INTRODUCTION

Since the beginnings of civilization, humans have used natural products to cure the series of diseases. Distinct investigations described that various pharmaceutical agents have been part of traditional healthcare for thousands of years and recently have gained a lot of importance in the field of pharmacological industries. The increasing interest in evaluating the antioxidant properties to the drug discovery process has been frequently focused on the screening of plant extracts as source of novel drug candidates that contribute to the development of many diseases [1 - 2]. In general natural oxidants or phytochemical antioxidants are secondary metabolites of plants. These metabolites have important biological and pharmacological activities, such as anti-allergic, antibiotic, anti-carcinogenic and anti-oxidative [3 - 7]. Numerous physiological and biochemical processes in the human may produce oxygen-centered free radicals and other reactive oxygen species. The over production of these components during the metabolism and other activities beyond can cause oxidative stress. This imbalance is developed in the organism is an important contributor to the pathophysiology of a variety of pathological conditions including cardiovascular dysfunction, atherosclerosis, inflammation, carcinogenesis, neurodegenerative disorders, aging, chronic degenerative diseases and inflammation [1 - 2] [8 - 9].

Antioxidants (AO) are vital substances which possess the ability to protect the body, cells and tissues from continuously threatened by the damage caused by free radical and reactive oxygen species which are produced during normal oxygen metabolism or are induced by exogenous damage [10]. The mechanisms and interferences with cellular functions are not fully understood, but one of the most important events seems to be the attenuation of the oxidative damage of a tissue can be indirectly, by enhancing natural defence of cell and/or directly by scavenging the free radical species [11]. These compounds are named in two categories which are the natural and synthetic which have some side effects [12]. Antioxidants components include carotenoids, vitamins, flavonoids and phenols that are microconstituents capable to prevent the destructive process caused by oxidative stress [13].

The phenolic compounds are secondary metabolites in plants and possess a wide and complex array of phytochemicals that exhibit several health beneficial activities such as antioxidant, anti-inflammatory, anti-atherosclerotic, antitumoral and antimicrobial [14 - 16]. Phenolic phytochemicals are known to exhibit several health beneficial activities such as compounds with an antioxidant activity, including phenolic acids and flavonoids. Flavonoids are naturally occurring in plants and are thought to have positive effects on human health and are the major group of free radical scavenging, inhibition of hydrolytic and oxidative enzymes and anti-inflammatory action [17 - 18].

More than 4000 varieties of flavonoids have been identified many of which are responsible for the attractive colours of flowers, fruit and leaves [10]. Some evidence suggests that the biological actions of these compounds are related to their antioxidant activity [19]. Natural antioxidants either in the form of raw extracts or their chemical constituents are very effective to prevent the destructive processes caused by oxidative stress [13]. The toxicity profile of medicinal plants have not been evaluated, it is generally accepted that medicines derived from plant products are safer than their synthetic counterparts [20]. Joshy et al., 2012 [21] have been described that medicinal plants which are a part of earth’s biodiversity, now a days the most important medicines are also based on more than 90 species of plants with distinguishable medicinal properties, among which one is Bougainvillea xbutiana.
An ornamental flowering plant from the genus of Bougainvillea and Nyctaginaceae family is widely distributed in Cuernavaca, Morelos and other states of Mexico, such as B. xbuttiana. Due to their pharmacological effects of this plant is widely used in traditional and modern medicine for preparation of cough, bronchitis, respiratory infection, gastritis hyperacidity, gastritis and duodenal ulcer, colic, diarrhoea, injury, diabetes, and stomach ache and it is also used as expectorant [18]. We are the first group that described the antinociceptive and anti-inflammatory activities of Bougainvillea xbuttiana [22]. The current study aimed to investigate and compare the antioxidant activity, phenolic and flavonoids contents and proliferative activity in various ethanolic extract from flowers of different colours of Bougainvillea xbuttiana.

MATERIAL AND METHODS

Chemicals, reagents and buffers

2,2’-diphenyl-1-picrylhydrazyl (DPPH), gallic acid, quercetin, Folin-ciocalteu reagent, aluminum chloride, ferric chloride, fetal calf serum (FCS), RPMI-1640 medium supplemented with 5% FCS and 1 mM/mL NH$_4$Cl were maintained as described above. For the vacuolation assay the cells were incubated with the RPMI-1640 medium supplemented with 5% FCS and 1 mM/mL NH$_4$Cl. Briefly, 0.5 ml of extract was added to 75 μl of 5% NaOH solution. After 6 min, 150 μl of a 10% AlCl$_3$-H$_2$O solution was added and the mixture was allowed to stand another 5 min. Then, 0.5 ml of 1 mol NaOH and 2.5 ml of distilled water was added. The solutions were mixed and its absorbance was determined at 510 nm using spectrophotometer. The experiments were carried out in triplicate. TFC was expressed in terms of gallic acid equivalents (mg of GA/g of extract) using the following equation based on the calibration curve: $Y = 2.2144x + 0.962$ ($R^2 = 0.9906$ where $x$ was the absorbance and $Y$ was the mgQE/g.

Determination of total flavonoid content (TFC)

Total flavonoids contents were estimated in different extracts from distinct colors of Bxb with the method described by Zhisen et al., 1999 [25]. Briefly, 0.5 ml of extract was added to 75 μl of 5% NaOH solution. After 6 min, 150 μl of a 10% AlCl$_3$-H$_2$O solution was added and the mixture was allowed to stand another 5 min. Then, 0.5 ml of 1 mol NaOH and 2.5 ml of distilled water was added. The solutions were mixed and its absorbance was determined at 510 nm using spectrophotometer. The experiments were carried out in triplicate. TFC was expressed in terms of mgQE/g of extract, using the following equation based on the calibration curve: $Y = 2.2224x + 0.982R^2 = 0.9917$ where $x$ was the absorbance and $Y$ was the mgQE/g.

Antioxidant activity

DPPH Radical Scavenging Activity

DPPH radical scavenging of Bxb extracts was measured by 2,2’- diphenyl-1-picrylhydrazyl (DPPH) assay according to the method of Milauskas et al., 2004 [26]. The stock solution was prepared by dissolving 24 mg of DPPH with 100 ml methanol and stored at 20°C until use. The working solution was obtained by diluting DPPH solution with methanol to attain an absorbance of about 0.98 ± 0.02 at 515 nm using the spectrophotometer. A 3 ml of this solution was mixed with 100 μl of the sample at various concentrations (2.9 to 940 μg). The reaction mixture was shaken well and incubated in water bath for 20 min at 37°C, and the decrease in absorbance at 515 nm was determined. The control was prepared as above without any sample. Percentage inhibition was estimated using the following equation: % inhibition = $[\frac{(A_{control} - A_{sample})}{A_{control}}] \times 100$. The IC$_{50}$ values were concentration providing 50% inhibition of DPPH radicals and were calculated from the regression equation, developed by plotting concentration of the samples versus percentage inhibition of free radicals. Here, all test analysis was run in triplicate and average values were reported, quercetin was used as positive control.

The antioxidant activity was expressed as the antioxidant activity index (AAI) calculated as follows equation: AAI = final concentration of DPPH in the control sample μg.mL$^{-1}$/IC$_{50}$ mg.mL$^{-1}$. Thus the AAI values was calculated considering the mass of DPPH and the mass of the tested compound in the reaction resulting in a constant for each compound independent of the concentration of DPPH and sample use. The AAI values were considered as poor, moderate, higher and strong antioxidant activity as described by Scherer and Godoy, 2009 [27].

Determination of ferric reducing ability of plasma (FRAP)

Antioxidant activity of different Bxb extracts colors was measuring colorimetrically using the ferric reducing ability of plasma assay described by Benzie and Striz, 1999 [28]. FRAP assay was determined based on the reduction of Fe$^{3+}$-TPTZ to a blue colored Fe$^{2+}$-TPTZ. In brief, the FRAP reagent was prepared fresh and was warmed to be used before using FRAP reagent (900 μl) was pipetted into test tubes.

A total of 30 μl of sample and 300 μl of distilled water was then added to the same test tubes, and incubated at for 30 min in the dark condition. Reading of the colored product was then taken at 595 nm. The standard curve was using iron (II) sulphate solution (0 – 2000 μM), and the reducing power were expressed as equivalents concentration (EC1). FRAP value was expressed in terms of mmol Fe$^{2+}$/g of sample using ferric chloride standard curve $Y = 1.8075x - 0.2121$, $R^2 = 0.9901$. All of measurement was taken in triplicate and the mean values were calculated.

STATISTICS

Statistical analyses was carried out using ANOVA with significance level set at $p < 0.05$. Differences among means were evaluated using a statistical program. All experiments were carried out in triplicate and repeated at least.
RESULTS

Effect of Bxb on HeLa cells.

To determine the effect of Bxb on HeLa cells that were exposed to different concentrations of Bxb extracts from flowers of distinct colors and incubated under the same conditions for 24, 48 and 72 hours. The effects of Bxb extracts were determined by measuring viability percentage and vacuoles formation.

Viability: To determine the viability percentage the HeLa cells were treated as described above. The cells were exposed during different periods of time to Bxb extracts showed cytotoxic effect that was dose and color-dependent manner (Fig. 1). The lowest viability percentage was observed in cultures of HeLa cells treated with 940 μg/ml of all color Bxb extracts for 72 hours. The viability percentage at this period was 80%, 78.6%, 78.5%, 72.8% and 70% for BxbV, BxbW, BxbR, BxbO1 and BxbO2, respectively (Fig. 1). The more high cytotoxicity percentage was observed in HeLa cells that were exposed during 72 hours to 940 μg/mL of extract for 48 hours. At this period and concentration used the percentages of vacuoles formation are 32.85%, 23.07%, 21.18% and 6.94%, for BxbR, BxbW, BxbO2 and BxbV, respectively (Fig. 3).

Antioxidant activity

DPPH radical scavenging

To determine the antiradical activities of Bxb extracts were assessed using 2,2-diphenyl-1-picrylhydrazyl radical scavenging assay. This method depends on the reduction of purple DPPH to a yellow colored diphenyl-pircyhydrazine and the remaining DPPH. The results of the assay for antioxidant activity are shown in Figure 4.
The examination of antioxidant activity of extracts from Bxb showed values varied from 8.72 to 81.03%.

The largest capacity to neutralize DPPH radicals was found for BxbO2 and BxbR, which neutralized 50% of free radicals at the concentration of 108 μg/mL and 235 μg/mL, respectively (Table 1). A moderate activity was found for BxbW extract, which neutralized 50% of free radicals at the concentration 429 μg/mL, respectively (Table 1). Due to low activity of BxbV and BxbO1 extracts, which were capable to neutralize 50% of free radicals at the concentration 750 μg/mL and 830 μg/mL, respectively (Table 1). The antioxidant activity index for extracts obtained from extracts of BxbO2 and BxbR was significantly higher when compared with those obtained for BxbW, BxbV and BxbO1 extracts (p< 0.001) (Table 1). Poor antioxidant activity when AAI values are < 0.5 observed in extracts of BxbO1 and BxbV. Moderate antioxidant activity when AAI values are between 0.5 and 1.0 obtained in the BxbW extract. Strong antioxidant activity when AAI values are between 1.0 and 2.0 showed in BxbO2 and BxbR extracts.

Ferric reducing ability of plasma values

Another method as the reduction power assay was used to confirm the Bxb extracts antioxidant activity. The reduction capacity of the ethanolic Bxb extracts is indicated in Figure 5. The FRAP values were 1.95 mmolFe$^{2+}$/g, 2.13 mmolFe$^{2+}$/g, 2.17 mmolFe$^{2+}$/g, and 2.28 mmolFe$^{2+}$/g for BxbO1, BxbV, BxbW and BxbO2 and BxbR, respectively (Fig. 5). The results obtained in this study are confirmed the antioxidant activity for these Bxb extracts and it is clear that existence of discrete differences between the extracts in a capacity of reducing power.

Total phenolic content

To determine the total phenolic contents in the examined ethanolic extracts using the Folin-ciocalteu’s reagent. The TPC was expressed in terms of galic acid equivalents (mg of GA/g of extract) using the following equation based on the calibration curve: Y = 2.2144x + 0.962 $R^2 = 0.9906$ where x was the absorbance and Y was the mgQE/g. The values obtained for the concentrations of total phenolic content are expressed as mg of GA/g of extract (Fig. 7).

The highest phenolic content was measured in BxbO2 extract with values of 28.89 mgGA/g and the lowest TPC values was observed in BxbO1 extract with 14.68 mgGA/g (Fig. 6). For the extracts from BxbR, BxbW and BxbV the phenolic contents were 23.67, 22.16 and 20.71 mgGA/g, respectively (Fig. 6).
In the present study, the ethanolic extract of B. xbuttiana was assessed for its effects on HeLa cells and antioxidant activity. Several evaluation protocols have been used to determine the antioxidant activity of the extracts and beverages based on different principles. The IC50 values ranged from 108 to 830 μg/mL for DPPH radical scavenging. The redox potential of the Fe3+/ferrocyanide complex to the ferrous form. Therefore, Fe3+ can be quantifying by absorbance measurement at 700 nm. The higher the absorbance of the reaction mixture indicates an increase in reducing power. The presence of reductants in Bxb extracts caused the reduction of the Fe3+ into Fe2+. Reducing power was found to be in order BxbO2 > BxbR > BxbO1 > BxbV > BxbW > BxbO1. It has been reported that the reducing power of substances is probably because of their hydrogen-donating ability.

The BxbO2, BxbR, and BxbW extracts might therefore, contain high amount of reductors than that observed in BxbO1 and BxbW extracts. Hence, ethanolic extracts of B.xbuttiana may act as electron donors and could react with free radicals to convert them into more stable products and them terminate the free radical chain reactions. Phenolic compounds are considered secondary metabolites and these phytochemical compounds derived from phenylalanine and tyrosine occur ubiquitously in plants and are diversified. The TPC assay is another that is commonly used in conjunction with either of both of the DPPH and FRAP assays, again presumably with the aim of increasing the antioxidant activity of the B. xbuttiana extract. Phenolic compounds may be responsible for the possible antioxidant activity of many plants because their hydroxyl groups confer scavenging ability. The results presented in this study indicate that high antioxidant activity is associated with a high phenolics content, a finding reported previously many times. The results presented in this study indicate that high antioxidant activity is associated with a high phenolics content, a finding reported previously many times.

To assess the toxicity of the extracts towards HeLa cells viability and vacuoles formation. In the literature are described that plant extracts contain a high concentrations of bioactive compounds and also several compounds which show cytotoxic activity. In this study the exposition of HeLa cells to B. xbuttiana extracts with 940 μg/mL caused lysis and morphological changes. We also observed that B. xbuttiana extracts mediated vacuole formation in HeLa cells and in a concentration-dependent manner. In this study we observed that the presence of large vacuoles and membrane projections were not damaging. The cells presented plasma membrane integrity and well preserved mitochondria profiles, characteristic of good cell viability.
manner color-dependent. Our results suggested that phenolic and flavonoids may be the major contributors for the antioxidant activity. In the present study analysis of free radical scavenging activity and total phenolic and flavonoids content from the B. xibutiana flowers can be the potent source of natural antioxidants.

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Competing Interest
The authors declare that they have no competing interests

REFERENCES