

IN VITRO ANTIOXIDANT ACTIVITY OF N-BUTANOL EXTRACT OF CURCUMA LONGA AND ITS POTENTIAL TO PROTECT ERYTHROCYTES MEMBRANE AGAINST OSMOTIC-INDUCED HAEMOLYSIS

¹Bulus, T., ¹David, S.I., ²Bilbis, L.S., ³Babando, A.

¹.Department of Biochemistry, Kaduna State University, Kaduna.

².Department of Biochemistry, Usman Danfodiyo University, Sokoto.

³. Department of Chemistry, Ahmadu Bello University, Zaria.

*Corresponding author's Email: tbulus@yahoo.com Phone: +2348051282789

ABSTRACT

Antioxidant activity of *Curcuma longa* rhizomes (Turmeric) has been widely investigated in Asia. This work evaluated the antioxidant potential of Nigerian variety of *Curcuma longa* with the main hypothesis that variation in plant phytochemicals in type and/or amount is a function of geographical location/soil type. The experimental models used for the antioxidant studies include: iron (III) reducing capacity, total antioxidant capacity, 1,1-diphenyl-2-picrylhydrazyl (DPPH) scavenging assay and *in vitro* inhibition of osmotic-induced haemolysis. The extract was found to reduce osmotic pressure-induced haemolysis of mammalian erythrocytes in a dose-dependent manner and also gave high total antioxidant capacity. At 1000 µg/mL concentration, its DPPH radical scavenging ability compared favourably well with those of the standard compounds - ascorbic and garlic acids. The implications of these findings in the usage of *Curcuma longa* as food additives and ethnomedicinal practice in Nigeria are discussed.

Keywords: *Curcuma longa*, *In vitro*, Antioxidant potential, mammalian erythrocytes.

INTRODUCTION

Oxidative stress occurs in cells and tissues of organisms wherever oxidation and reduction processes abound. This has been attributed to production of reactive oxygen species (ROS) beyond the ability of the system to neutralize them (Halliwell, 1996). Oxidative stress has been incriminated in the pathogenesis of several disease conditions such as cancer, cardiovascular, liver injury, myocardial infarction, Alzheimer's disease, Parkinsonism as well as in the ageing process (Grzegorzczak et al., 2007). Continuous exposure to synthetic compounds, which is characteristic of orthodox medicine, could lead to increase in the amount of free radicals in the body and hence cause different tissue and/or biochemical lesions. This, among other factors of toxicity and cost, has caused significant increase in research interests towards medicinal plant research in recent years.

A lot of scientific researches has focused on screening plants and herbs for their potential antioxidant properties (Matterlini, 2000) because active principles from these plants are thought to play important roles in preventing diseases caused by oxidative stress. Again, since about 80% of the world's population is thought to rely on traditional medicines, which is mainly the use of plant extracts, for some aspect of their primary health care needs (WHO, 1993) and that traditional medicine may be the surest means to

achieving total health care coverage in the world (WIPO, 1998), it seems justifiable that the search for antioxidants potentials of plants should continue.

Curcuma longa which is also known as 'turmeric', is a herbaceous perennial plant belonging to the ginger family, Zingiberaceae (Figures 1). It is extensively cultivated in China, India and other countries with tropical climate (Paramasivam et al., 2009) including Nigeria where it is commonly called 'Zabibi' or 'Magina' among Hausa speaking Nigerians. It is extensively used as spice, food preservative and colouring material in India, China and South-East Asia (Neeta et al., 2007). It has been used in traditional medicine as a household remedy for various diseases, including biliary disorders, anorexia, cough, diabetic wounds, hepatic disorders, rheumatism and sinusitis (Araujo and Leon, 2001). Literature review revealed that curcumin (diferuloylmethane), the main yellow bioactive component of turmeric, possesses wide spectrum of biological properties including anti-inflammatory (Matterlini et al., 2000; Jureka, 2009), antioxidant (Matterlini et al., 2000), anticarcinogenic and antimutagenic (Ammon and Wahl, 1991; Regunathan and Panneerselvam, 2007), antiulcer (Natchaya et al., 2004), and antiprotozoal (Mazumdar et al., 1995; Reddy et al., 2005), hypotensive and hypocholesteremic activities (Chattopadhyay et al., 2004).

Of all these antioxidant studies, almost all were conducted with curcumin/curcumin-rich ethanolic extracts obtained from *Curcuma longa* cultivated in Asian countries with virtually few studies on extracts from Nigerian cultivars. This is important when one considers that variation in phytochemicals such as phenols and flavonoids has been established among same plant cultivated in different geographical locations (Ndhlala et al., 2014). There is therefore, the need to investigate the antioxidant potentials of Turmeric cultivated in Nigeria where there is paucity of literature on the antioxidant potentials of this rhizome and where a lot of emphasis has been given to its role in dyeing of various local fabrics. This paper investigated the antioxidant properties of n-butanol extract of Nigerian variety of *Curcuma longa* and determined the ability of the extract to protect, *in vitro*, mammalian erythrocytes against lysis induced by osmotic pressure.

In Nigeria, the plant is locally cultivated in the Northern parts of the country and the colour pigment is used mainly in traditional dyeing industry.



Fig. 1. *Curcuma longa* Rhizomes (A) and shoot (B)

MATERIALS AND METHODS

Plant material

Rhizomes of turmeric (*Curcuma longa*) were obtained from Kaura village in Kaura Local Government Area of Kaduna State, Nigeria and taken to the herbarium at the Department of Biological Sciences, Ahmadu Bello University, Zaria for identification. The specimen voucher number of the plant is 1071.

Blood Sample

Fresh blood samples were obtained from healthy sheep, cattle and goat animal after slaughter in Kakuri Abattoir, Kaduna State, Nigeria.

Chemicals

DPPH (1,1- diphenyl- 2- picrylhydrazyl), Trichloroacetic acid (TCA), Ammonium Molybdate, Ascorbic acid, Gallic acid were purchased from Sigma Co. U.S.A. All other chemicals used were of analytical grade.

METHODS

Extraction

Fresh rhizomes (200g) of turmeric was pounded to puree and extracted with 200ml of petroleum ether at 40°C using soxhlet apparatus, after which residual puree was re-extracted with 200ml of n-butanol at 60°C. The extract was concentrated by removing the solvent with the aid of a rotary evaporator (NYC R- 205D) and subsequently dried in a water bath. The crude extract, a brownish gummy exudates, was then stored in the refrigerator until required for the experiment.

Preparation of erythrocyte suspension

Fresh blood samples from sheep, cattle and goat were collected in separate EDTA bottles from Kakuri abattoir (in Kaduna metropolis, Nigeria). After swirling to mix, 2ml of each blood sample was transferred into centrifuge tubes using a sterile glass pipette. Five (5) ml of phosphate buffer saline (PBS), pH 7.4, was thereafter added to each tube and centrifuged at 2000 r.p.m. for 10 min. The supernatant was discarded and 5 ml of the buffer (PBS) was added to the washed erythrocytes pellet. This process was repeated twice (the supernatant for the third washing is usually free of haemoglobin). The washed cells (RBCs) were finally made ready for use by suspension in 4 ml of PBS buffer. The cell density of the suspension was determined using haemocytometer.

In Vitro Antioxidant Activity of N-Butanol Extract Of Curcuma Longa and Its Potential to Protect Erythrocytes Membrane Against Osmotic-Induced Haemolysis

Fragility Test

The method described by Tizard et al.(1978) was adopted. Briefly, to each set of test tubes containing 5 ml of different concentrations (0.00- 0.85%) of saline, 10^5 washed RBCs was added. The mixture was incubated in water bath at 37°C for 30 minutes, centrifuged at 2000 r.p.m. for 10 minutes, the supernatant fluid removed and its haemoglobin content estimated spectrophotometrically at 540 nm against 0.85% buffered saline (the blank). Appropriate positive and negative controls were employed at the same time. Percentage haemolysis (mean \pm standard deviation) was calculated as shown below:

$$\% \text{ Haemolysis} = \frac{[\text{Absorbance of individual fluid} - \text{Absorbance with 100\% haemolysis}]}{\text{Absorbance with 100\% haemolysis}} \times 100$$

Study on the effect of aqueous butanol extract of *C. longa* on haemolysis induced by osmotic-pressure

From the fragility curve, the effective concentration that will cause 50% haemolysis (EC_{50}) was extrapolated. This EC_{50} was used to incubate the washed RBCs of each animal with varying concentrations of aqueous butanol extract of *C. longa*. The test tubes were set as follows:

- positive controls: tubes contained 10^5 washed RBCs in distilled water.
- Negative controls: tubes contained 10^5 washed RBCs in phosphate buffer, pH 7.4.
- Extract-treated groups: tubes contained 1 mls of the extract solution with 10^5 each of washed RBCs. The total volume was made up to 5ml by adding phosphate buffer, pH 7.4, to give different active concentrations of 100, 200, 400, 800 and 1600 $\mu\text{g/mL}$. The extract solutions were prepared with the same buffer, and each experiment was carried out in triplicate.

The incubation was done at 37°C for 60mins at the end of which the tubes were centrifuged at 2000 r.p.m. for 10 mins and the haemoglobin content of the supernatant was determined spectrophotometrically (540 nm) as described above.

The ameliorative potential of the extract was expressed as Percent retardation (mean \pm standard deviation) and was calculated using the formula below:

$$\% \text{ Retardation} = \frac{A - B}{A} \times 100$$

Where A is haemolysis by EC_{50} saline concentration while B is haemolysis caused by the concurrent addition of extract.

Determination of Total antioxidant capacity

The antioxidant activity of butanol extract of *C. longa* was evaluated by the phosphomolybdate method of Prieto et. al (1999). The assay measures the reduction of Mo(VI) – Mo (V) by the extract and the subsequent formation of a green phosphate/ Mo (V) complex at acid pH. Briefly, a 0.3 ml extract solution was combined with 3 ml of reagent solution (0.6 M sulfuric acid, 4mM ammonium molybdate and 28mM sodium phosphate). The tubes containing the reaction solution were incubated at 95°C for 90 min. The absorbance of the solution at 695 nm was the measured after cooling to room temperature using spectrophotometer (Jenway UV/Vis 6505) against blank (0.3 ml butanol extract in phosphate buffer). The antioxidant activity is expressed as the number of gram equivalents of ascorbic acid.

Reducing Power assay

Different concentrations aqueous butanol extract of *C. longa* (31.25- 1000 $\mu\text{g/mL}$) in 1ml distilled water with 2.5ml phosphate

buffer (pH 6.6, 0.2M) and 2.5ml of potassium ferricyanide [1% $K_3Fe(CN)_6$] were incubated at 50°C for 20mins. A portion (2.5 ml) of 10 % trichloroacetic acid (TCA) was added to the mixture and then centrifuged at 3000 r.p.m. for 10 min. The upper layer of the solution (2.5 ml) was mixed with 2.5 ml distilled water and 0.5 ml of iron (III) chloride (1% $FeCl_3$). The absorbance was measured at 700 nm using spectrophotometer (Jenway UV/Vis 6505), increased absorbance of the reaction mixture indicated increased reducing power. Ascorbic acid and garlic acid were used as standards. Phosphate buffer, pH 6.6 was used as blank solution. Each test was carried out on triplicate and absorbances of the final reaction mixtures were expressed as mean \pm standard deviation. This procedure was described by Saba et al., (2008).

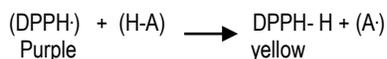
DPPH radical scavenging activity

The determination of the free radical scavenging activity of butanol extract of *C. longa* using DPPH (1,1- diphenyl - 2- picrylhydrazyl) as assay as described Blois (1958) with slight modification.

Various concentrations (1000- 31.25 μ g/mL) of butanol extract in methanol were prepared. 1ml of 0.3mM DPPH in methanol was added to 2ml solution of the extract and standards, and allowed to stand at room temperature in a temperature in a dark chamber for 30mins. The change from deep violet to yellow was then measured at 518nm on a spectrophotometer (Jenway UV/Vis 6505). The decrease in absorbance was then converted to percentage scavenging activity (% SA) using the formula;

$$\% \text{ Scavenging Activity} = \frac{100 - [(Absorbance \text{ of sample} - Absorbance \text{ of blank}) \times 100]}{Absorbance \text{ of control}}$$

Blank is methanol (1ml) plus sample solution (2.0ml). Negative control is DPPH solution (1ml, 0.25mM) and methanol (2ml), ascorbic acid and garlic acid were used as standards. The scavenging reaction between (DPPH) and an antioxidant (H- A) can be written as;



Results and Discussion

Herbs and plants have been used traditionally as food and as sources of medicine and thus providing strong link between nutrition and chemotherapy. In *C. longa*, such connection is easily seen as the rhizome of the plant is use by local dwellers as part of their staple food and also in the management of local ailments (Narasinga, 2003; Ishita et al., 2004). The aim of this study was to investigate the potential antioxidant properties of Nigerian turmeric and to assess the ability of the extract to protect mammalian erythrocytes from haemolysis caused by osmotic pressure.

The n-butanol extract showed high DPPH scavenging activity at 1000 μ g/ml comparable to standard Ascorbic and Gallic acids. It means that the extract exhibited free radical scavenging activity but it is not known, by this work, if the antioxidant potential in Nigerian Extract will surpass that seen the Asian Turmeric (Fig. 3). The reducing capacity of compounds could serve as indicator of potential antioxidant property (Meir et al., 1995). However, the n-butanol extract of *C. longa* revealed poor reducing power, which

indicates low ability to reduce $FeCl_3$ to $FeCl_2$ (Fig. 4). This could be attributed to poor solubility of the extract's active components in aqueous solvent. The total antioxidant capacity of the extract based on the phosphomolybdate protocol exhibits high values with increasing concentration in Ascorbic acid equivalents (Table 1). This corroborate the DPPH scavenging activity in this study and agrees with observation made on turmeric extract from Asia in which curcumin has been identified as the active principle (Narasinga, 2003; Ishita et al., 2004). The high antioxidant activity may be due to the presence of phytochemicals such as phenols, flavonoids and anthocyanins (Aderogba et al., 2004). The antioxidant mechanism of curcumin is attributed to its unique conjugated structure, which includes two methoxylated phenols and enol form of β - diketone; the structure shows typical radical-trapping ability as a chain- breaking antioxidant (Ishita et al., 2004).

Fragility test conducted showed that the n-butanol extract of *Curcuma longa* lowered haemolysis of sheep, goat and bovine erythrocytes under saline-induced stress, thus suggesting a degree of protection of the erythrocytes against damage of their membrane integrity. Bovine erythrocyte was most protected from this stressor. This could be partly attributed to the variation in composition among various erythrocytes membranes an observation that has already been made (Lanham and Godfrey, 1970). This implies that the extract may possess principles that could protect the erythrocytes from damages due to stressors, at least *in vitro*. Similar observation has been made by Neeta and Rantej (2007) using aflatoxin-induced haemolysis model.

Our results showed that Nigerian turmeric also showed tremendous antioxidant properties but whether these properties are due to curcumin or other cucurminoids is yet to be ascertained. This finding will be useful to both food and drug industries. Curcumin is known to be a component ingredient of curry powder. But the component curcuminoids may vary in chemical structure and is possible that the chemical and colour characteristics as well as the functional properties will vary (Paramasivam et al., 2009). If this study is consolidated with further researches, it may provide strong basis to discontinue importation of food condiment so that production and local utilization will be encouraged

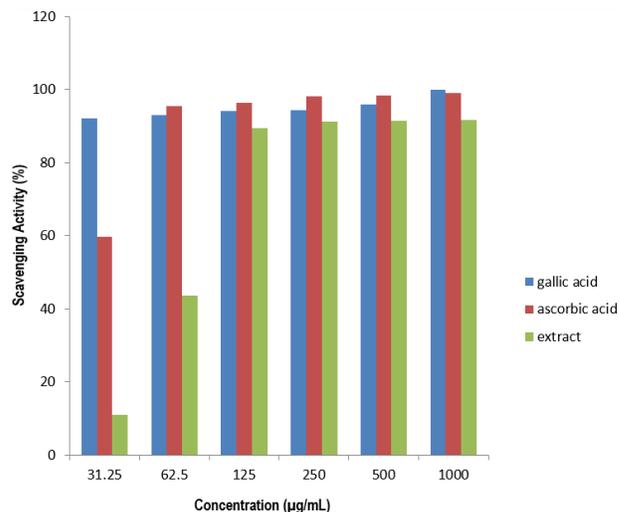


Fig. 3. DPPH free radical scavenging activity

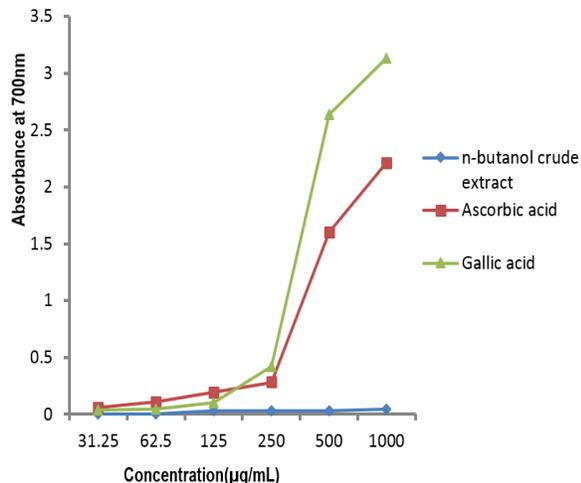


Fig. 4. Reducing power assay of butanol of *Curcuma longa*

Table 1: Total antioxidant capacity (TAC) of n- butanol extract of *C. longa*.

Concentration (µg/mL)	TAC of extract (Equivalent to ascorbic acid)
1000.00	370
500.00	210
250.00	100
125.00	60
62.50	30
31.25	15

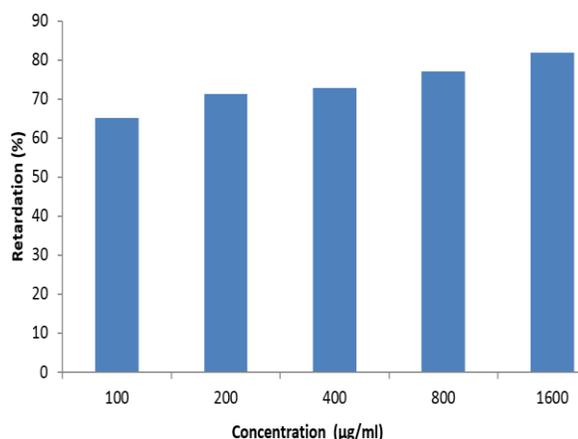


Fig. 5. Retardation of osmotic induced haemolysis on sheep erythrocytes by aqueous butanol extract of *C. longa*

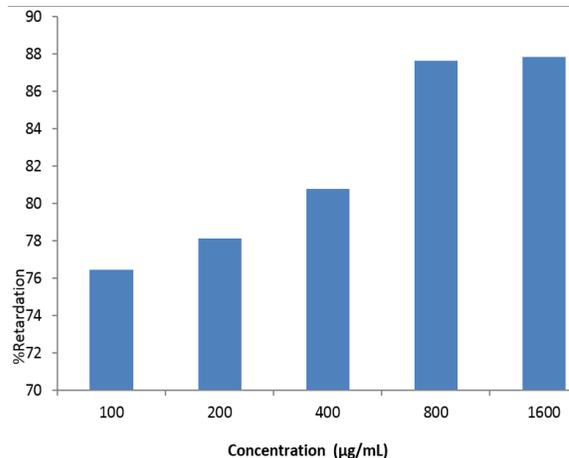


Fig. 6. Retardation of osmotic induced haemolysis of aqueous butanol extract on bovine erythrocytes

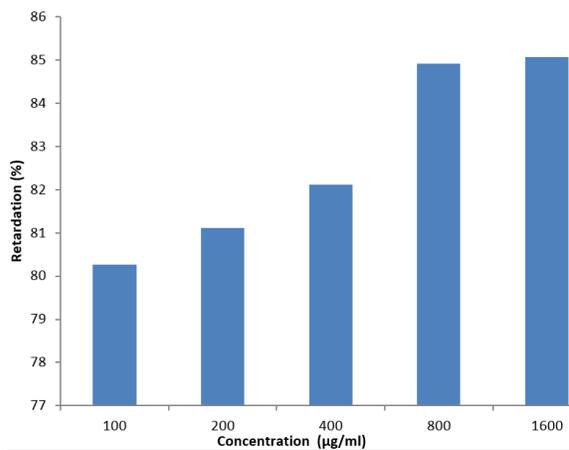


Fig. 7. Retardation of osmotic induced haemolysis on goat erythrocytes by aqueous butanol extract

Acknowledgement

The Authors acknowledged the contribution of Kaduna State University, (KASU) Kaduna, for providing the facilities for conducting the research

REFERENCES

- Aderogba, M.A., Okoh, E.K., Adelanwa, T.A. and Obuotor, EM (2004) Antioxidant properties of the Nigerian *Piliostigma* species. *Journal of Biological Sciences* 4(4): 501-503.
- Amon, H.P.T. and Wahl, M.A. (1991) Pharmacology of *Curcuma longa*. *Planta Med.* 57:1-7.
- Araujo, C.A.C. and Leon, L.L. (2001) Biological activities of *Curcuma longa* L. *Memórias do Instituto Oswaldo Cruz* 96:723-723.
- Blois, M.S. (1958) Antioxidant determinations by the use of stable free radical. *Nature* 26:1199-1200.
- Chattopadhyay, I., Biswas, K., Bandyopadhyay, U. and Banerjee, R.K. (2004) Turmeric and Curcumin: Biological actions and medical applications. *Current Science* 87:44-53.

- Grzegorzczak, I., Matkowski, A. and Wysokinska, H. (2007) Antioxidant activity of extracts from *in vitro* cultures of *Salvia officinalis* L. Food Chemistry 104:536-541.
- Halliwell, B. (1996) Oxidative stress, nutrition and health: Experimental strategies for optimization of nutritional antioxidant intake in humans. Free Radical Research 25:1-32.
- Ishita C., Kaushik, B., Uday, B. and Ranajit, K.B. (2004) Turmeric and curcumin: Biological actions and medicinal applications. Current Science 87(1):44-53.
- Jurenka, J.S. (ASCP). (2009). Anti-inflammatory Properties of Curcumin, a major constituent of Curcuma longa: A review of Preclinical and Clinical Research. Alternative Medicine Review. 14(2): 141-152.
- Lanham, S.M. and Godfrey, D.G. (1970) Isolation of salivarian *Trypanosome* from man and other mammals using DEAE-Cellulose. Experimental Parasitology 28:521-534.
- Mazumdar, A., Regharavan, K., Wenistein, J., Kohn, K.W. and Pommer, Y. (1995) Inhibition of human immunodeficiency Virus Type-1 integrase by curcumin. Biochemical Pharmacology 49:1165-1170.
- Meir, S., Kanner, J., Akiri, B. and Hada, S.P. (1995) Determination and involvement of aqueous reducing compounds in oxidative defense system of various senescing leaves. Journal of Agriculture Food and Chemistry 43:1813-1819.
- Motterlini, R., Foresti, R., Bassi, R. and Green, C.J. (2000). Curcumin, an antioxidant and anti-inflammatory agent, induces heme oxygenase-1 and protects endothelial cells against oxidative stress. Free Radical Biology and Medicine. 28(8):1302-1312.
- Narasinga, B.R. (2003) Bioactive phytochemicals in Indian foods and their potential in health promotion and disease prevention. Asian Pacific Journal of Clinical Nutrition 12 (1):9-22.
- Natchaya, W., Sridulyakul, P., Jariyapongskul, A., Suksamrain, A. and Patumraj, S. (2009) Effects of curcumin and tetrahydrocurcumin on diabetes-induced endothelial dysfunction. African Journal of Biochemistry Research 3(5):259-265.
- Ndhilala, A. R., Mulaudzi, R., Ncube, B., Abdelgadir, H.A., du Plooy, C.P. and Staden, J.V. (2014). Antioxidant, Antimicrobial and Phytochemical Variations in Thirteen *Moringa oleifera* Lam. Cultivars. Molecules. 19:10480-10494.
- Neeta, M. and Ramtej, J.V. (2007) Aflatoxin induced- haemolysis and its amelioration by turmeric extracts and curcumin *in vitro*. Drug Research 64:165-168.
- Paramasivan, M., Poi, R., Banerjee, H. and Bandyopadhyay, A. (2009) High-performance thin layer chromatographic method for quantitative determination of curcuminoids in Curcuma longa gerplasm. Food Chemistry 1133:640-644.
- Prieto, P., Pineda, M. and Aguilar, M. (1999) Spectrophotometric quantification of antioxidant capacity through the formation of a phosphomolybdenum complex: specific application to the determination of vitamin E. Analytical Biochemistry 269:337-341.
- Ragunathan, I. and Panneerselvam, N. (2007). Antimutagenic Potential of Curcumin on chromosomal aberrations in *Allium cepa*. Journal of Zhejiang University Science B. 8(7):470-475.
- Reddy, R. C., Vatsala, P.G., Keshamouni, V.G., Padmanaban G. and Rangarajan P.N. (2005). Curcumin for malaria therapy . Biochemical and Biophysical Research Communications. 326(2):472-474.
- Saba, M.R., Hasan, S.M.R., Akter, R., Hossain, M.M., Alam, M.A. and Mazumder, M.E.H. (2008) *In vitro* free radical scavenging activity of methanolic extract of the leaves of *Mimusops elengi* Linn. Bangladesh Journal of Veterinary Medicine 6(2):197-202.
- Tizard, I.R., Holmes, W.L. and Nielsen, K. (1978) Mechanisms of the anemia in trypanosomiasis: studies on the role of the hemolytic fatty acids derived from *Trypanosoma congolense*. Tropenmed. Parasitology 29:108-114.
- World Health Organization (1993) Summary of W.H.O. guidelines for the assessment of herbal medicine. Herbal Gram 28:13-14.
- World Intellectual Property Rights Organization (WIPO) (1998) Asian Regional Seminar on intellectual Property issues in the field of traditional medicine. New Delhi. Pp. 2-3.