dead cells. After the expansion of cultures to approximately 75 - 80% confluence, cells were trypsinized by (0.025% trypsin and 0.0025% EDTA; Biowest) and incubated at 37 °C for 1-2 min. The action of trypsin was stopped by addition of 5ml of serum-containing medium to prevent over-digestion of cell membranes. Cells suspension was collected into 15 ml sterile Falcon tubes and centrifuged at 2000 rpm for 5-10 minutes. Supernatant fluid was discarded and the cell pellets were dissolved in an appropriate amount of fresh media without serum. Cells were seeded in 24 well culture plates at a concentration of (2 x 104 cell/well).

Then wells were subjected to different treatments with various concentrations of *H. sabdariffa* extracts (0 - 600 ng/ml). Serial dilutions as (100, 200, 300, 400, 500, 600 ng/ml) of the extracts were prepared in DMSO with a final DMSO concentration of 0.5%. The herbal drug concentrations prepared in DMSO were diluted in fresh media without serum as in cells to obtain the required concentration. Positive and dimethylsulfoxide (DMSO) cytotoxicity controls were run in each plate. DMSO controls (cells with dimethylsulfoxide (0 - 600 ng/ml) only; untreated cells). Cells subjected to Doxorubicin were taken as positive controls (zero viability). The morphological changes of the cells were investigated by phase contrast microscopy (40× magnification). The cells with the herbal drugs were incubated for 48 h in a serum free medium prior to the MTT assay. After incubation, media were carefully removed, 40 µL of MTT (5 mg/ml) were added to each well and then incubated for an additional 4 h. The purple formazan dye crystals were solubilized by the addition of 200 µL of acidified isopropanol. The absorbance was measured at 570 nm using a microplate ELISA reader (Biorad, USA). Cell viability (%) was expressed as the optical density ratio of treated samples to control and was calculated by:

$$\text{Cell viability (\%)} = A_{test}/A_{control}*100$$

Where [A_{test}] is the absorbance of tested sample and [$A_{control}$] is the absorbance of control sample. Each result was the average of three wells, and 100% viability was determined from the untreated cells.

g. Statistical analysis

One-way ANOVA was used to test the effect of Roselle extracts on total flavonoids, antioxidant activity, $A\beta_{24}$ production and Notch intracellular domain responsive gene. After a significant factor effect by ANOVA, individual group differences were analyzed using Duncan procedure for multiple comparisons ($p < 0.05$ as statistically significant). Exact probabilities (P) were reported for multiple comparisons. Calculations were done with the statistical software IBM SPSS Statistics Base 21.0 for windows. All experiments were conducted in triplicate (n=3). Data are presented either as individual values or as mean ± S.D.M. (standard deviation of mean).

3. Results and Discussion

3.1. Phytochemical investigation

a. In-vitro antioxidant activity using DPPH Free Radical Scavenging Assay

In present study, the resulting absorbance was converted as previously described into percentages for antioxidant activity determination. Then a graphs were plotted between the concentrations of the dark red calyces within the two solvent system and the corresponding antioxidant activity percentage. The graph presenting the concentration versus antioxidant activity percentages resulted in a regression pattern which was statistically analyzed to determine the IC_{50}.

In current research, the antioxidant activity was expressed as IC_{50}; the amount of extract (µg/ml) needed for 50 % quenching of the UV absorption of DPPH. The IC_{50} values of dark red extracts and standards were listed in table (2). The antioxidant

activity of our extracts was in the range of (45.5 - 88.5 µg/ml) among the four Roselle extracts within the two solvent system. The lower IC_{50} value was corresponding to the larger scavenging activity. The DPPH radical-scavenging activity of the dark red calyces extract was observed to be occurred in a dose dependent manner. All Extracts concentrations showed a good inhibitory activity against the DPPH free radical.

Table 2. IC_{50} values (µg/ml) of the dark red calyces from *Hibiscus sabdariffa* L. within two solvent system compared to Gallic acid. Antioxidant activity was based on the ability to scavenge DPPH free radicals.

Extract name	IC_{50} (µg/ml)
DR_1	45.5 ± 6.4 ^{A}
DR_2	88.5 ± 11.3 ^{B}
DR_3	57.4 ± 3.3 ^{A}
DR_4	47.5 ± 3.5 ^{A}
Gallic acid	47.5 ± 2.9 ^{A}

Numbers shown are means ± standard deviation of three replicates. Values bearing different letters in the same column are significantly different at level of probability ($p < 0.001$) according to Duncan's Multiple Range Test.

Dark red calyces extracts showed to an extent similar results as previously reported [16] who determined the average IC_{50} value of *H. sabdariffa* calyces was 37.15 when treated with 0.1 mM DPPH while it has significantly higher antioxidant activity compared to that detected in literature [17] by 240 µg/ml as IC_{50} value when investigated by 0.15 mM DPPH.

In the presented research, the highest antioxidant activity was observed in DR_1 extract (IC_{50} 45.5 µg/ml) compared to the standard gallic acid (IC_{50} 47.5 µg/ml) while the lowest was detected in DR_2 extract (IC_{50} 88.5 µg/ml). As seen in table (2), the dark red calyces extracted by two solvent system exhibited varying degrees of antioxidant activity with a significant variation at $p > 0.001$. At hot temperature, the aqueous ethanol extraction exhibited higher value in antioxidant activity (45.5 µg/ml) compared to the hot water extraction (57.4 µg/ml). While at cold temperature, the aqueous extraction was significantly higher (47.5 µg/ml) compared to the aqueous ethanol extraction (88.5 µg/ml).

This variation may be due to the chemical properties of the solvent that could affect which compounds are extracted from the plant crude extract. This phenomenon can be elucidated by a change in polarity of the antioxidant compounds caused by the solvent used for extraction. As the polarity of the solvent increases, higher antioxidant compounds produced [18]. As well as, the water extract of Roselle calyces also showed good hydrogen donating abilities, indicating that they had effective activities as radical scavengers [19].

b. Total flavonoids

In our research, total flavonoid content was calculated from the calibration curve and expressed as µg rutin equivalents. Analysis of total flavonoid in the dark red calyces within the two solvent system showed their contents to be in the range of (6.17 - 15.94 µg rutin equivalent/ml) as shown in table (3) that is equivalent to the following percentage (0.13 - 0.32 %) of the total dry extract.

Table 3. Total flavonoid content (µg/ml) of the dark red calyces from *Hibiscus sabdariffa* L. within two solvent system expressed as Rutin equivalent.

Extract name	Total flavonoid content (µg/ml)	Flavonoid (%)
DR_1	11.41 ± 0.67 ^{B}	0.23 ± 0.014 ^{B}
DR_2	10.81 ± 0.56 ^{B}	0.22 ± 0.007 ^{B}
DR_3	6.17 ± 0.26 ^{C}	0.13 ± 0.007 ^{C}
DR_4	15.94 ± 1.93 ^{A}	0.32 ± 0.042^{A}

Numbers shown are means ± standard deviation of three replicates. Values bearing different letters in the same column are significantly different at level of probability ($p < 0.01$) according to Duncan's Multiple Range Test.

The presence of total flavonoids as traces in our study (0.13 - 0.32 %) was also observed previously to some extent in a similar quantity as 0.5 % of total extract [20]. Better quantities of total flavonoid in *H. sabdrriffa* extracts were previously reported with varying degree in its amount; 1.23 % [21], 2.1 % [22], 3.3 % [23] and the highest quantity was reported previously in amount of 10.5 % [16]. The variation in results from this work and other studies are may be due to environmental factors and variety of Roselle, as well as sample: solvent ratio, extracting method, and type of solvent used.

The extraction methods and type of solvent significantly influenced ($p < 0.001$) the total flavonoid content as shown in table (3). At hot aqueous ethanol extraction, the total flavonoid (11.41 µg/ml) was slightly higher in comparison to the cold

extraction (10.81 µg/ml) however the highest quantity was observed at cold aqueous extraction (15.94 µg/ml) compared to the hot aqueous extract (6.17 µg/ml). This may be attributed to presences of flavonoid compounds as glycosides which could explain the high percentage yield obtained in aqueous extracts, due to the ability of these solvents to dissolve endogenous compounds [23].

3.2. Biological investigation

a. In-vitro Aβ42 modulation

The potential application of our Roselle extracts for cellular studies was explored. First, their potency to inhibit APP processing and generation of Aβ, particularly the Aβ_{42} species was examined. A dose-dependent inhibition of Aβ42 secretion was shown at figure (1 - 2). No toxicity was detected by standard MTT assay on H4 cells treated with Roselle extracts by concentrations up to 600 ng/ml. In addition, no inhibition activity was observed against COX-1 and COX-2 up to 600 ng/ml concentration.

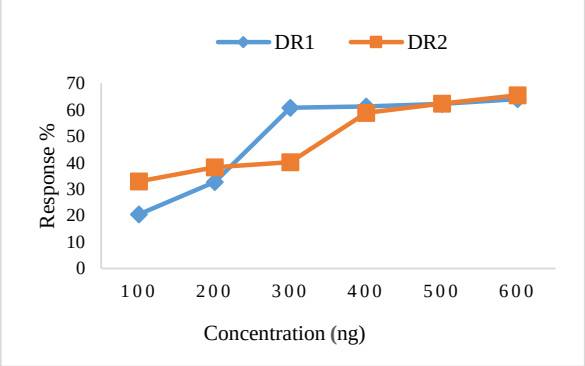

Fig. 1. Dose response activities of dark red Roselle at aqueous ethanol extraction against formation of Aβ_{42} peptide

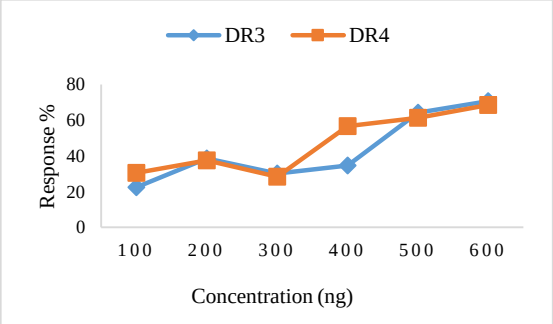

Fig. 2. Dose response activities of dark red Roselle at aqueous extraction against formation of Aβ_{42} peptide

In the present study, the reduction in Aβ_{42} concentration was achieved at all concentrations from each of the investigated extracts compared to the reference drug, *R*-flurbiprofen. The effect of the dark red calyces on APP processing and generation of Aβ, particularly the Aβ_{42} species was summarized in table (4) as IC_{50} values (the required concentration of Roselle extracts for 50% inhibition of Aβ_{42} production).

The IC_{50} values for Aβ_{42} inhibition estimated for *R*-flurbiprofen was 800.4 ng/ml that is equivalent to 3276.89 nM and was consistent with what previously reported by other authors using identical human neuroglioma cell line [12]. *R*-flurbiprofen was chosen as a reference drug because it reduces Aβ_{42} levels by targeting γ-secretase without any side effects related to inhibition of cyclooxygenase (COX) as it lacks COX activity, and in humans, undergoes very limited chiral inversion to the S-enantiomer that is active against COX [24] as well as, it was suggested that the high dose regimen may significantly slow the cognitive and functional decay of mild affected patients [25].

Table 4. IC_{50} values (ng/ml) of dark red calyces from *Hibiscus sabdariffa* L. within two solvent system compared to *R*-flurbiprofen. In-vitro Aβ_{42} modulation based on the ability of dark red calyces to inhibit Aβ_{42} production.

Extract name	IC_{50} (ng/ml)
DR_1	246.47 ± 6.43 ^{A}
DR_2	336.74 ± 8.44 ^{B}
DR_3	467.64 ± 9.43 ^{F}
DR_4	378.53 ± 12.34 ^{E}
R-flurbiprofen	800.4 ± 0. 012^{G}

Numbers shown are means ± standard deviation of three replicates. Values bearing different letters in the same column are significantly different at level of probability ($p < 0.01$) according to Duncan's Multiple Range Test.

In our study, inhibition of Aβ_{42} was recognized in our red calyces for both water and aqueous ethanol extracts with concentrations less than the reference drug by approximately one and half to three times. Under experimental conditions the relative potencies of Roselle extracts were in the order: DR_1 > DR_2 > DR_4 > DR_3 > R-flurbiprofen. Among the four Roselle extracts, (DR_3) extract with IC_{50} 467.64 ng/ml was the less potent while (DR_1) extract was the most potent extract with IC_{50} 246.47 ng/ml demonstrated the strongest anti-Alzheimer activity in our study.

Within the two solvent system, the aqueous ethanol extract was the more potent anti-Alzheimer extracts with IC_{50} (on average 291.6 ng/ml)

particularly at hot temperature. Among the distilled water extracts, the cold extract was the more potent in inhibiting Aβ_{42} secretion compared to the extraction at hot temperature with IC_{50} (378.53 and 467.64 ng/ml respectively). This finding may be correlated to the high total flavonoid at cold temperature for the aqueous extraction (15.94 µg/ml) compared to the hot temperature (6.17 µg/ml) and its presence also in a high quantity in the hot aqueous ethanol extraction (11.41 µg/ml) compared to the cold extraction (10.81 µg/ml) as shown in table (3). The positive correlation (r = 0.436) observed in our statistical analysis between total flavonoid and Aβ_{42} modulation by the four Roselle extracts also confirmed the significant role of total flavonoid in alteration of Aβ_{24} production.

In literature, it was reported that the diversity of structural variations within flavonoids opens avenues for their development as chemical tools in the treatment of AD by tackling the formation and distribution of neurotoxic oligomers species through reducing Aβ burden by relieving Aβ deposition, decreasing insoluble Aβ levels, and inhibiting β-amyloidogenic processing pathway involving downregulation of β-secretase and β-C-terminal fragment in the brain [26, 27].

Furthermore, the antioxidant activity was also detected in a high activity in the cold aqueous extraction by (IC_{50}, 47.5 µg/ml) compared to (IC_{50}, 57.4 µg/ml) in the hot extraction. In addition to the high antioxidant activity at hot aqueous ethanol extraction (45.5 µg/ml) compared to the cold extraction (88.5 µg/ml) as shown in table (2). This mean that the antioxidant activity of our Roselle extracts has a significant role in modulation of Aβ_{42} production but no statistically correlation (r = 0.055) was observed in our analysis.

The variation in the pharmacological function of Roselle calyces as anti-Alzheimer from IC_{50} of 246.47 to 467.64 ng/ml may be due to extraction methods and type of solvent used. In statistical analysis, there is a significance difference between the four Roselle extracts within the two solvent system and *R*-flurbiprofen in the concentration required for 50% inhibition of Aβ_{42} production at P value < 0.01 using One-way ANOVA. Duncan test were adopted for the post hoc analyses to the One-way ANOVA.

b. Results of Notch-Mediated Hes1-Luciferase Reporter Assay

The effect of the dark red calyces on Notch cleavage mediated transcription of Hes1 gene (Notch-Hes1) was evaluated. A Hes1-luciferase reporter system was used to investigate γ-secretase-mediated Notch signaling pathway. The background luciferase activity is identified as the activity in the presence of 600 ng/ml from each of our Roselle extracts. Each tested extract modulated γ-secretase through Notch ΔE cleavage-mediated luciferase activity and has no significantly effect as reported in table (5) in the range of (15.56 - 64.65 RLU) and allow Notch processing and signaling to continue

R-Flurbiprofen also has no effect on Notch-mediated luciferase activity in the Notch intracellular domain-transfected cells as shown in table (5). This consistent with that previously reported about non-steroidal anti-inflammatory drugs (NSAIDs) that can preferentially reduce the generation of the highly amyloidogenic Aβ_{42} peptide without affecting Notch cleavage [28] and also close to that obtained in literature [29].

Table 5. The effect of dark red calyces from *Hibiscus sabdariffa* L. on Notch intracellular domain responsive genes.

Extract name	Luciferase activity (RLU)
DR_1	24.65 ± 3.4 ^{B}
DR_2	35.74 ± 3.7 ^{B}
DR_3	26.84 ± 4.8 ^{B}
DR_4	25.63 ± 4.6 ^{B}
R-flurbiprofen	Nil ^{A}

Result were expressed as mean (RLU) value ± standard deviation of three replicates. Values bearing different capital letters in the same column are significantly different at level of probability ($p < 0.01$) according to Duncan's Multiple Range Test.

Anyway, both R-Flurbiprofen and the four Roselle extract allowed for potential inhibition of Aβ42 levels among our experimental samples without inducing potential side effects related to complete inhibition for γ-secretases in the cell. As the existence of more than one γ-secretase in the cell was previously elucidated that distinguished between the cells surface γ-secretase and the intracellular γ-secretase [30]. The first one is responsible for Notch cleavage

and the latter for APP processing and recycling. This may have important consequences in terms of safety, since the interference with Notch is a potentially harmful effect of traditional γ-secretase inhibitors [28,31]. The proposed mechanism for this activity may be due to an allosteric modulation of presenilin-1, the major component of the γ-secretase complex, that is responsible for the formation of Aβ42 as reported previously for some NSAIDs [32,33].

4. Conclusions

The findings from our in-vitro study for $A\beta_{42}$ alteration indicated that the pharmacological action of dark red calyces extracts is via the functional modulation of γ-secretase mediated APP processing pathway without any alteration related to Notch signaling pathway. And this pharmacological action could be related to the amount of total flavonoid and not for its significant antioxidant activity without any detectable side effect related to inhibition of cyclooxygenases (COX-1 and COX-2). The hot aqueous ethanol extraction was detected to be the best Roselle extraction method for alteration of $A\beta_{42}$ in a human neuroglioma model. The promising results suggest that more research into Roselle extract may lead to the development of a new complementary drug for Alzheimer's disease.

5. Conflicts of interest

"There are no conflicts to declare".

6. References

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