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In vitro antioxidant activity of *Bixa orellana* (Annatto) Seed Extract

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ABSTRACT

The seeds of *Bixa orellana* (Annatto, family *Bixaceae*), have been used in food coloring for over 50 years. With the aim of introducing its extracts as pharmaceutical colorant, there is the need to investigate the biological and pharmacological activities of the extract. This study was designed to develop extraction protocols for annatto coloring fraction with potential for pharmaceutical application and evaluate the antioxidant activity of the extracts *in vitro*. Powdered seed material was extracted using acid-base protocols and the crystals obtained were washed with deionized water, oven-dried for about 12 hours at 45 °C and stored in air-tight containers. The *in vitro* antioxidant activity was tested via 2,2-diphenyl-1- picrylhydrazyl (DPPH) radical scavenging activity and iron (III) oxide reducing power using ascorbic acid (vitamin C) as a reference standard. The free radical scavenging activity of *annatto* extract ranged from 5.5 % to 48.9 % relative to ascorbic acid (2.9 % to 41.5 %) at respective concentrations between 0.25 and 2.5 µg/ml. Similarly, iron (III) oxide reducing power shows good linear concentration-dependent relation (R^2 = 0.9986) comparable with ascorbic acid (R^2 = 0.9934). Results generally indicated that *Bixa orellana* seed extract is a potential source of antioxidants of natural origin.

INTRODUCTION

An antioxidant is a substance which, at low concentrations, significantly delays or prevents oxidation of cell components such as proteins, lipids, carbohydrates and deoxyribonucleic acid (DNA). Free radicals are the major cause of chronic and degenerative diseases such as coronary heart diseases, inflammatory stroke, diabetes and cancer (Scalbert *et al.,* 2005). Free radicals are fundamentals to any biochemical process and represent an essential part of aerobic life and metabolism (Tiwari, 2001). The most common reactive oxygen species (ROS) include superoxide anion, hydrogen peroxide (H_2O_2), peroxyl (ROO^-) radicals, and reactive hydroxyl (HO^-) radicals. The nitrogen derived free radicals are nitric oxide (NO_2) and peroxynitrite anion $(ONOO)^-$. Reactive oxygen species (ROS) are continuously being generated inside of human body while the generated ROS are being detoxified by the antioxidants present in the body. However, over production of ROS or inadequate antioxidant defense can produce oxidative damage to various biomolecules including proteins, lipids, lipoproteins and DNA. Due to their redox properties, antioxidants act as reducing agents, hydrogen donors, singlet oxygen quenchers and chelating metal(Tung *et al.,* 2009; Lauro and Francis, 2000). Although several synthetic antioxidants such as butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) are commercially available, their toxicity has always been a concern and strong restrictions have been placed on their application in food and pharmaceuticals. There is therefore a need for more effective, less toxic and cost effective antioxidants. Recently, there has been an upsurge of interest in the therapeutic potentials of plant-derived antioxidants in reducing free radical-induced tissue injury and the current trend is to substitute synthetic with naturally occurring antioxidants (Barlow, 1990). Several biologically active compounds of plant origin (phytochemicals) have been found to possess antioxidant, free-radical scavenging activity and many are being applied therapeutically for free radical associated disorders (Lee et al, 2000). Natural antioxidants are found in various parts of plants such as leaves, fruits, seeds, roots and bark (Mathew and Abraham, 2006; Chanda and Dave, 2009). Antioxidants especially phenolics and flavonoids from tea, wine, fruits, vegetables and spices are already being exploited commercially either as antioxidant additives or as nutritional supplements (Schula, 1990).

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Recently, more medicinal plants are being investigated for potent antioxidant activities due to the promise of no or relatively lower side effects and economic viability (Auddy *et al.*,2003). One such plant is *Bixa orellana*, commonly known as annatto. Annatto belongs to the *Bixaceae* family and is a profusely fruiting shrub, reaching 6 - 20 feet tall and could thrive for up to 50 years. The prickly heart shaped pods contain small reddish-orange seeds, each pod containing approximately 50 seeds. It is found mainly in the forest ecosystem of the Brazilian humid tropics but now cultivated in the tropics throughout the world (Mello and Lima, 1990). It is extensively used as colourant in food and cosmetic industry, condiment and as a remedy in traditional medicine. The objective of the present study was to develop extraction protocols for annatto coloring fraction and **e**valuate the antioxidant activity of *the extract* using two *in vitro* methods, DPPH and iron (III) oxide reducing power assay.

MATERIALS AND METHODS

Plant material

Fresh plant materials were collected in batches from a single source (farmer) in the parish of Manchester, Jamaica, West Indies during the peak fruiting season between August and January each year. Plant samples were identified with Rural Agricultural Development Authority (RADA) and confirmed by a botanist at the Botany Department of the University of the West Indies Mona Campus. Each batch of plant material was air-dried on the laboratory bench for about 2 weeks; the seeds were freed from the pod and dried in an oven at 45 °C for 7 days

Preparation of plant extract

The plant extracts were prepared using the modified method (Ruiz and Wood, 1971; Batista, 1994; Pimentel, 1995). Briefly, three 250-g of annatto seed were soaked in 900 ml of 1 M KOH and stirred for 30 minutes at 70 °C on a magnetic stirrer in a Labconco protector laboratory hood (Labconco Corporation, USA). The mixture was filtered using cheesecloth and the residue was washed with fresh 900 ml of 1 M KOH and stirred for 30 minutes and then filtered. A fresh 700 ml of 1 M KOH was used to wash the residue while stirring for 15 minutes and the mixture was filtered. The filtrates were combined and 3 M HCl was used to acidified the mixture and precipitate crystals of extract. The precipitate was allowed to settle overnight and the supernatant was decanted and washed repeatedly with distilled water. The wet mass was dried in the oven at 45 °C for about 72 hours. The lumps were pulverized in a mortar with pestle and the resulting powder was stored in airtight container until used for further studies.

UV-VIS Spectrophotometric analysis of extract

Preparation of 0.02 %$^{w}/_{v}$ stock solution of annatto extract

Annatto extract (20 mg) were weighed into a small beaker using OHAUS digital balance (OHAUS Corp., U.S.A). A small amount of ethanol was added to the beaker. The mixture was then stirred with a glass rod to dissolve the extract. The solution was carefully transferred into a 100 ml volumetric flask. Ethanol was used to rinse the beaker and transferred to the flask. The volume in the flask was made up to 100 ml mark with ethanol. The flask was then covered and placed in a hot water bath at 50 ^{0}C with periodic shaking until all the extract was completely dissolved. The solution was allowed to cool at room temperature and stored for further use. Serial dilutions of 0.0001 % to 0.001 % from 0.1 % w/v annatto extract was prepared and, using ethanol as blank, the absorbance were read on UV-Vis Diode array spectrophotometer (Agilent, Santa Clara, USA) and the procedure was replicated at least thrice. A graph of absorbance against wavelength was plotted and lambda maximum (λ_m) was determined. This procedure was repeated with extraction in 0.1 M of potassium hydroxide.

Thin Layer Chromatography of annatto extract

TLC plate was activated for one hour at 110 ^{0}C. Ten (10 µL) of 5% solution of the annatto extract in 95% ethanol was spotted on the plate and 10 µL was added to the plate. The plate was allowed to dry and develop in a mixture of n-butanol, methylethylketone and 10% aqueous ammonia (3:2:2 by volume) until the solvent front had ascended to 10cm. It was allowed to dry and the retention factors of the color spots were determined.

Antioxidant assay

Free radical scavenging activity of 2,2-diphenyl-1-picrylhydrazyl (DPPH)

The antioxidant potential of extract was determined via scavenging activity of stable 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical as described by (Sahdu *et al.*,2003). DPPH, a stable free radical at room temperature, produces a violet color in methanol. When the free radical reacts with an antioxidant, its free radical property is lost due to chain breakage and its color changes to light yellow (Badarinath *et al.*,2010). Extent of color change in presence of annatto extract was monitored by UV absorption at its absorption maxima in methanol of 517 nm. The scavenging reaction between (DPPH) and an antioxidant (H-A) can be represented as (Eq. 1):

$$DPPH + (H-A) \rightarrow DPPH-H + A \quad (1)$$

(Purple) (Yellow)

Antioxidants react with DPPH, which is a stable free radical and is reduced to the DPPH-H and, as a result, the absorbance is decreased. The degree of discoloration indicates the scavenging potential of the antioxidant compounds or extracts in terms of hydrogen donating ability. Stock solution of sample were prepared by dissolving 1 mg of dried annatto powder in 10 ml of methanol to give a concentration of 0.1 mg/ml. Dilutions of the stock solutions were made at concentrations of 0.25, 0.5, 0.75, 1.0,1.25, 1.5,1.75, 2.0, 2.25, and 2.5 µg/ml. One milligram of ascorbic acid was dissolved in methanol and suitably diluted to give concentrations of 0.25, 05,075, 1.0, 1.25, 1.5, 1.75, 2.0, 2.25, and 2.5 µg/ml. About 13.1 mg of DPPH was dissolved in 100 ml

methanol and the solution was protected from light by wrapping the flask with aluminum foil. Various concentrations of annatto extract (3.0 ml) were mixed with (1.0 ml) of 0.1 mM DPPH solution and the mixture was shaken vigorously and allowed to stand in the dark at room temperature for 30 minutes. The absorbance was measured at 517 nm on UV-Vis Diode array spectrophotometer (Agilent, Santa Clara, USA) along with the absorbance of ascorbic acid reference solution. The free radical scavenging activity was calculated. The absorbance of the negative control (blank; methanol instead of the 1 ml DPPH and 3 ml methanol solution) was also read along with test and standard, each in triplicate. The percentage inhibition (ability to scavenge the DPPH radical) by the samples was calculated with reference to control absorbance using the equation:

$$DPPH\ Scavenged\ (\%) = \frac{A_{control} - A_{test}}{A_{control}}\ X\ 100 \qquad (2)$$

where $A_{control}$ is the absorbance of the control and A_{test} is the absorbance of the extract or reference standard. The percentage of DPPH radical scavenging activity was plotted against the sample concentration.

Reducing power assay

The reducing power of annatto extract was determined according to the method previously described by Oyaizu (1986) with some modifications. Substances which have reduction potential react with potassium ferricyanide (Fe^{3+}) to form potassium ferrocyanide (Fe^{2+}), which then reacts with ferric chloride to form ferric-ferrous complex that has an absorption maximum at 700 nm. The equation defining the reaction is:

$$FeCl_{3(aq)} + K_4\,[Fe(CN)_{6\,(aq)} \rightarrow KFe[Fe(CN)_6]_{(aq)} + 3KCl_{(aq)}$$

Various concentrations (5- 37.5 µg /ml) of the extracts in methanol were mixed with 2.5 ml of phosphate buffer (pH 6.6) and 2.5 ml of potassium ferricyanide. This mixture was kept in water bath at 50 ^{o}C for 20 minutes, the reaction was terminated by adding 2.5 mL aliquots of tricholoroacetic acid and the mixture was centrifuged at 3000 rpm for 10 minutes. The clear supernatant (2.5 mL) solution was mixed with 2.5 ml of distilled water and 0.5 ml of freshly prepared ferric chloride solution and the absorbance was measured at 700 nm using phosphate buffer and distilled water as blank. Ascorbic acid at various concentrations (5- 37.5 µg /ml) was also analyzed concurrently. The reducing power was estimated as increase in absorbance of the reaction mixture.

RESULTS AND DISCUSSION

Phytochemical screening

Annatto pigments produced an average yield of 11.9 ± 2.6% of the crude extract. Preliminary phytochemical screening of the *Bixa orellana* (Annatto) seed extract revealed the presence of different compounds as presented in Table 1. The major components for which it tested positive were saponins, steroids and terpenoids. The fatty δ-T3 and γ-T3, which have been reported as components of annatto extracts (Pierpaoli *et al.*,2013), were not found in the extract obtained using the extraction protocol employed in this study. Raga *et al.*,(2011) reported a bioactive sesquiterpine from annatto.

Table. 1: Preliminary phytochemical screening of *Bixa orellana* (Annatto) seed extract.

S/N	Phytochemical test for compounds	Result
1	Saponins	++
2	Tannins	+
3	Terpernoind (Salkowski test)	++
4	Steroid	++
5	Glycoside	+
6	Carbohydrate	+
7	Falconoid	+
8	Resins	-
9	Alkaloids	-

(+) = Present; (-) = absent

UV-VIS spectrophotometric scan of absorption wavelength (λ) of annatto seed crude extract in potassium hydroxide and ethanol

Scanning of annatto seed crude extract in potassium hydroxide and ethanol was done in a Diode array spectrophotometer within the visible range (800 – 400 nm) gives a highest peaks (λ_{max}) at 453nm in potassium hydroxide and 458 nm in ethanol (Figs. 1 & 2).

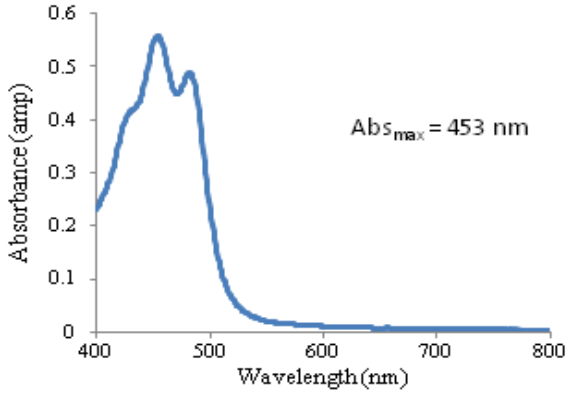

Fig. 1: Absorbance vs. wavelenght of ananatto seed extract in KOH.

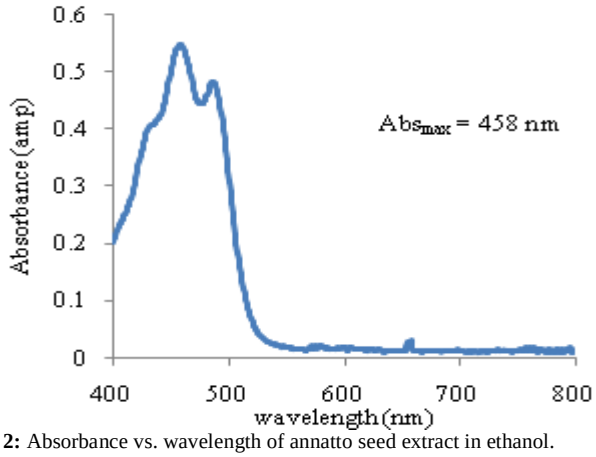

Fig. 2: Absorbance vs. wavelength of annatto seed extract in ethanol.

The standard curve of the crude extract was then prepared within the concentration range 0.001 %/w/v to 0.0001 %/w/v, which gives a straight line with regression coefficient (r^2) of 0.9864 in potassium hydroxide and 0.9857 in ethanol respectively (Fig. 3 & 4).

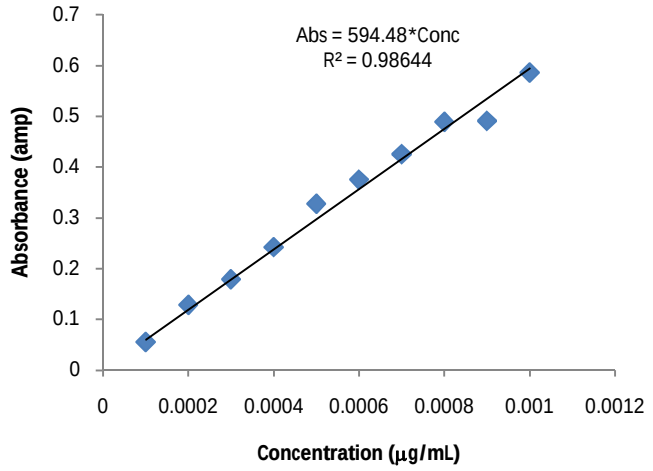

Fig. 3: Absorbance versus concentration (Beer's plot) for annatto extract in potassium hydroxide. solution.

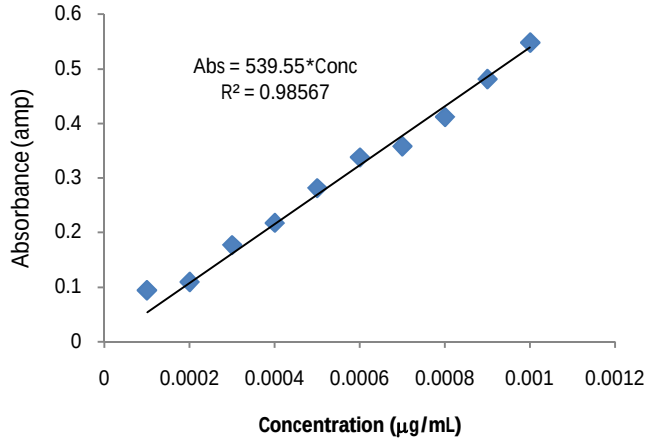

Fig. 4: Absorbance versus concentration (Beer's plot) for annatto seed extract in ethanol.

Thin Layer Chromatography of annatto seed crude extract

The TLC analysis of annatto crude extract shows presence of bixin and norbixin with retention factor (R_f) values of 0.6 and 0.4 respectively. A typical TLC chromatograph is shown in Fig. 5. The spots for bixin and norbixin from annatto extract are quite similar to those of the reference substances.

DPPH Free radical scavenging activity

Antioxidant DPPH radical scavenging ability is shown in Fig. 6 as decrease in absorbance with concentration of anxtioxidant at 517 nm. In the free radical scavenging activity, 2,2-diphenyl-1-hyrazyl (DPPH), a stable radical at room temperature accepts an electron or hydrogen radical to become stable 2,2-diphenyl-1-hyrazine (DPPH-H) molecule (Eq. 1). The decrease in absorbance of DPPH radical is caused by antioxidants reaction with the radical through donation of hydrogen (H^+). This was manifested visually as color change from violet to yellow. Annatto extract exhibited a comparable antioxidant activity ($p < 0.5$) with ascorbic acid as the standard at various concentrations (0.25, 0.5, 0.75, 1.0, 1.25, 1.5, 1.75, 2.0, 2.25 and 2.5 µg/ml).

Results showed that the annatto extract has a significant free radical scavenging activity on DPPH owning to its hydrogen donating ability. The comparison of the antioxidant activity of annatto extract and ascorbic acid as loss of light absorption and DPPH scavenging activity is shown in Figs. 6 and 7. The percentage inhibition activity of ascorbic acid and annatto extract at concentration range of 0.5- 2.5 µg/ml showed a moderate anti-radical activity against DPPH radical with percentage inhibition of about 5.5 % to 48.9% and 2.9 % to 41.5 % respectively.

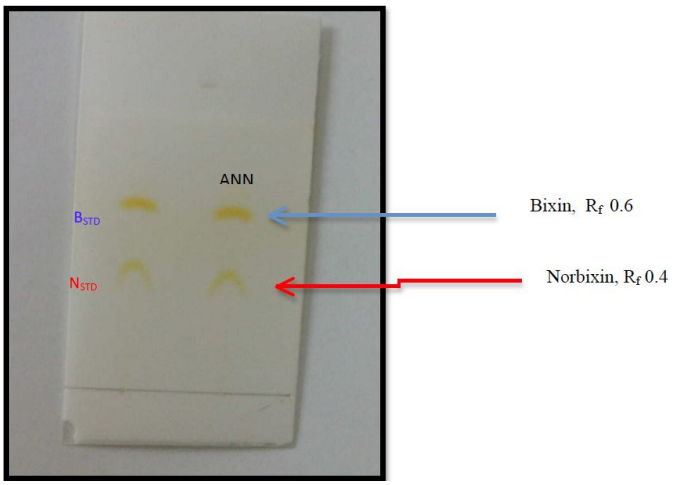

Fig. 5: TLC of Annatto crude extract; B_{STD} = Bixin standard; N_{STD} = Norbixin standard.

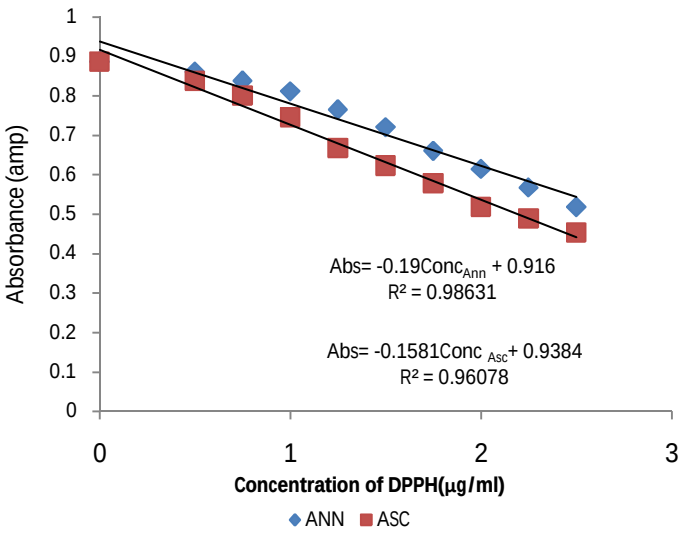

Fig. 6: Reduction of oxidizing ability of DPPH by Annatto seed extract (as function of decrease in absorbance).

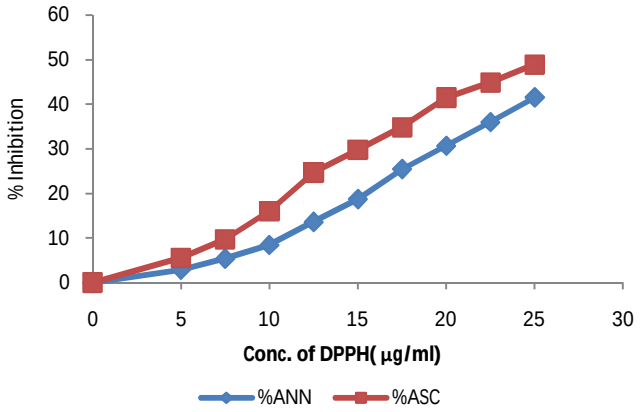

Fig. 7: % Inhibition of oxidant activity of DPPH by annatto seed extract and ascorbic acid.

Reducing power assay

The reducing power of both ascorbic acid and annatto extract is shown in the Fig. 8. The reducing power of annatto extract as increase in absorbance at 700 nm correlates well with increasing concentrations. The plot shows that the annatto extract exhibit maximum a bsorbance of 0.863 at a concentration of 37.5 µg/ml and the minimum absorbance of 0.553 at a concentration of 5 µg/ml whereas ascorbic acid maximum absorbance of 0.8857 at a concentration of 37.5 µg/ml and the minimum absorbance of 0.7373 at a concentration of 5 µg/ml (Fig. 8). The reducing power shows good linear relation (Fig. 9) in both ascorbic acid (R^2 = 0.9934) as well as annatto extract (R^2 = 0.9986). The amount of Fe^{3+} remaining in 20 min at 50 ^oC shows good linear relation in both ascorbic acid (R^2 = 0.9885) as well as annatto extract (R^2 = 0.9908). Annatto has been reported to contain tocotrienols (T3), a less prominent isomer of vitamin E which has been reported to possess in vitro and in vivo anti-cancer activity in mutagenic rodents and this was recently confirmed via oxidative effect, senescent-like growth inhibition and immune modulation effect as well as in tumoral mammary glands of transgenic mice expression of HER-2/neu (Pierpaoli *et al.*,2013). Anti-apoptotic effect of δ-T3 and γ-T3 components of annatto have been established in vitro in human and mice tumor cell lines. The acid-base protocol for the extraction of annatto seeds may be responsible for the absence of δ-T3 and γ-T3 in the extracts obtained in this study. The protocol therefore appears to be suitable for producing annatto extract which does not contain some fatty constituents of annatto seeds.

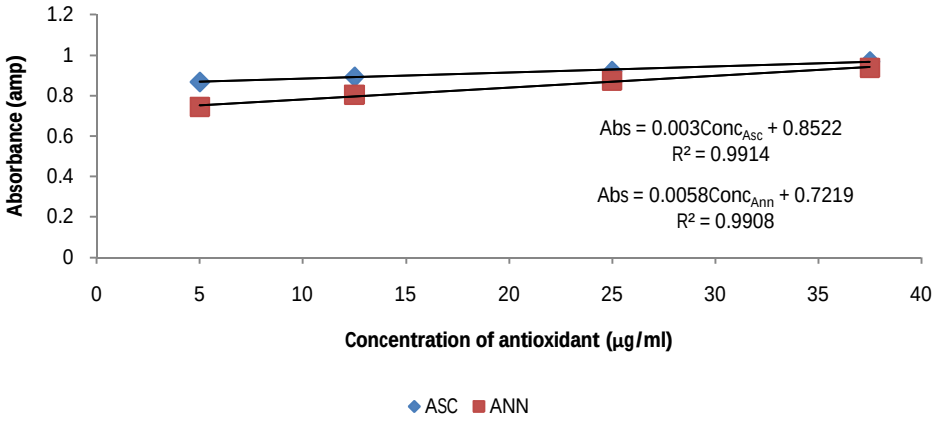

Fig. 8: Iron (III) oxide reducing power of annatto seed extract as function of increased absorbance.

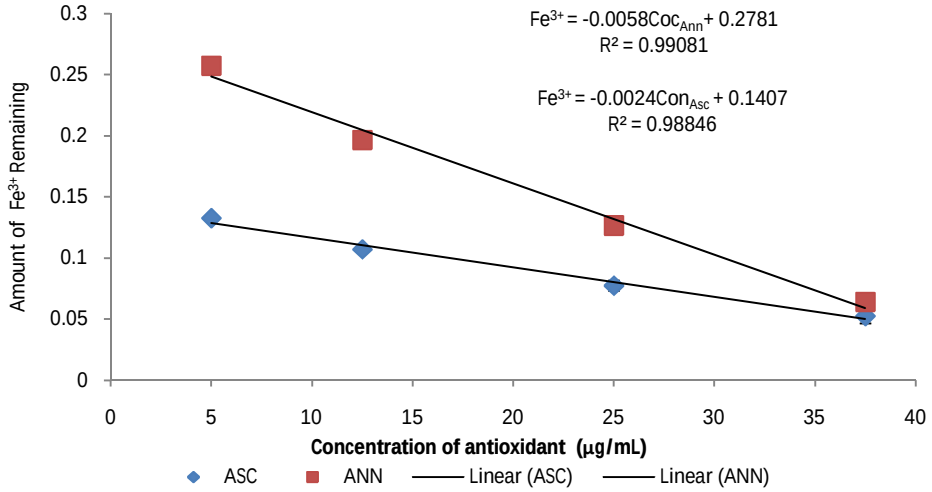

Fig. 9: Amount of Fe^{3+} remaining vs. concentration of antioxidant at 50 ^oC for 20 minutes exposure.

CONCLUSIONS

Results of *in vitro* antioxidant assay of *Bixa orellana* (Annatto) seed extract using DPPH and Ferric iron reducing power models show antioxidant activity at low concentrations and is comparable with those of ascorbic acid. The activity may be due to the presence of tannins and flavonoid found in the preliminary analysis. Annatto extract holds some promise for use as a plant-derived antioxidant for medicinal application. Its suitability as pharmaceutical colorant however requires careful evaluation as this may be affected by its biological activity as antioxidant. Development of suitable extraction protocol that eliminates much of the tocotrienols and other potent bioactive principles may produced annatto extracts suitable for use as pharmaceutical colorant.

REFERENCES

Auddy B, Ferrira M, Blasima F, Lafon L, Arredondo F. Screening of antioxidant activity three indian plants traditionally used for the management of neurodegenerative diseases. J Ethnopharmacol 2003;73: 429-436.

Badarinath AV, Madhu C, Ramkanth SA. Review on *In-vitro* antioxidants methods: Comparison, Correlations and Considerations. Intl J Pharm Tech Res, 2010; 2: 1276-1285.

Barlow SM. 1990. Toxicolgical aspect of antioxidants used as food additives: In Hudson BJF (ed.) Food antioxidants. London, UK: Elsevier. 253 - 307

Batista CLLC. 1994. Production and evaluation of the stability of water-soluble annatto Dissertation (Master of Science and Food Technology) University of Federal Vicosa 71.

Chanda E, Dave R. *In vitro* mo*del* for antioxidant aactivity evaluationand some medicinal plants possessing antioxidant properties: An overveiw. Afr J Microbiol, 2009; 3: 981-996.

Lauro GJ, Franscis FJ. 2000. Natural Food Colurants. New York, USA: Marcel Dekker 1- 10

Lee YM, Kim H, Hong EK, Kang BH, Kim SJ. Water extract of 1:1 mixture of phellodendron cortex and Aralia cortex has inhibitory effects on oxidative stress in kidney of diabetic rats. J Ethnopharmacol, 2000;73: 429-436.

Mathew S, Abraham TE. *In vitro* antioxidant activity and scavenging effects of *Cinnamomu verum* leaf extract assayed by different methodologies. J Food Chem Toxicol, 2006; 44: 198- 206.

Mello AAA, Lima LCF. 1990. Situation of culture in Brazil annatto and perspectives. In: Saint Joseph AR, Reboucas TNH, eds. The culture of annatto in Brazil. Bahia: State University of Southwest Bahia. Pp. 88-90

Oyaizu M. studies on product of browning reaction prepared from glucose amine. Japan J Food Chem, 1986; 44: 307- 315.

Pierpaoli E, Viola V, Baruccal A, Orlando F, Galli F, Provincalli M. 2013. Effect of annatto-tocotrienols supplementation on the development of mammary tumors in HER-2/neu transgenic mice. Carcinogenesis, 2013; 34: 1352-1360.

Pimentel, FA. 1995. Evaluation of methods for obtaining and stability of pigments of annatto seeds. Dissertation (Master of Science and Food Technology) University of Federal Vicosa 132.

Raga DD, Espiritu RA, Shen C, Ragasa CY. A bioactive sesquiterpene from *Bixa orellana*. J Natural Med, 2011; 65: 206 – 211.

Ruiz HV, Wood GP. The separation of annatto pigments by sephadex. Tropical Sci 1971; 13: 211-214

Sahdu SK, Okuyama E, Fujimoto H, Ishibashi M. Separation of Leucas aspera, a medicainal plant of Bangladesh,giuded by prostaglandin inhibitory and antioxidant activities. Chem Pharm Bull, 2003; 51: 595 - 598.

Scalbert A, Manach C, Remecsy C, Morand, C. Dietary polyphenols and the prevention of diseases. Crit. Rev. Food Sci. and Nutri.2005; *45*: 287-306.

Schular P. 1990. Natural antioxidants exploited commercially, In Hudson B.J.F, ed. Food Antioxidants. *London:* Elsevier. Pp. 99 - 170

Tiwari A. Imbalance in antioxidant defense and human diseases: Multiple approach of natural antioxidants therapy. Curr. Sci, 2001; 81: 1179 -1187.

Tung YT, Wub JH, Huang CY, Chang ST. Antioxidant activites and Phytochemical Characteristic of Extract of Acacia Confuse Bark. J. of Biosource Technol. 2009;100: 509-514.

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