Damascus University Journal for the Basic Sciences
Vol 40 (2024): 120-136

مجلة جامعة دمشق للعلوم الأساسية
مجلد 40- (2024): 120-136

Successive Solvent Soxhlet Extraction and Phytochemical Screening and in Vitro Evaluation of Antioxidant of *Bauhinia Variegate* Leaves Extracts

[1]Dr. Mohamad Jawad Khubeiz*
[1]Department of Chemistry, faculty of Science, Damascus University Corresponding author
Mohamad.khubeiz@damascusuniversity.edu.sy

Abstract
The effect of the extraction by Soxhlet Apparatus of the leaves of *Bauhinia variegata* was studied for free radical scavenging and antioxidants activities in vitro. The extracts were quantitatively analyzed for total phenolic and flavonoid contents using spectrophotometric methods. In vitro free radical scavenging activity of extracts were studied for DPPH (2,2-diphenyl-1-picryl-hydrazyl), Reducing Power (RPA), ferric reducing antioxidant power using TPTZ (2.4.6-tripyridyl-striazine), Hydrogen Peroxide, hydroxyl radicals, Ferric thiocyanate assay (FTC) scavenging activities using Gallic acid, BHT and ascorbic acid as standard were examined. Maximum phenolic content showed by Me.fr, followed by Et.fr, Aqu.fr and Cl.fr then Hx.fr which showed the lowest phenolic content. Cl.fr showed highest flavonoid content against other fractions whose flavonoid content reported as the following order (Hx.fr > Et.fr > Me.fr > Aqu.fr). The extracts showed antioxidant activity with 50 % inhibitory concentration (IC_{50}), for DPPH radical-scavenging, Reducing power, TPTZ assay, Hydrogen Peroxide, Hydroxyl radical's and FTC Assay. In conclusion, Finally, the different extracts from *Bauhinia variegata* leaves and specifically the Me.fr extract reveal several properties such as higher DPPH (IC_{50} = 77.22), RPA (IC_{50} = 95.43), TPTZ (IC_{50} = 33.106) significant antioxidant capacities, whereas the Cl.fr had a higher H_2O_2 scavenging activity (IC_{50} = 43.76) and the Et.fr had a higher hydroxyl radicals scavenging activity (IC_{50} = 133.07) significant antioxidant capacities, while, It was observed that Cl.fr possessed the highest value FTC inhibition activity (55.15 ± 0.99%). This study contributed to the knowledge of the chemical and biological properties of *Bauhinia variegata* and will subsidize future studies of this species.

Keywords: *Bauhinia variegata*, antioxidants, Phenols, flavonoids, DPPH, H_2O_2, OH^*, reducing power, FTC.

Received :2022/07/19
Accepted:2022/10/23

ISSN (online): 2789-6366
http://journal.damascusuniversity.edu.sy/index.php/index/index

الاستخلاص المتتالي بالمذيبات العضوية بطريقة "سيكسولييه" وتحري الخواص الكيميانباتية والتقدير الكمي المخبري لمضادات الأكسدة في مستخلصات أوراق نبات البوهينيا المبرقشة

أ.م.د محمد جواد خبيز[1]

قسم الكيمياء، كلية العلوم، جامعة دمشق. Mohamad.khubeiz@damascusuniversity.edu.sy

الملخص:

درس تأثير الاستخلاص باستخدام جهاز سيكسولييه لأوراق نبات البوهينيا المبرقشة من أجل كسح الجذور الحرة والفعالية المضادة للتأكسد في المختبر. حللت المستخلصات كمياً لتعيين الفينولات والفلافونيدات الكلية باستخدام طرائق القياس الطيفي، درس نشاط كسح الجذور الحرة في المختبر للمستخلصات لـ DPPH (2,2-diphenyl-1-picryl-hydrazyl) والقوة الإرجاعية RPA، والقوة الإرجاعية المضادة للتأكسد للحديد الثنائي باستخدام TPTZ (2.4.6- tripyridyl-striazine)، وبيروكسيد الهدروجين، وجذور الهدروكسيل، واختبار ثيوسيانات الحديد الثنائي (FTC) باستخدام حمض الغاليك، وBHT (بوتيلتيد هيدروكسي تولوين)، وحمض الأسكوربيك كمعيار. فقد أظهر جزء المستخلص الميتانولي قيمة أعظمية للمحتوى الفينولي، يليه خلات الايتيل ثم المائي ثم الكلوروفورم ثم الهكسان الذي أظهر أخفض محتوى فينولي. أظهر مستخلص جزء الكلوروفورم أعلى محتوى من الفلافونيدات مقابل الأجزاء الأخرى، والتي رتب محتواها من الفلافونيدات حسب التالي: (Hx.fr > Et.fr > Me.fr > Aqu.fr). أظهرت جميع المستخلصات فعالية مضادة للتأكسد عند نسبة كسح 50% من التركيز (IC_{50}) لكل من جذور DPPH، والقوة الإرجاعية، واختبار TPTZ، وبيروكسيد الهدروجين، وجذر الهدروكسيل. أظهرت المستخلصات المختلفة لأوراق نبات البوهينيا المبرقشة لاسيما مستخلص الجزء الميتانولي العديد من الخصائص، مثلاً قيمة اعلى لكسح جذور الـ DPPH (IC_{50} = 77.22)، وRPA (IC_{50} = 95.43)، وTPTZ (IC_{50} = 33.106)، مع مقدرة مضادة للتأكسد عالية، بينما أعطى مستخلص جزء الكلوروفورم الفعالية الأعلى في فعالية كسح بيروكسيد الهدروجين (IC_{50} = 43.76)، بينما يمتلك جزء خلات الايتيل فعالية عالية لكسح جذور الهدروكسيل (IC_{50} = 133.07)، بينما لوحظ أن لجزء الكلوروفورم قيمة كسح عالية لـ FTC تبلغ (55.15±0.99%). أسهمت هذه الدراسة في التعرّف على الخصائص الكيميائية والبيولوجية لنبات البوهينيا المبرقشة مما يدعم الدراسات المستقبلية لهذا النوع.

الكلمات المفتاحية: بوهينيا مبرقشة، مضادات تأكسد، فينولات، فلافونيدات، قوة ارجاعية، DPPH، OH^*، H_2O_2، FTC.

تاريخ الإيداع: 2022/05/19
تاريخ الموافقة: 2022/10/23

Introduction

Various plants have been used by traditional herbal medicinal practitioners. Medicinal plants are the source of synthetic and traditional herbal medicine [3], as they contain some organic compounds which produce definite physiological action on the human body and these bioactive substances include tannins, alkaloids, carbohydrates, terpenoids, steroids and flavonoids [4]. The excessive exposure to free radicals and other reactive oxygen species (ROS) has made our body highly susceptible to ROS-mediated tissue and genetic damage. Generation of these free radicals causes lipid peroxidation, DNA damage, and may lead to carcinogenesis [5]. Therefore, to counteract the effect, antioxidants generated inside the body quench the free radicals and prevent the oxidation of substrates, thus preventing oxidative stress and DNA damage. Exposure to various environmental and food contaminants resulted in shifting of the balance toward the accumulation of free radicals, necessitating the need of dietary supplements rich in antioxidants. Plants being highly rich in metabolites that are involved in resisting the generation of free radicals are considered to be best source of antioxidant dietary supplements. Several plants and their isolates have been reported to possess various antioxidants [6,7] and thus prevent the loss of DNA integrity induced by genotoxic ants [8].

Available data indicate that natural products have beneficial effects on human health, notably in relation to their antioxidant activity. This property is particularly important for phenolic compounds because of their ability to scavenge free radicals originating from different oxygen and nitrogen species (ROS/RNS), such as superoxide anion ($O_2^{\bullet -}$), hydroxyl radical (HO•) peroxyl radical (ROO•), nitric oxide (NO•), hypochlorite ion ($ClO^{\bullet -}$), hydrogen peroxide (H_2O_2), singlet oxygen ($^1O_2$) and peroxynitrite ($ONOO^{\bullet -}$). For most living organisms, ROS are produced continuously during normal physiological events but their overproduction can affect and damage essential biomolecules such as nucleic acids, lipids, proteins and carbohydrates. ROS can also easily initiate the peroxidation of membrane lipids, which leads to the accumulation of lipid peroxides [9–11]. Recently, there is a growing interest in the discovery of natural antioxidants, mainly for there are epidemical and clinical evidences suggesting that consumption of vegetables and fruits reduces the risk of developing chronic disease, e.g. Cancer, and phytochemicals are generally safer than synthetic chemicals. Therefore, there is a need to find nontoxic and inexpensive drugs for clinical chemo protection. Recent studies have indicated that some of the commonly used medicinal plants may be good source of potent but nontoxic chemoprotectors [12,13].

Bauhinia is a large genus belonging to the subfamily *Caesalpinioideae* (*Leguminosae*). It consists of about 300 species and is distributed in pantropical regions of the world. Plants in the genus *Bauhinia* have characteristic butterfly shaped leaves [14]. Also, this species is widely cultivated as a common ornamental plant in Syria and other countries. The phytochemical screening revealed that *Bauhinia variegata* contained terpenoids, flavonoids, tannins, saponins, reducing sugars, steroids and cardiac glycosides. Pharmacological studies showed that *Bauhinia variegate* exerted anticancer, antioxidant, hypolipidemic, antimicrobial, anti-inflammatory, nephroprotective, hepatoprotective, antiulcer, immunomodulating, and wound healing effects. Previous phytochemical studies on the leave of this species have led to the isolation of several flavonoids [15, 16]. There are also a few reports of antitumor activity of *B. variegata* ethanolic extract against Dalton's ascetic lymphoma (DAL) in Swiss albino mice [17] and N-nitrosodiethylamine induced experimental liver tumors in rats and human cancer cell lines [18].

These properties of *Bauhinia Variegata*, prompt us to carry out the phytochemical screening and antioxidant properties of the plant leaves. From this viewpoint the present study was carried out to evaluate the polyphenols, flavonoids and in *vitro* free radical scavenging activity of leaves extracts. As far as we could ascertain this is the first study in Syria of its kind on this plant.

Materials and Methods

Collection and Identification of Plant Materials

The leaves of *B. variegata* L. was collected in June 2021, from Damascus University campus, Syria. The plant material was taxonomically identified by a botanist. The fresh leaves collected were washed with distilled water to remove dust and was shade dried, pulverized by a mechanical grinder and stored in airtight containers for further use.

Preparation of Extracts

One hundred gr powdered leaf material was successively extracted with organic solvents, with increasing polarity index using Soxhlet apparatus continuously for 12 h with 500 ml of the various solvents each. The powdered plant sample was extracted sequentially with n-hexane (Hx.fr), chloroform (Cl.fr), ethyl acetate (Et.fr) and methanol (Me.fr), and the air-dried residue was further extracted with hot water (Aqu.fr) at 24 hours by the method of maceration. Each time before extracting with the next solvent, the material was dried in a hot air oven at 40 ^{o}C. The solvents were evaporated using a rotary vacuum-evaporator at 50 ^{o}C and later dried in a desiccator.

The dried extracts were weighed to determine the percentage yield of the soluble constituents using the formula, % Yield: (Weight of dry extract/ Weight taken for extraction) × 100. The dried extracts thus obtained were dissolved in the respective solvents at the concentration of 1 mg/mL and used for assessment of antioxidant capacity through various chemical assays, and was kept in a refrigerator until further use.

Determination of Total Phenolic Content (TPC)

Total phenolic content (TPC) of each of extract obtained by hot extraction with a Soxhlet apparatus was estimated according to the method of Lapornik, B *et al.* [19] with slight modification. The mixture was sonicated for 5 min to obtain a homogenized solution. To 300 µL of this solution taken in a test tube, 1 mL methanol, 3mL distilled water and 200 µL Folin-Ciocalteu reagent were added. Then, after an 5-8 min incubation at room temperature, 800 µL sodium carbonate solution (20%) was added and the test tube was covered with aluminum foil and incubated in a hot water bath at 40 °C for 45 min. A blank was prepared using the same procedure but replacing the plant extract with an equal volume of methanol. The absorbance of the sample was determined using a UV visible spectrophotometer at 765 nm. Different concentrations of Gallic acid (12.5 - 300 µg/mL) were prepared in methanol, the standard curve of gallic acid was obtained using the same procedure (Fig. 1a). Total phenolic content was expressed as mg of gallic acid equivalents (G.acid) per gram extract, which was calculated using the formula, $y = 0.0038x + 0.024$ $R^2 = 0.9982$, where, *y* is the absorbance at 765 nm and *x* is the amount of gallic acid equivalent (mg G.acid/g.Ext). Data presented are average values of four measurements for each sample.

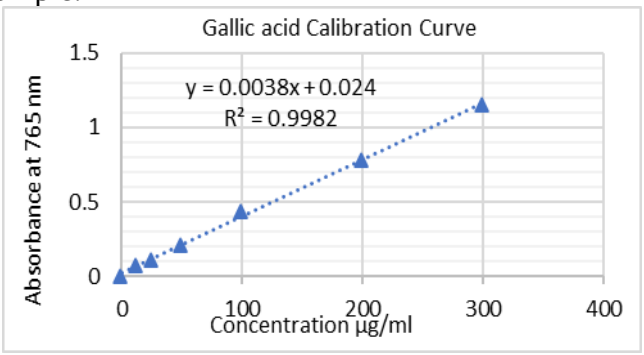

Fig.1a Gallic Acid Calibration Curve.

Determination of Total Flavonoid Content (TFC)

Aluminium-chloride colorimetric assay was used to determine the total flavonoid content in the extracts as previously reported protocol [20]. Briefly, (250 µL) of each extract (1 mg/mL) was diluted with 1250 µL of distilled water followed by the addition of 75 µL of a 5% $NaNO_2$ (w/v) solution. After 6 minutes, 150 µL of a 10% $AlCl_3.6H_2O$ (w/v) solution was added, and the blend was permitted to remain for another 5 minutes. Five hundred microliters of 1 M NaOH was added, and the aggregate

was made up to 2.5 ml with distilled water. The absorbance was measured against the blank at 415 nm utilizing a UV-Vis spectrophotometer (Double beam UV-visible Spectrophotometer SYSTRON-ICS 2201) in correlation with known Quercetin standard.
A set of standard solutions of quercetin (12.5, 25, 50, 100, 200, and 300 µ*g*/mL) were prepared in the same manner as described for the extracts. The total flavonoid content was determined from the calibration curve (Fig. 1b) and expressed as milligram of quercetin equivalent (Que) per gram of extracts, calculated using the formula, y = 0.0038x + 0.024 R^2 = 0.998, where, *y* is the absorbance at 415 nm and *x* is the amount of quercetin equivalent (µg/mL). Where; x was the absorbance and Y the quercetin equivalent (mg Que/g. Ext.). Measurements were conducted in triplicate and values are expressed in mean ± SD. The determinations of total flavonoid in the extracts and standards were carried out in triplicates.

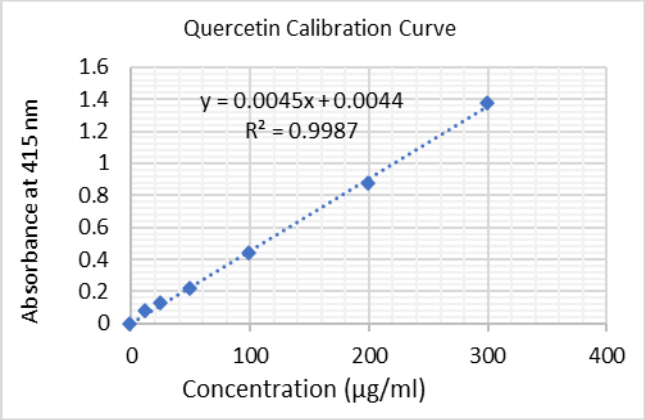

Fig.1b Quercetin Calibration Curve

DPPH free radical scavenging activity

The DPPH method is based on the capture of the purple-colored DPPH radical (2,2-diphenyl-1-picrylhydrazyl), which absorbs at 517 nm. By the action of an antioxidant or a radical species, the DPPH• is reduced creating 2,2- diphenyl-1-picrylhydrazyl, of a yellow color, and consequent absorption disappearance, so it can be monitored by the decrease of absorbance. Based on the results obtained it is possible to obtain the free radical antioxidant or scavenging activity percentage and/or the DPPH remaining percentage in the reaction environment [21]. The antioxidant activity of the plant extracts and the standard was evaluated on the basis of the radical scavenging effect of the stable (DPPH)-free radical activity by the method of [22] with slight modification. The stock solution of crude extracts (1 mg/mL) was prepared by dissolving a known amount of dry extract in 10% DMSO of methanol. The working solution (100, 200, 400, 600, 800, 1000 µg/mL) of the extracts were prepared from the stock solution using suitable dilution. Ascorbic acid was used as standard in 50-250 µg/mL solution. A quantity of 0.1 ml of the sample or standard was added to a 2.9 ml DPPH (Sigma-Aldrich, Germany) radical solution at 100 µM, dissolved in methanol at 80%. It was care fully homogenized and stored in the dark at room temperature (30 ^oC ± 1^oC) for 20 minutes. The absorbance was measured using a Spectrophotometer at a 517 nm wave length. The tests were performed in triplicate and based on the data obtained the percentage of DPPH consumed was calculated, with the help of Equation:

DPPH scavenging activity (I %) = $[(A_C - A_S / A_C) \times 100]$

where A_C = absorbance of the control and A_S = absorbance of tested samples. Then, curves were constructed by plotting percentage of inhibition against concentration in µg/mL. The equation of this curve allowed to calculate the IC_{50} corresponding to the sample concentration that reduced the initial DPPH• absorbance of 50 %. A smaller IC_{50} value corresponds to a higher antioxidant activity.

Reducing Power Activity (RPA)

The reducing power of the extracts was evaluated according to the protocol of Hseu et al. [23]. Briefly, 0.1 ml of different concentration sample (100, 200, 400, 600, 800, 1000 µg/mL) was mixed with 1.25 ml of a 0.2 M phosphate buffer (pH 6.6) and 1.25 ml of a 1% (w/v) solution of potassium ferricyanide $K_3Fe(CN)_6$. The mixture was incubated in a water bath at 50°C for 20 min. Afterwards, 1.25 ml of a 10% (w/v) trichloroacetic acid solution was added and the mixture was then centrifuged at 4000 rpm for 10 min. A 1.25 ml aliquot of the upper layer was combined with 1.25 ml of distilled water and 0.25 ml of a 0.1% (w/v) solution of ferric chloride $FeCl_3$, and absorbance was measured spectrophotometrically at 700 nm against a blank sample. Ascorbic acid at different concentrations (15.625, 31.25, 62.5, 125, 250 ,500 and 1000 µg/ml) was used as standard, and phosphate buffer was used as blank solution. Increase in the absorbance of the reaction mixture indicated increase in the reducing power. The percentage inhibition was calculated by using the following equation:

$$\text{I\% RPA} = \left[\frac{A_S - A_C}{A_S}\right] \times 100$$

where A_s and A_c are the absorbance of the sample and the control, respectively. The IC_{50} value (µg Fe^{2+}/mL) is the effective concentration giving an absorbance of 0.5 for reducing power and was estimated from the % inhibition versus concentration sigmoidal curve, using a non-linear regression analysis by the software GraphPad prism 8.0.2 .

Ferric reducing antioxidant power (FRAP) by TPTZ Method

Total Antioxidant Capacity mM/g was determined chemically using FRAP method. TPTZ method depend upon the reduction at low pH of ferric tripyridyltriazine complex to the ferrous tripyridyltriazine by a reductant antioxidant. This ferrous tripyridyl-triazine complex has an intensive blue color and can be monitored at 595 nm [24].

Reagents: acetate buffer, 300 mM/L, pH 3.6 (3.1g sodium acetate-$3H_2O$ and 16 mL/L glacial acetic acid); 10 mM/L TPTZ 2.4.6-tripyridyl-striazine in 40 mM/L HCl; 20 mM/L $FeCl_3 6H_2O$ in distilled water. FRAP working solution: 25 mL acetate buffer, 2.5 mL TPTZ solution and 2.5 mL $FeCl_3$ solution. It is necessary to prepare freshly the working solution. Aqueous solution of known Fe^{2+} concentration was used for calibration, in a range of 0.05 - 1.0 mM/L. For the preparation of calibration curve 0.5 mL aliquot of 0.05, 0.1, 0.2, 0.4, 0.6, 0.8 and 1.0 mM/mL aqueous Fe^{2+} were mixed with 2.5 mL FRAP working solution. FRAP reagent was used as blank. The absorption was read after 40 minutes at room temperature and 595 nm. All solutions were prepared using deionized water. For all the samples the determination was made in triplicate and the mean values were reported. Total antioxidant capacity in Fe^{2+} equivalents was calculated. Correlation coefficient R^2 for calibration curve was 0.998 (fig. 1c). Absorption determination for FRAP was made using UV-VIS spectrophotometer. The values obtained were expressed as mM of ferrous equivalent Fe^{2+} per 1 gram of dry extract.

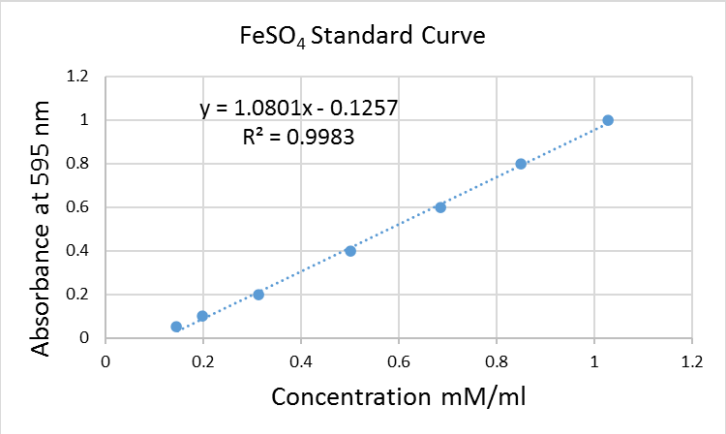

Fig.1c $FeSO_4$ Calibration Curve

Hydrogen Peroxide Scavenging Assay

The free radical scavenging activity was determined by hydrogen peroxide assay [25] certain modifications. Hydrogen peroxide (40 mM) solution will be prepared in phosphate buffered saline (0.05M, pH 7.4). 1ml of the extract containing samples of different concentration (25, 50, 100, 150, 200 and 250 µg/ml) will be rapidly mixed with 2ml of hydrogen peroxide solution. The absorbance will be measured at 230 nm in the UV spectrophotometer after 10 minutes of incubation at 30°C against a blank (without hydrogen peroxide). All tests were run in triplicate and the percentage of scavenging of hydrogen peroxide will be calculated using the formula:

Percentage scavenging (H_2O_2) = ((A_C - A_S) / A_C) x 100

A_C - Absorbance of control; A_S - Absorbance of sample. Ascorbic acid has been taken as standard. IC_{50} values of the extract i.e., concentration of extract necessary to decrease the initial concentration of hydrogen peroxide by 50 % were calculated.

Hydroxyl Radical Scavenging Activity

The scavenging activity for hydroxyl radicals was measured with Fenton reaction performed in accordance with the method described by [26], with slight modifications. Both 1, 10-phenan-throline (1.5 mM) and $FeSO_4$ (1.5 mM) were dissolved in phosphate buffer (pH 7.4) and mixed thoroughly. A total of 1 mL H_2O_2 (0.01%) and 1 mL of various concentrations of extracts (25, 50, 100, 150, 200, and 250 µg/mL) were dissolved in methanol. Subsequently, the volume was adjusted to 10 mL. The mixture was then vortexed vigorously and left for 60 min at 37 ^oC in the dark; the absorbance was measured at 512 nm. Hydroxyl radical scavenging activity is calculated using the following equation: Hydroxyl radical scavenging activity (HRSA) % = (As -A_1)/ (A_0-A_1) x100 where As is the absorbance of the sample; A_1 is the absorbance of control solution containing 2 mL 1, 10-phenanthroling, $FeSO_4$, and 1mL H_2O_2, and A_0 is the absorbance of blank solution containing 1, 10-phenanthroline and $FeSO_4$. Each sample was examined in three times and averaged. Gallic Acid was used as the positive control.

Ferric Thiocyanate (FTC) Assay

The antioxidant activities of various extracts of plant on inhibition of linoleic acid peroxidation were assayed by thiocyanate method (FTC) with a modification of the procedure reported previously [27]. The 0.1 mL of each of sample solution (1.0 mg/ mL) was mixed with 2.5 mL of linoleic acid emulsion (0.02 M, pH 7.0) and 2.0 mL of phosphate buffer (0.02 M, pH 7.0). The linoleic emulsion was prepared by mixing 0.28 g of linoleic acid, 0.28 g of Tween-20 as emulsifier and 50.0 mL of phosphate buffer. The reaction mixture was incubated at 40 for 6 days at 40^{o}C. The mixture without extract was used as control. The 0.1 mL of the mixture was taken and mixed with 5.0 mL of 75% ethanol, 0.1 mL of 30% ammonium thiocyanate and 0.1 mL of 20 mM ferrous chloride in 3.5% HCl and allowed to stand at room temperature. Precisely 4 min. after addition of ferrous chloride to the reaction mixture, absorbance was recorded at 500 nm. The antioxidant activity was expressed as percentage inhibition of peroxidation:

$$\text{Inhibition (\%)} = Ac - As / Ac \times 100$$

Where, As is the absorbance of the sample on the day when the absorbance of the control is maximum and Ac is the absorbance of the control on the day when the absorbance of the control is maximum. The antioxidant activity of BHT was assayed for comparison as reference standard. The experiment was performed in triplicate.

Statistical Analysis

The experiments were carried out in triplicate or more and results are given as the mean ± standard deviation SD. Average, standard deviation calculation and graphs were performed with Microsoft Excel (2013). IC_{50} values were generated with GraphPad Prism v 8.0.2 .

Results and Discussion

Several methods have been developed to evaluate total antioxidant capacity of herbal extracts. Nevertheless, few of them have been used widely due to the difficulty of measuring total antioxidant

capacity as result of limited methodological protocol and free radical sources [28]. Understanding of the chemical composition and potential biological properties of plant extracts is of essential importance for their further use in the food industry or alternative therapy. A range of spectrophotometric assays was applied to create a data base which could be used to evaluate the potential of *Bauhinia Variegate* leaves as an inexpensive and accessible natural resource for the production of polyphenol-rich extracts with strong antimicrobial and antioxidant activity.

Extract Yields, Total Phenolic and Total Flavonoid Contents

The yield percentage of *B. Variegate* leaves is shown in (Fig. 2a). The extract yield percentage of all the extracts was found to be in the order of: Me.fr > Aqu.fr > Hx.fr > Et.fr > Cl.fr. polar solvents show high yield of extraction when compared to Nonpolar solvents.

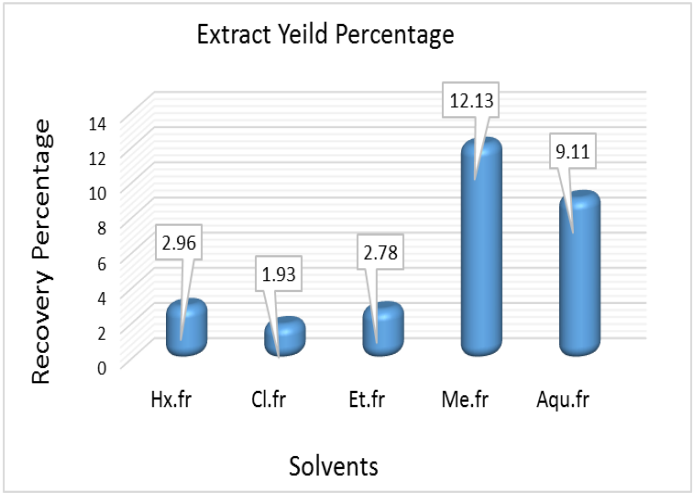

Fig. 2a Percentage of Yield.

Phenolics are the widest spread secondary metabolite in plant kingdom. These diverse groups of compounds have received much attention as potential natural antioxidants in terms of their ability to act as efficient radical scavengers. Total Phenolic Content TPC activity is the process to figure out the amount of phenolic content in the samples. Phenolic compounds that contained in the plants have redox properties, and the properties allow them acting as antioxidants. The total phenolic content for *B. Variegate* leaves extracts were estimated by Folin Ciocalteu's method using gallic acid as standard. The reagent is formed from a mixture of phosphotungstic acid and phosphomolybdic acid which after oxidation of the phenols, is reduced to a mixture of blue oxides of tungsten and molybdenum. The blue coloration produced has a maximum absorption in the region of 760 nm and proportional to the total quantity of phenolic compounds originally present. The relationship between the content of gallic acid and absorbance is expressed as the equation $y = 0.0038x + 0.024$ with a correlation coefficient ($R^2 = 0.9982$).

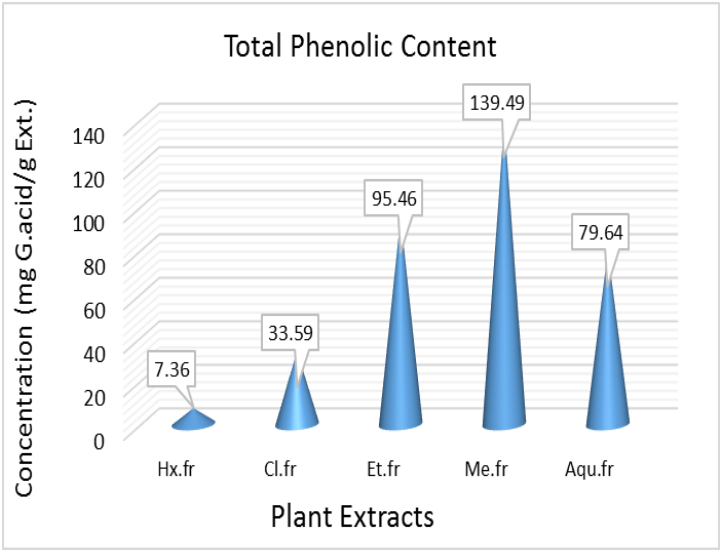

Fig.2b Total Phenolic Content

According to the results obtained from the determination of total phenolic contents, it was found that Met.fr contained more phenolic contents than the other fractions (139.49 ± 0.65 mg G.acid /g ext.), followed by Et.fr (95.46 ± 61 mg G.acid /g ext.), Aqu.fr (79.64 ± 0.31 G.acid /g ext.), Cl.fr (33.59 ± 0.87 mg G.acid E/g ext.), while Hx.fr recorded the lowest phenolic contents (7.36 ± 0.76 mg G.acid/g ext.) (Fig. 2b). The flavonoid content was obtained using aluminum chloride method and is based on the formation of a complex between the aluminum ion, Al (III), and the carbonyl and hydroxyl groups of flavones and flavanols that produce a yellow color. Quantitative estimation of total flavonoid was done on the basis of a standard curve of quercetin and linearity of the calibration curve was achieved between 12.5 to 300 µg/ml concentration of quercetin ($y = 0.0038x + 0.024$ $R^2 = 0.996$) (fig. 2c).

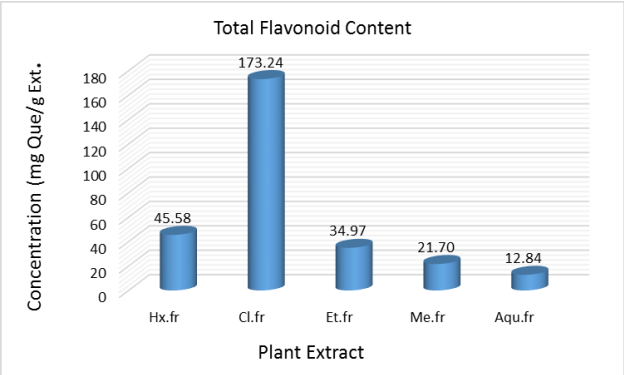

Fig.2c Total Flavonoid Content

The results showed that the total flavonoid contents in the selected plant for the study varied considerably and ranged from 12.84 to 173.24 mg of Que/ g ext. (Fig. 2c). The highest flavonoid content was observed in Cl.fr extract i.e. 373.24 mg Que/g ext. and lowest flavonoid content was observed in Aqu.fr extract i.e. 12.84 mg Que/g ext.

DPPH radical scavenging activity

DPPH, a commercially available radical serves as the oxidizing radical to be reduced by the antioxidant and as the indicator for the reaction. DPPH is relatively stable nitrogen centered free radical that easily accepts an electron or hydrogen radical to become a stable diamagnetic molecule. DPPH radicals react with suitable reducing agents as a result of which the electrons become paired off forming the corresponding hydrazine. The reduction capability of the DPPH radical is determined by the decrease in its absorbance at 517 nm, induced by antioxidants. Results were reported as IC_{50}, which is the amount of antioxidant necessary to decrease the initial DPPH• concentration by 50%. The lower the IC_{50}, higher the antioxidant power [29]. In this study, the DPPH radical scavenging activities of

extracts therefore increased gradually in a dose concentration dependent manner (100-1000 µg/ml). A variation in antioxidant activities ranging from 11.05 to 90.88% At the high dose of 250 µg/ml, Ascorbic acid exhibited very high scavenging effect (95.02 %) (Fig.3a). The IC_{50} of extracts of Hx.fr, Cl.fr, Et.fr, Me.fr and Aqu.fr was 738.57, 628.88, 104.85, 77.22 and 307.79 µg/mL, respectively. Whereas the IC_{50} of As. acid. (standard) was 15.76 µg/mL (Fig. 3b).

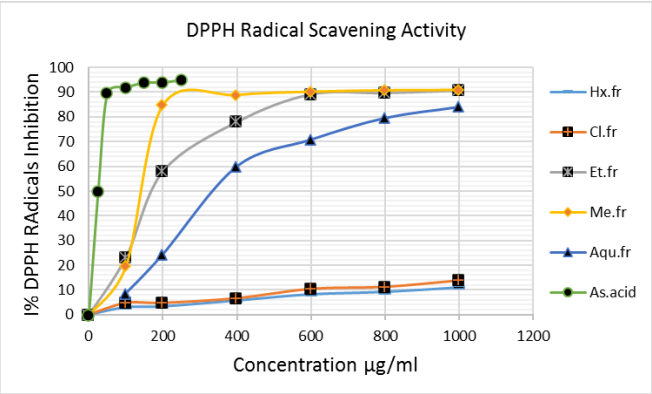

Fig.3a DPPH Scavenging Activities of Different Extracts

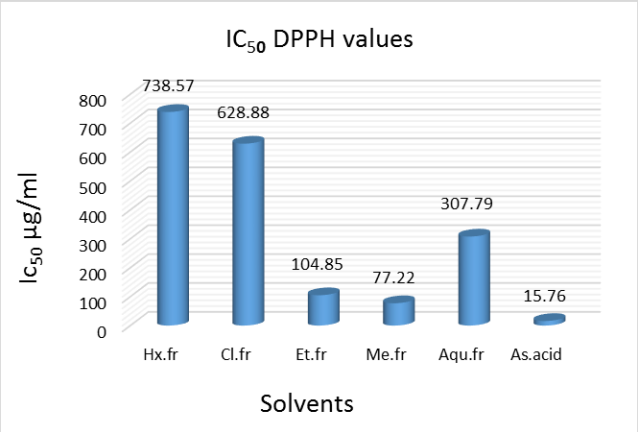

Fig.3b IC_{50} Value for DPPH Scavenging Activities

Reducing Power Activity (RPA)

The reducing properties are generally depending on the presence of reductions, which have been shown to exert antioxidant activity by breaking the free radical chain by donating a hydrogen atom. In this assay, the ability of extracts to reduce Fe^{3+} to Fe^{2+} was determined. The presence of antioxidants in the extracts resulted into reduction of the ferric cyanide complex (Fe^{3+}) to the ferrous cyanide form (Fe^{2+}) [30]. The assay is based in the following chemical reactions:

$$Fe(CN)_6^{3-} + Ar\,OH \longrightarrow Fe(CN)_6^{4-} + Ar\,O^{\bullet} + H^+$$

$$Fe(CN)_6^{4-} + Fe^{3+} + K^+ \longrightarrow K\,Fe[Fe(CN)_6]$$

The results of reducing power activity of the extracts were compared with standard ascorbic acid at 700 nm. Like the scavenging activity, the reducing power of all the extracts increased with increasing concentration. (Fig. 4a) showed the reducing activities of various extracts of *B. Variegate* leaves in comparison with ascorbic acid as standard. The higher the absorbance of the reaction mixture, the higher would be the reducing power. When the results are evaluated, there is significantly difference between all the compounds tested at all concentrations. However, Me.fr showed higher reducing power activity than the others but not as efficient as standard ascorbic acid. The reducing power of the extract was in an order of As.acid. > Me.fr > Et.fr > Aqu.fr > Cl.fr > Hx.fr. In order to compare of reducing ability of samples can be used a factor as IC_{50} value (Fig. 4b).

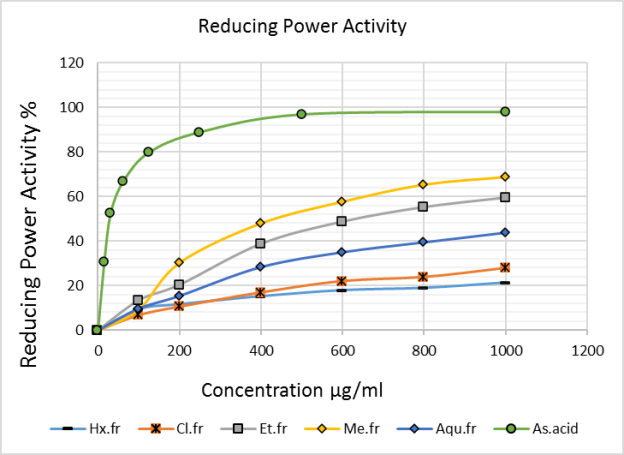

Fig.4a Reducing Power Assay

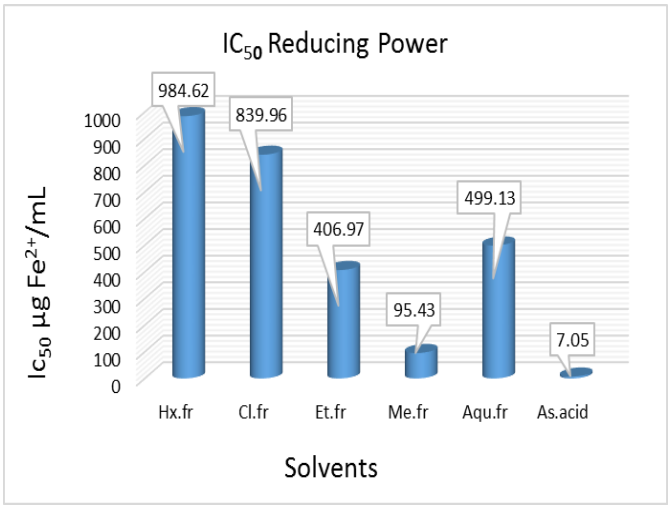

Fig.4b IC_{50} Value for Reducing Power Activity

Ferric Reducing Antioxidant Potential (FRAP) Assay

FRAP assay measures the reducing potential of an antioxidant reacting with a ferric tripyridyltriazine (Fe^{3+}-TPTZ) complex and producing a colored ferrous tripyridyl-triazine (Fe^{2+}-TPTZ). The reducing properties associated with the presence of compounds exert their action by breaking the free radical chain through donating a hydrogen atom. FRAP assay showed positive correlation between reducing power and phenolic content in *B. Variegate leaves* extracts. Based on (Fig.5 a), at the highest concentration, each sample had high activity of FRAP reduction (Et.fr = 674.64, Me.fr = 729.73, Aqu.fr = 648.72, and G.acid = 714.61 (standard) µM Fe^{++}/g ext.), when Cl.fr = 386.26 and Hx.fr = 293.01 mM Fe^{++}/g ext. which were significant. On the other hand, at the lowest concentration, each sample had the activity values was in an order of (G.acid > Me.fr > Et.fr > Cl.fr > Aqu.fr > Hx.fr) (Table 2). In order to compare of FRAP activity of samples can be used a factor as IC_{50} value (Fig.5 b).

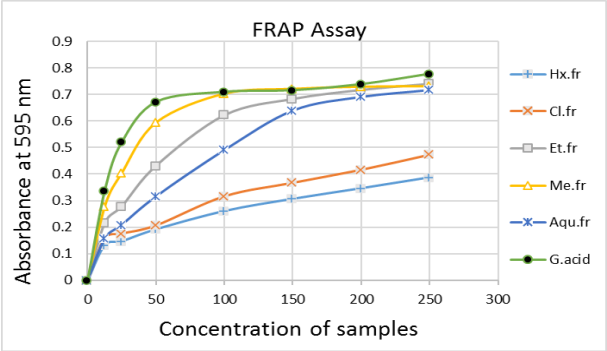

Fig.5a FRAP Radical Scavenging Activity

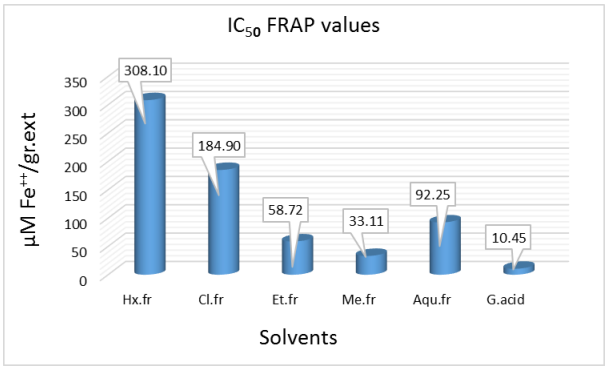

Fig.5b IC_{50} Value for FRAP Activity

Hydrogen Peroxide Scavenging Activity

H_2O_2 is highly important because of its ability to penetrate into biological membranes. H_2O_2 itself is not very reactive, but it can sometimes be toxic to cell because it may give rise to hydroxyl radicals in the cells [31]. Scavenging of H_2O_2 by extracts may be attributed to their phenolics, which can donate electrons to H_2O_2, thus neutrali-zing it to water. The results show the all the extracts had potent H_2O_2 scavenging activity which may be due to the antioxidant compounds. As the antioxidant components present in the extracts are good electron donors, they may accelerate the conversion of H_2O_2 to H_2O. (Fig. 6 a) reports the H_2O_2 scavenging activity of various leaf extracts of *B. Variegate*. The Cl.fr was shown to scavenge the H_2O_2 radicals with an excellent inhibition percentage of (43.90 - 73.84 %). The IC_{50} values of Cl.fr, Et.fr, Me.fr, Hx.fr and Aqu.fr were (43.76, 86.11, 74.1, 82.14, 120.73) mg/ml respectively. All the leaf extracts of exerted moderate scavenging activity when compared to ascorbic acid with IC_{50} value of 99.76 mg/ml (fig. 6 b). The scavenging activity of the extracts in high dose were effective in the order Cl.fr > Et.fr > Me.fr > Hx.fr > Aqu.fr.

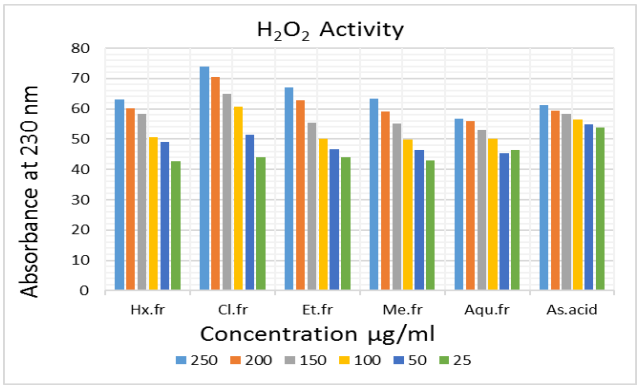

Fig.6a Hydrogen Peroxide Scavenging Activity

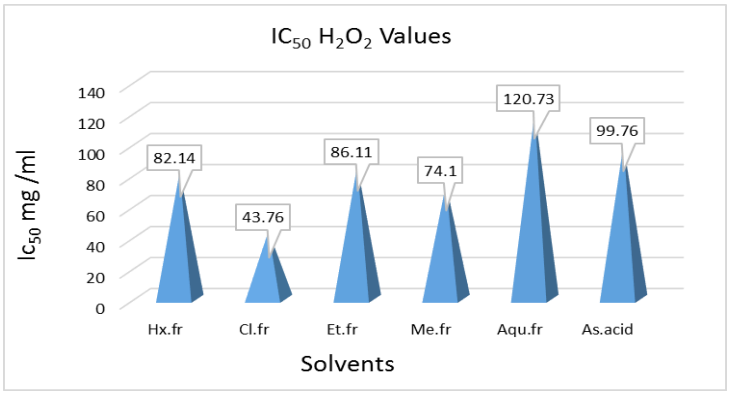

Fig.6b IC_{50} Value for H_2O_2 Scavenging

Hydroxyl Radical Scavenging Activity

The hydroxyl radical is an extremely reactive free radical formed in biological systems and has been implicated as a highly damaging species in free radical pathology, capable of damaging almost every molecule formed in living cells Hydroxyl radicals are also known to initiate peroxidation of lipid membranes [32]. The hydroxyl radical scavenging activity of the various extracts of *B. Variegate* leaves was dose dependent. Our result in (fig. 7 a) shows that Et.fr extract has a low hydroxyl radical scavenging activity which compared favorably with G.acid. In other tests; among the extracts, Et.fr had higher activity than that of the other extracts. At a concentration of 250 µg/ml, the scavenging activity of Et.fr, Me.fr, Aqu.fr, Cl.fr and Hx.fr was 60.14 ± 0.97, 55.23 ± 1.08, 38.22 ± 0.16, 34.49 ± 0.16 and 22.28 ± 1.33 %, respectively, whereas at the same concentration, G.acid was 98.56 ± 0.74 %. The IC_{50} of Et.fr, Me.fr, Aqu.fr, Cl.fr and Hx.fr was 133.07, 163.61, 286.32, 328.32 and 916.24 µg/ml, respectively, Whereas the IC_{50} of G.acid (standard) was 43.41 µg/mL (Fig. 7 b).

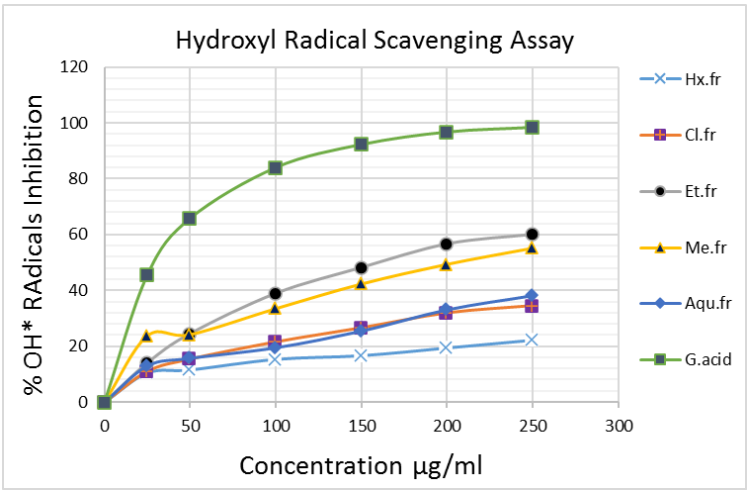

Fig. 7a Hydroxyl Radical Scavenging Activity

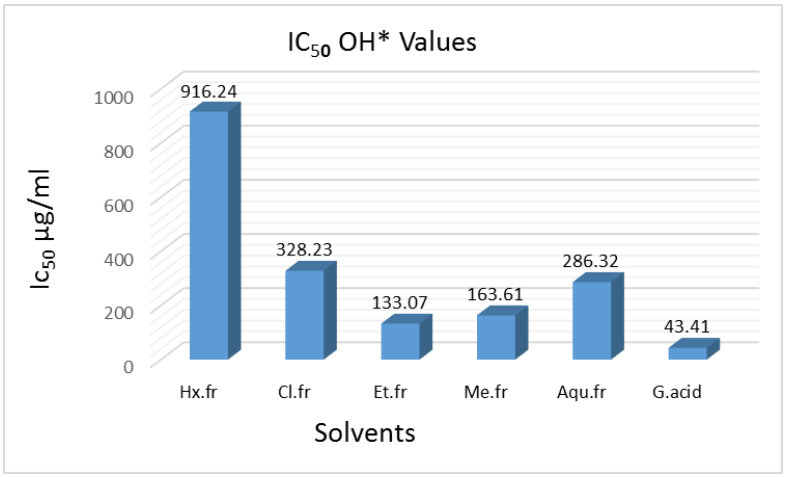

Fig. 7b IC_{50} value for Hydroxyl Radical Scavenging

Ferric Thiocyanate (FTC) Assay

Reactive and cytotoxic products are produced in vitro due to peroxidation of lipids. These products give modified or damaged DNA due to malfunctioning of the normal cell. Lipid hydroperoxides and free radicals are generating when oxygen reacts with double bond of unsaturated lipid. Hydrogen donating antioxidants breakdown the process of production of new radicals when react with lipid peroxyl radicals. In FTC method peroxide will react with $FeCl_2$ and form Fe^{3+} which in turn react with NH_4SCN and produce reddish ferric thiocyanate pigment. This process is used to calculate the quantity of peroxide in lipid peroxidation [33]. It was observed that Cl.fr possessed the highest value of lipid peroxidation inhibition $55.15 \pm 0.99\%$. Hx.fr displayed the lowest percentage of lipid peroxidation inhibition $17.16 \pm 0.49\%$. Aqu.fr, Et.fr and Me.fr also displayed % inhibition of lipid peroxidation having value of $54.25 \pm 1.33\%$, $45.43 \pm 0.13\%$ and $39.48 \pm 0.61\%$, respectively. The result was compared with a standard BHT having inhibition of lipid peroxidation value $63.39 \pm 0.60\%$ (Fig.8). Significantly lower absorbance as compared to control was observed, which indicate that these fractions have greater antioxidant activities. The fractions which showed greater values of percent inhibition of lipid peroxidation might contain primary antioxidant compounds, which are able to react aggressively with free radicals, particularly hydroxyl radicals, thereby terminating the radical chained reaction and retarding the formation of hydroperoxides [34].

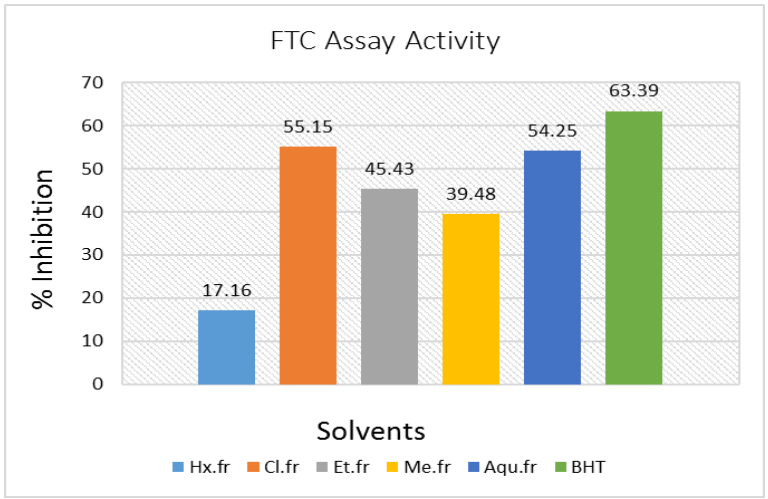

Fig.8 Ferric Thiocyanate (FTC) Assay Activity

Soxhlet extraction is a classical technique for the solvent extraction of obtaining polyphenolic compounds from plant sources. Even though, some of the heat sensitive compounds may decompose in the Soxhlet technique [35,36]. However, thermolabile-thermostable compounds cannot be dehydrolyzed due to the stability of compounds. From the result, thermolabile compounds from the Soxhlet extraction method showed good antioxidant properties. The present study revealed that the Soxhlet method of extraction had a big influence on the antioxidant properties of obtained extracts. These results showed that *B. Variegate* leaves could be a potential natural source of antioxidants and could have greater importance as therapeutic agent in preventing or slowing oxidative stress, inflammation and diabetic related disorders. Further studies are currently underway to assess the in vivo biological activities and to identify the active component responsible for their antioxidant, antidiabetic and anti-inflammatory properties. This study showed that methanol was the best solvent for the extraction of polyphenols of *B. Variegate* leaves, in terms of high extract and component yield. Chloroform, Ethyl acetate and aqueous were also found to be able to extract antioxidants component, but at lower yield; n-hexane, ethyl acetate and chloroform might be the good solvents to remove the unwanted components. However, the use of large amounts of unsafe organic solvents may not be a good choice for the processing of high-value phytochemicals, especially for the use in the pharmaceutical industry. Present data are in line with the observation of many scientists who documented the relationship of antioxidant activity with total phenolic compounds [37]. The present results are unable to compare with other results due to the usability of the literature on the selected plant extract methods. But there are several reports are available in the literature on the other methods and species belong to this family. The total phenols content and antioxidants activity of other species also showed the good amount present in the plant extracts reported.

Conclusion

Based on the present results, it has been concluded that *B. Variegata* leaves can be used as a natural source of antioxidants and its continued use can provide health benefits for humans by protecting against oxidative stress, liver injury, and other similar liver disorders. The plant extracts studied could be an answer to the people seeking for better therapeutic agents from natural sources which is believed to be more efficient with little or no side effects when compared to the commonly used synthetic chemotherapeutic agents. The present study verified the traditional use of *B. Variegata* for various human ailments especially for various infectious diseases. Further studies are needed to isolate, characterize and elucidate the structure of the bioactive compounds of this plant for antimicrobial drug formulation. More details in studies based on the in vitro and in vivo association with Separation of active ingredients is required to unravel new approaches to treating diseases caused by free radicals.

References

1. Gyawali R (2013a); General steps for the development of new drugs from herbs. *Handbook of pharmacognosy.* 1st ed. Bagbazar, kathmandu, Nepal: Nabodit Hamro pustakk bhandar.
2. Kumar PV, Chauhan SN, Padh H, Rajani M (2006); Search for antibacterial and antifungal agents from selected Indian medicinal plants. J. Ethnopharmacol. 107:182-188.
3. Gami B, Parabia MH. (2010); Pharmacognostic evaluation of bark and seeds of mimusopselengi. International journal of pharmacy and pharmaceutical sciences; 2(suppl 4): 110-113.
4. Edoga HO, Okwu DE, Mbaebie BO. (2005); Phytochemicals constituents of some Nigerian medicinal plants. Afr J Biotechnol; 4(7):685-688.
5. Zama D, and others *et al*. (2007); Chlorpyrifos-induced oxidative stress and tissue damage in the liver, kidney, brain and fetus in pregnant rats: The protective role of the butanolic extract of *Paronychia argentea*. Indian J Pharm; 39:145-50.
6. Khan MS, Yusufzai SK, Ying LY, Zulnashriq W. (2018); GC-MS based chemical profiling and evaluation of antioxidant potential of leaves and stems of *Alternanthera sessilis red* from Sabah, Malaysia. Int J Pharm Pharm Sci;10:4-9.
7. Balakrishnan BB, Krishnasamy K. (2018); Evaluation of free radical screening and antioxidant potential of *Moringa concanensis* nimmo-a medicinal plant used in Indian traditional medication system. Int J Pharm Pharm Sci;10:91-7.
8. Fierascu I, and others, *et al*. (2018); Geno protective, antioxidant, antifungal and anti-inflammatory evaluation of hydroalcoholic extract of wild-growing *Juniperus communis* L. (*Cupressaceae*) native to Romanian Southern Sub-Carpathian hills. BMC Complement Altern Med; 18:3.
9. Gulcin Ak, T.; I. (2008), Antioxidant and radical scavenging properties of *curcumin*. Chem. Biol. Interact. *174*, 27–37.
10. Royer, M.; Diouf, P.; Stevanovic, T. (2011); Polyphenols contents and radical scavenging capacities of red maple (Acer rubrum L.) extracts. Food Chem. Toxicol., 49, 2180–2188.
11. Saurai, P. (2015); Silymarin as a natural antioxidant: An overview of the current evidence and perspectives. Antioxidants, 4, 204–247.
12. K. Dasmalchi, H. J. Dorman and M. Kosar, (2007); Chemical Composition and In Vitro Antioxidant Evaluation of a Water-Soluble Moldavian Blam Extract, Food Sci. Technol., 40, 239-248.
13. G. K. Jayaprakasha, T. Selvi and K. K. Sakariah, (2003). Antibacterial and Antioxidant Activities of Grape (*Vitis vinifera*) Seed Extract, Food Res. Int., 36, 117-122.
14. Ramadan MF, and others. (2006); Characterization of fatty acids and bioactive compounds of kachnar (*Bauhinia purpurea* L.) seed oil. Food chemistry. 98(2):359-65.
15. Singh RS. Pandey, HS, Ghanshyam. (2006); Two new long chain compounds from *Bauhinia variegata* Linn. Ind J Chem. 45B:2151–3
16. Hirano T, Oka K, Akiba M. (1989); Antiproliferative effect of synthetic and naturally occurring flavonoids on tumour cells of human carcinoma cells lines. Res Comm Chem, Pathol Pharmacol. 64:69–78.
17. Rajkapoor B, Jayakar B, Murgesh N. (2004); Sub chronic toxicity of plant extract *Bauhinia variegata* on rats. J Ecotoxicol Env Monitor. 14:71–4.
18. Rajkapoor B, and others. (2006); Chemoprevention and cytotoxic effect of *Bauhinia variegata* against N-nitrosodiethylamine induced liver tumors and human cancer cell lines. J Ethnopharmacol. 104:407–9.
19. Lapornik, B., Prosek, M. and Wondra A. G.,(2005). Comparison of extracts prepared from plant by-products using different solvents and extraction time. Journal of Food Engineering, 71(2): 214-222.
20. Ahmed, D.; Fatima, K.; Saeed, R. (2014)**,** Analysis of phenolic and flavonoid contents, and the anti-oxidative potential and lipid peroxidation inhibitory activity of methanolic extract of *Carissa opaca* roots and its fractions in different solvents. *Antioxidants*, 3, 671–683.
21. Sharma, O.P. and Bhat, T.K. (2009) DPPH Antioxidant Assay Revisited. *Food Chemistry*, **113**, 1202-1205.
22. M.J Khubeiz (2021). Phytochemical and Antioxidant Activity of various solvent extract from *Brachychiton populneus* leaves Deployed in Syria.
مجلة جامعة دمشق للعلوم الأساسية. المجلد (37). العدد 2. 2021
23. Hseu YC, and others (2008). Antioxidant activities of *Toona sinensis* leaves extract using different antioxidant models. Food Chem. Toxicol. 46: 105-114
24. Benzie, I.F.F., Strain, J.J., (1999). Ferric reducing/antioxidant power assay: direct measure of total antioxidant activity of biological fluids and modified version for simultaneous measurement of total antioxidant power and ascorbic acid concentration. Methods Enzymol. 299, 15– 27.
25. Ruch RJ, Cheng SJ, Klaunig JE. 1(989); Prevention of cytotoxicity and inhibition of intercellular communication by antioxidant catechins isolated from Chinese green tea. Carcinogenesis 10(6):1003-8.
26. Zhang T, Mu W and Liu J. (2008), Antioxidant and free radical scavenging activities of chickpea protein hydrolysate (CPH). *Food Chem.* 106: 444-450.
27. Osawa T, NamikIi M. (1981); A novel type of antioxidant isolated from leaf wax of Eucalyptus leaves. Agr Biol Chem, 45: 735-739.
28. Minnoti G, Aust SD. (1987); An investigation into the mechanism of citrate –Fe^{2+} dependent lipid peroxidation. Free Rad Biol Med, 3: 379 – 387

29. Schauss AG, Wu X, Prior RL, ou B, Huang D, owens J, et al. (2006), Antioxidant capacity and other bioactivities of the freeze-dried Amazonian *palm berry*, Euterpe *oleraceae* mart. (acai). J Agric Food Chem; 54(22):8604–10.
30. Apak R. and others, (2013), Methods of measurement and evaluation of natural antioxidant capacity/activity. Pure Appl. Chem., Vol. 85, No. 5, pp. 957–998.
31. Jayaprakasha GK, Singh RP, Sakariah KK, (2001), Antioxidant activity of grape seed (*Vitis vinifera*) extracts on peroxidation models in vitro, Food Chem, 73, 285–90
32. Gulcin I, Huyut Z, Elmastas M, Aboul-Enein HY (2010) Radical scavenging and antioxidant activity of tannic acid. Arab J Chem 43-53.
33. Ryter SW, Tyrrell RM. (2002), The heme synthesis and degradation pathways: role in oxidant sensitivity: Heme oxygenase has both pro- and antioxidant properties Free Radic. Biol.Med. 28 (2): 289-309
34. Aqil F, Ahmad I, Mehmood Z. (2006); Antioxidant and free radical scavenging properties of twelve traditionally used Indian medicinal plants. Turk J Biol 30:177–83.
35. Ahmed S. and others, (2017), Acta Poloniae Pharmaceutica - Drug Research, Vol. 74 No. 1 pp. 173-178.
36. Wan, Lijun, Weller, Curtis L., (2006). Recent advances in extraction of nutraceuticals from plants. Trends. Food Sci. Technol. 17, 300– 312.
37. Zheng W, Wang SY, (2001), Antioxidant activity and phenolic compounds in selected herbs. Journal of Agricultural and Food Chemistry 49: 5165–5170.