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Following the abstract, about 3 to 10 key words that will provide indexing references should be listed.

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Chikere CB, Omoni VT and Chikere BO (2008). Distribution of potential nosocomial pathogens in a hospital environment. Afr. J. Biotechnol. 7: 3535-3539.

Moran GJ, Amii RN, Abrahamian FM, Talan DA (2005). Methicillinresistant Staphylococcus aureus in community-acquired skin infections. Emerg. Infect. Dis. 11: 928-930.

Pitout JDD, Church DL, Gregson DB, Chow BL, McCracken M, Mulvey M, Laupland KB (2007). Molecular epidemiology of CTXM-producing Escherichia coli in the Calgary Health Region: emergence of CTX-M-15-producing isolates. Antimicrob. Agents Chemother. 51: 1281-1286.

Pelczar JR, Harley JP, Klein DA (1993). Microbiology: Concepts and Applications. McGraw-Hill Inc., New York, pp. 591-603.

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Full Length Research Paper

Food and nutrition education; elaboration of a guide for children of 6 to 12 years

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Accepted 25 October, 2011

A qualitative description of the food situation for 2196 Algerian healthy school children aged 6 to 12 years (Constantine, Jijel, Touggourt) by a qualitative meta-analysis. Our aim is to compare the observed and recommended diet (2001) to correct some food errors (quantity, quality, behaviour) with recommendations for nutrition education as a guide for children to prevent communicable diseases related to diet; in Algeria this type of study is rare due to lack of work on this age and because of lack of access to previous studies. The results obtained are: the decline of daily caloric intake, an unbalanced distribution of macro and micronutrients, a high intake of starch products, an average consumption of vegetables, fruit and fish rich in antioxidants biomolecules, breakfast is neglected, the morning snack and snack time food are formed by foods of high energy density, the snacking food type 'junk food' practiced the whole time watching television and a decrease in the practice of regular physical activity.

Key words: Algeria, nutrition education, children of 6 to 12 years, quantity, quality and behavioural food, guide.

INTRODUCTION

The increase in chronic no communicable diseases related to diet seems to initiate an epidemiological and nutritional transition, exacerbated by demographic changes that combine energy-dense diet to a life of increasingly sedentary-fed refined products too fat, too salty and too sweet (Maire et al., 2002), it is urgent to establish an appropriate prevention. Currently, nutrition education is difficult to apply because the child's diet depends on not only family but also society. Our work aims to compare between observed and advised diet in quantity, quality and behavioural for safe Algerian children at school in 6 to 12 years to correct errors with recommendations for nutrition education as a guide.

MATERIALS AND METHODS

Our study is a qualitative description of the food situation for 2196 Algerian healthy children attending school 6 to 12 years (Constantine, Jijel and Touggourt). In our study we used a qualitative data analysis (summary of study results on the same issue); only seven (7) studies of 67 ones that talk about the nutritional status food of Algerian children aged 6 to 12. Then a comparison between the observed powers (the result of the meta-analysis) and the recommended diet (Martin, 2001) to correct some aspects as food quantity, quality and behavioural recommendations for nutrition education as a guide for children. We used Microsoft Excel 2007 in our study. The distributions were presented as percentages, averages and standard deviations.

RESULTS

Quantitative aspect of food

The results obtained are reduction of daily energy intake of children in relation to recommended daily allowance. Figures 1 and 2 shows the decrease in daily energy intake of children in relation to recommended dietary allowances according to sex. Figures 3 and 4 show the share of energy meals that are lower for breakfast (a tendency to obesity) and lunch but high for afternoon snack and dinner with the practice of snacking. Snacking, representing a significant share of 2.06% among children aged 7 to 9 years and 1.29% among children 10 to 12

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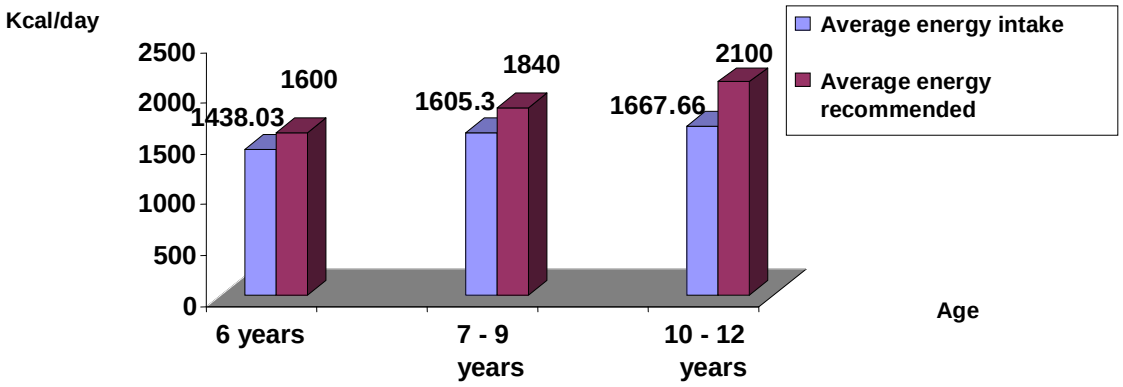

Figure 1. Comparison between average daily energy intake and recommended among girls aged 6 to 12 years.

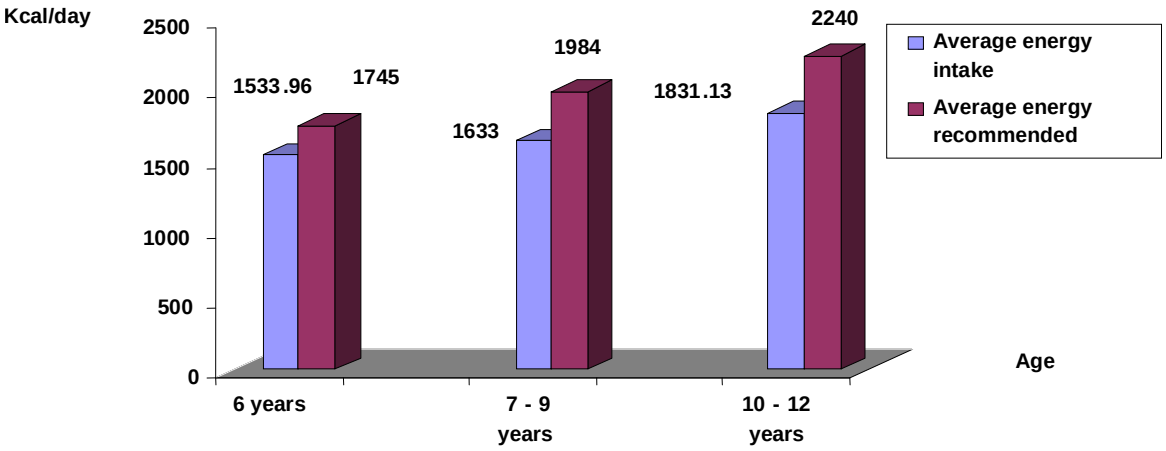

Figure 2. Comparison between average daily energy intake and recommended among boys aged 6 to12 years.

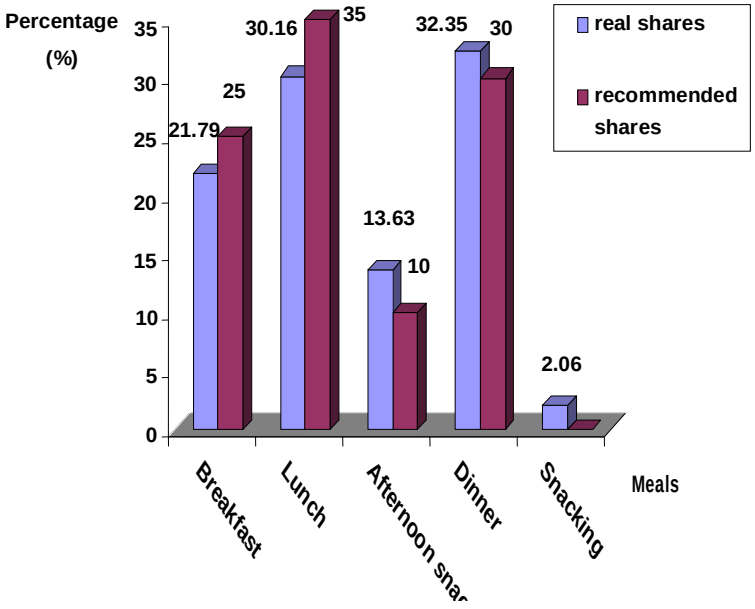

Figure 3. Comparison between energy real and recommended shares for children aged 7 to 9 years.

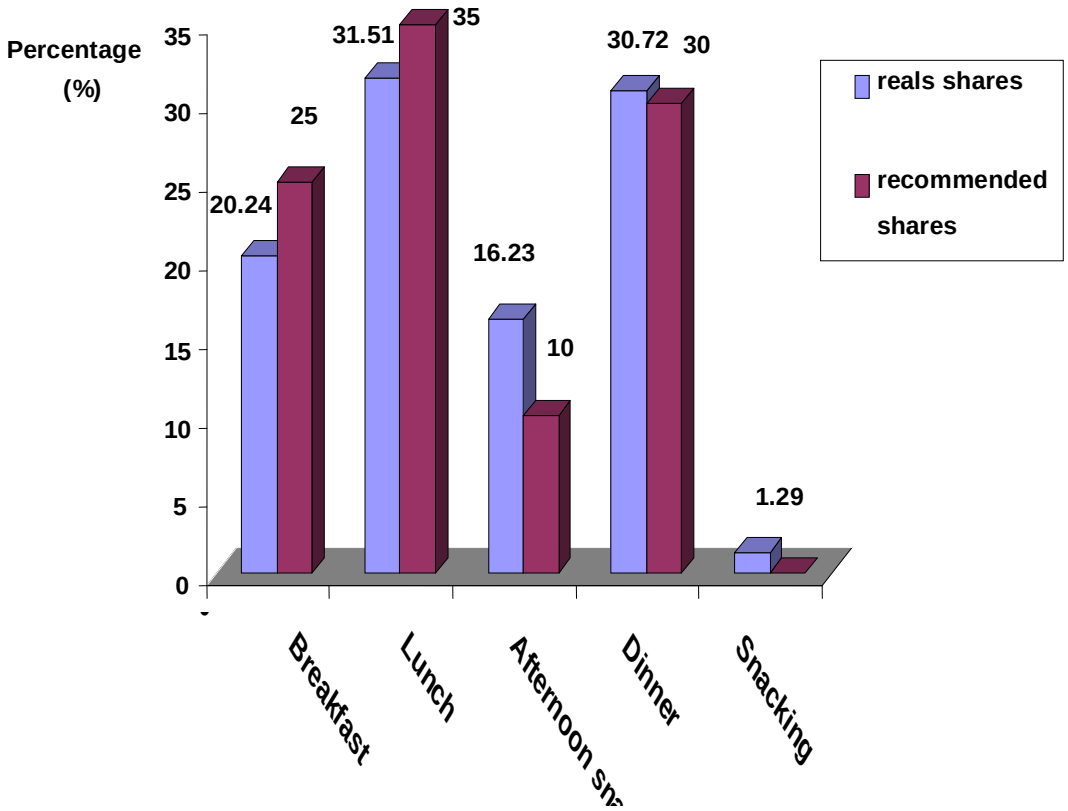

Figure 4. Comparison between energy real and recommended shares for children aged 10 to12 years.

Table 1. Distribution of recommended macronutrients.

Proteins 11 - 15 (%)	Lipids 30 - 35 (%)	Carbohydrates 50 – 55 (%)
½ animal protein (5.5 – 7.5%)	1/3 saturated fatty acids (8 à 10%)	1/5 simple sugar (10%)
½ plant protein (5.5 – 7.5%)	2/3 unsaturated (20 - 25%): 1/3 monounsaturated fatty acids (10- 15%), 1/3 polyunsaturated fatty acids (5 à 10%)	4/5 sugar complex (starch) (40 – 45%)

Table 2. Distribution of observed macronutrients.

Proteins 16.34% (15.34 – 17.56)	Lipids 22.49 % (20.32 – 24.96)	Carbohydrates 60.92% (57.78 – 63.87)
43% animal protein (7.02%)	9% saturated fatty acids	15.49% simple sugar
57% plant protein (9.31%)	11.67% unsaturated (8% monounsaturated fatty acids, 3.67% polyunsaturated fatty acids	45.43% sugar complex (starch)

years for the body compensates for its daily energy expenditure.

Qualitative aspect of food

An imbalanced distribution of macro and micronutrients.

Macronutrient intakes

According to Tables 1 and 2, the children's food is characterized by a total protein intake in excess consists of too many foods containing proteins of plant origin in relation to animal protein. A lack of overall consumption of total lipids with saturated fatty acid intake is sufficient,

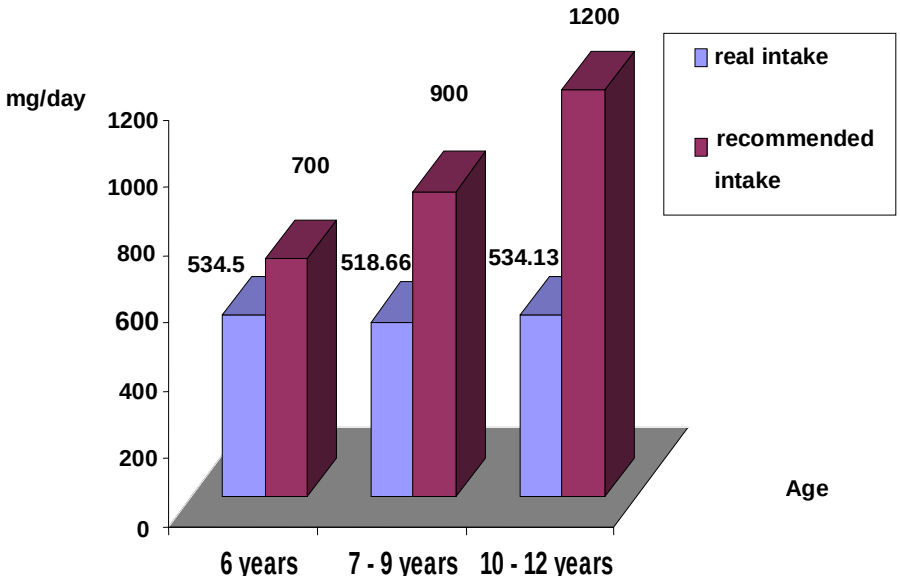

Figure 5. Comparison between real and recommended calcium intakes for children aged 6 to 12 years.

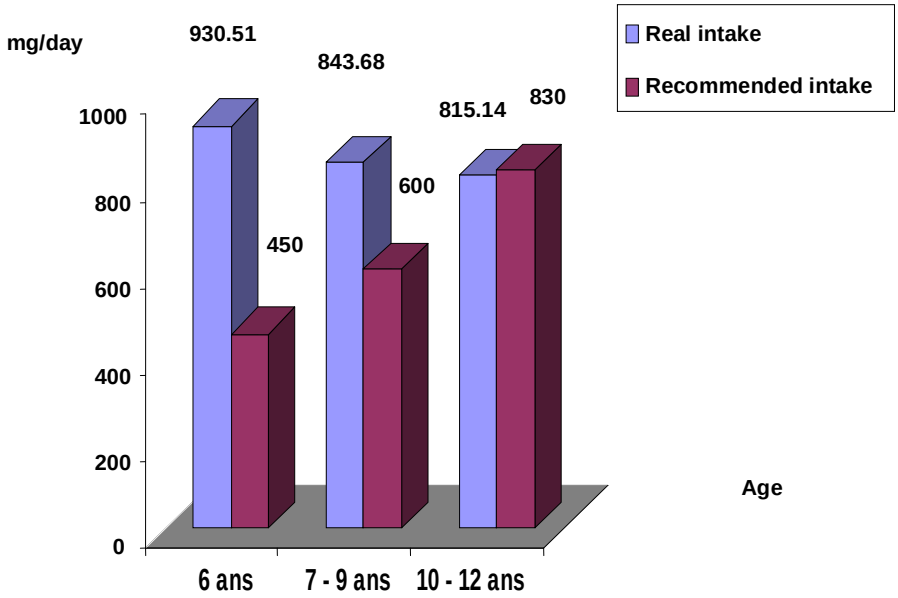

Figure 6. Comparison between real and recommended phosphorus intakes for children aged 6 to 12 years.

fewer foods containing monounsaturated fatty acids and polyunsaturated fatty acids. An overall excess carbohydrate intake, too many foods high in simple sugars and complex sugars.

Micronutrient intakes

According to Figure 5, all children suffer from severe deficiency in calcium during growth. According to Figure 6, phosphorus inputs are covered extensively for children aged 6 to 9 but a slight deficiency has occurred for children 10 to 12 years.

According to the Table 3, reports of Ca/P children are out of balance for all ages. According to Figure 7, iron intake is largely covered for children 6 to 9 years, but children aged 10 to 12 mild deficiency is notable in early adolescence especially for girls.

Table 3. Comparison of Ca/P real l and recommended for children 6 to 12 years.

Ages (years)	Report real Ca/P	Report recommended Ca/P
Children 6	0.57	
Children 7 - 9	0.63	1.2 to 1.6
Children 10 - 12	0.6	

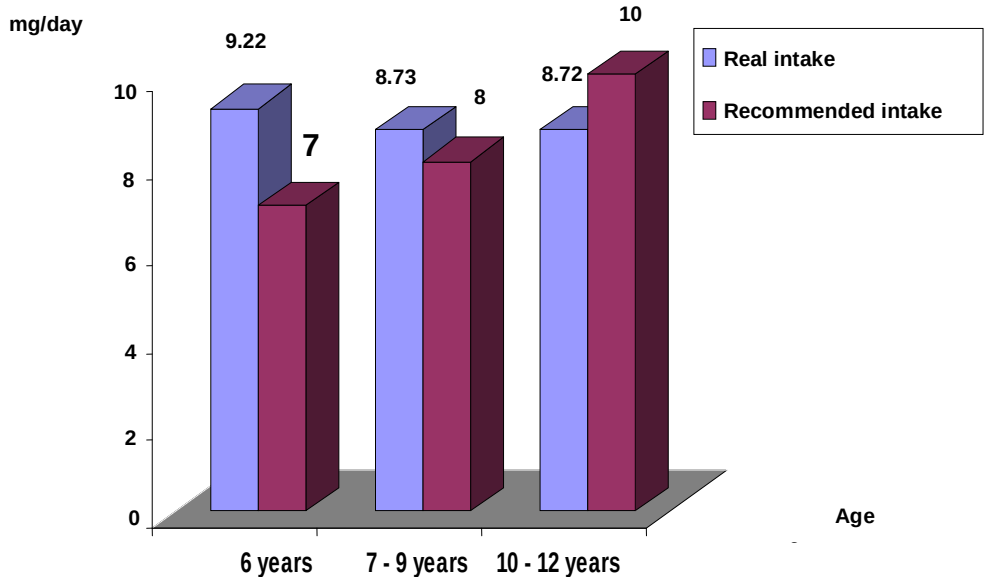

Figure 7. Comparison between real and recommended iron intakes apports for children aged 6 to12 years.

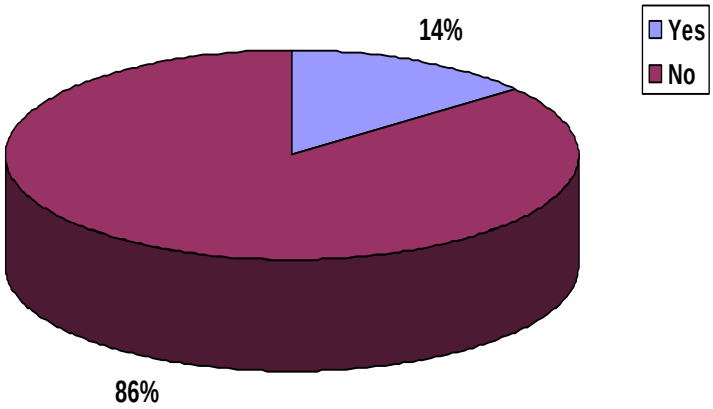

Figure 8. Distribution of children aged 6 to 8 years depending on the sport outside school.

Behavioural aspect of food

Practice of physical activity

According to Figure 8, 86% of children have no regular physical activity promote obesity and diabetes. Reduction in the practice of regular physical activity.

Getting breakfast

Meals are taken on a regular basis, we noted that about

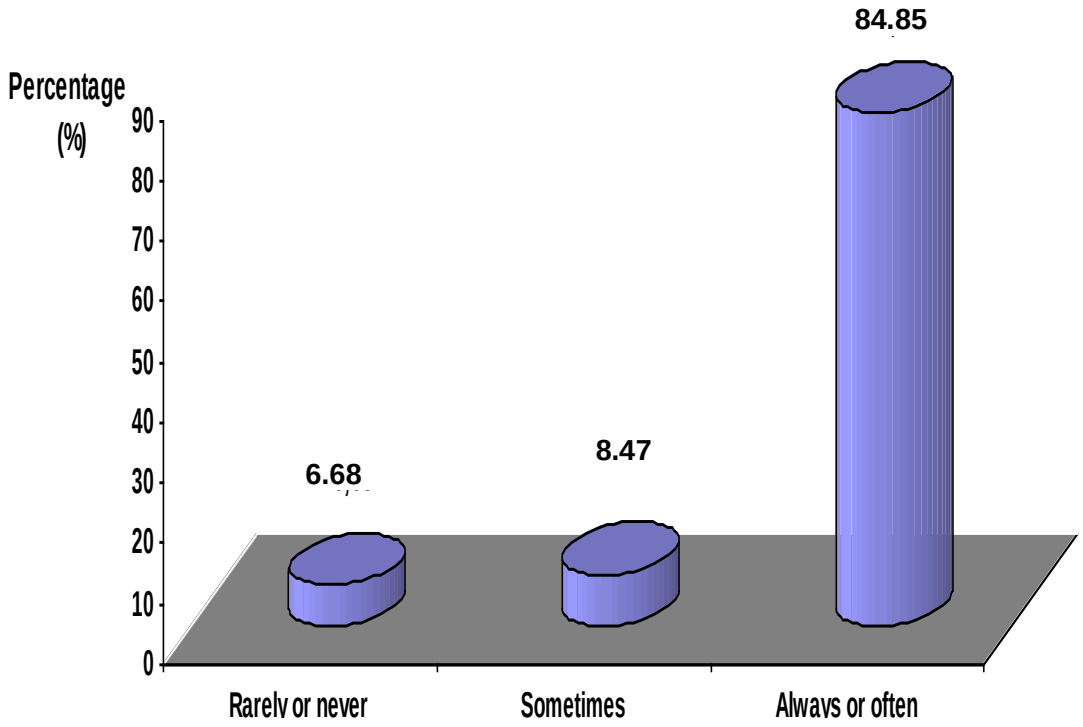

Figure 9. Average frequency per week of taking breakfast in children 6 to 12 years.

6.68 per 100 skip breakfast (tendency to obesity) (Figure 9) breakfast is skipped.

Composition of the morning snack and afternoon snack

The food component the morning snack and afternoon snack (Figures 10 and 11) are composed by candy, cookies and chocolate which is a very important energy supplement accompanied by an almost total absence of fruits rich in antioxidants biomolecules (Vitamin A, C and β carotene) against free radicals promote cancer. The composition of the morning snack and afternoon snack for energy-dense foods.

Taken in snacking

According to Figure 12, snacking is widespread among children; they nibble all the time watching TV (loss of attention to internal cues of satiety).

Frequency of consumption of foods such as junk food

The snack food of choice of food types are 'junk food' with high energy density (high in carbohydrates and lipids) (Figure 13).

DISCUSSION

Quantitative aspect of food

Our results have highlighted the importance of protein energy malnutrition in Algeria. In general, by sex and age group, the average daily energy intake of all children boys or girls are below the recommended daily caloric intake by (Martin, 2001), this can be explained by diet which does not suffice to meet the nutritional needs of children. That under nutrition caused the standard of living: the development of poverty which results in a decrease of purchasing power and the average socioeconomic status if they say no bottom of the Algerian population that relies primarily on its power grains (bread) and milk. These results are similar to results found by the national survey on the goals of the late decade health mother and child (MICS 2, 2000) and the National Statistics Office and Ministry of Health (MICS 3 Algeria, 2006).

Qualitative aspect of food

Excessive consumption of carbohydrates can be determined especially when added sugar which increases the energy density of food. The sweet part in hedonic food especially when combined with fat, it may well induce a passive over consumption (Simon, 2003). The high consumption of sugar induced lipogenesis and

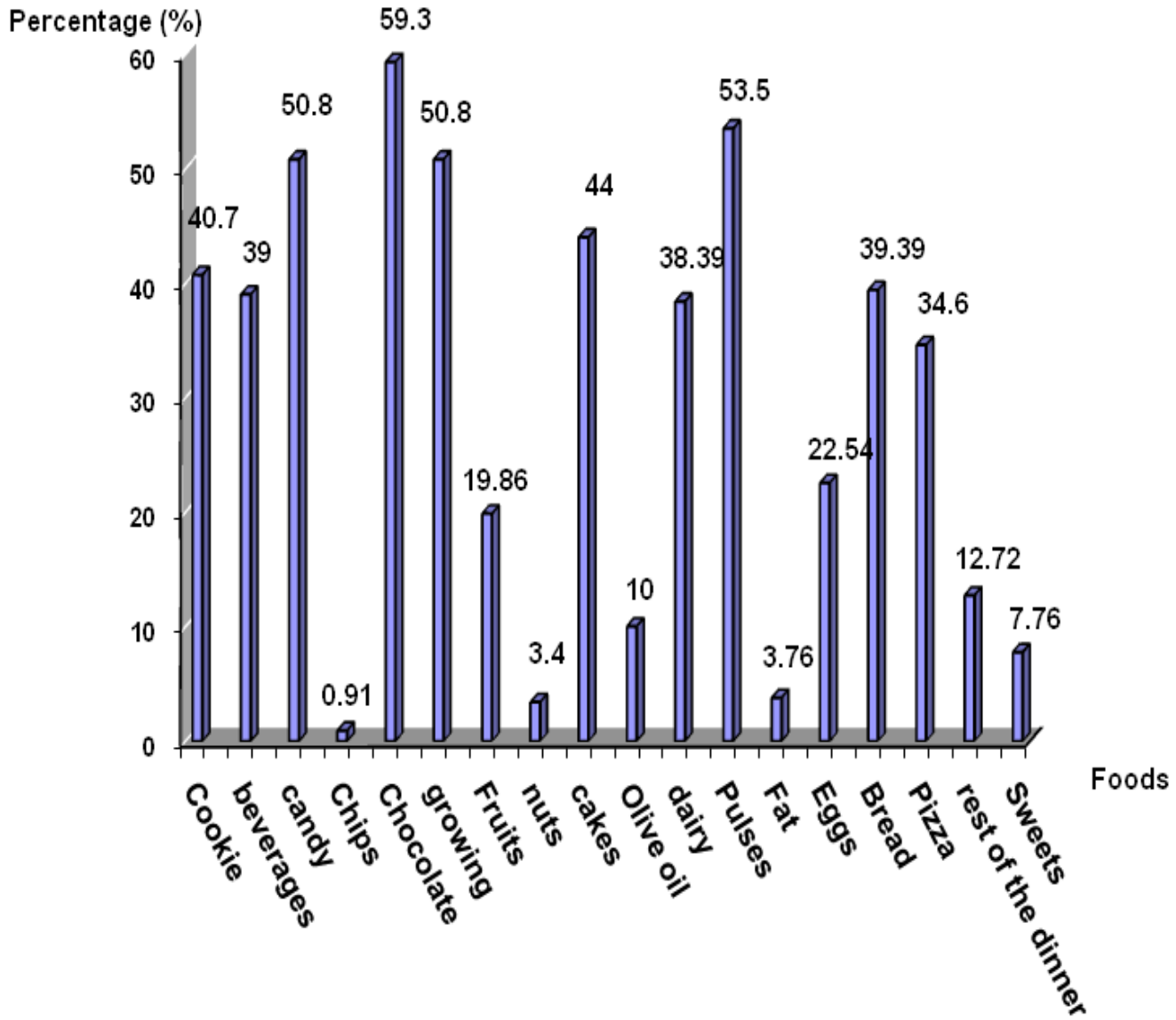

Figure 10. Average frequency of consumption of foods making up the morning snack among children 6 to 12 years.

thus obesity later. A high intake of protein during infancy induces adipocyte proliferation early (Rolland et al., 1995); also in our study, the vegetable protein consumed over 50% with small value biological compared to animal proteins that are a high biological value and very rich in Fe and B12; they can be explained by the cheret meat, offal and fish and reducing the power of purchase of the Algerian people. The fat intake is lower than the recommended dietary allowances due to the decreases consumption of food rich in animal and vegetable fats. All calcium intakes are very low capacity to recommended dietary reports by Maire et al. (2002) because of depletion of infant food sources of calcium are dairy products like milk, cheese, yogurt; this is due either because of the socio economic situation of households is that children do not consume milk.

Behavioral aspect of food

Practice of physical activity

Many studies suggest the existence of an association between increased prevalence of childhood obesity and the trend towards greater settling (Leynaud and Berthier, 1992).

Getting breakfast

Food must represent 25 per morning percent of daily

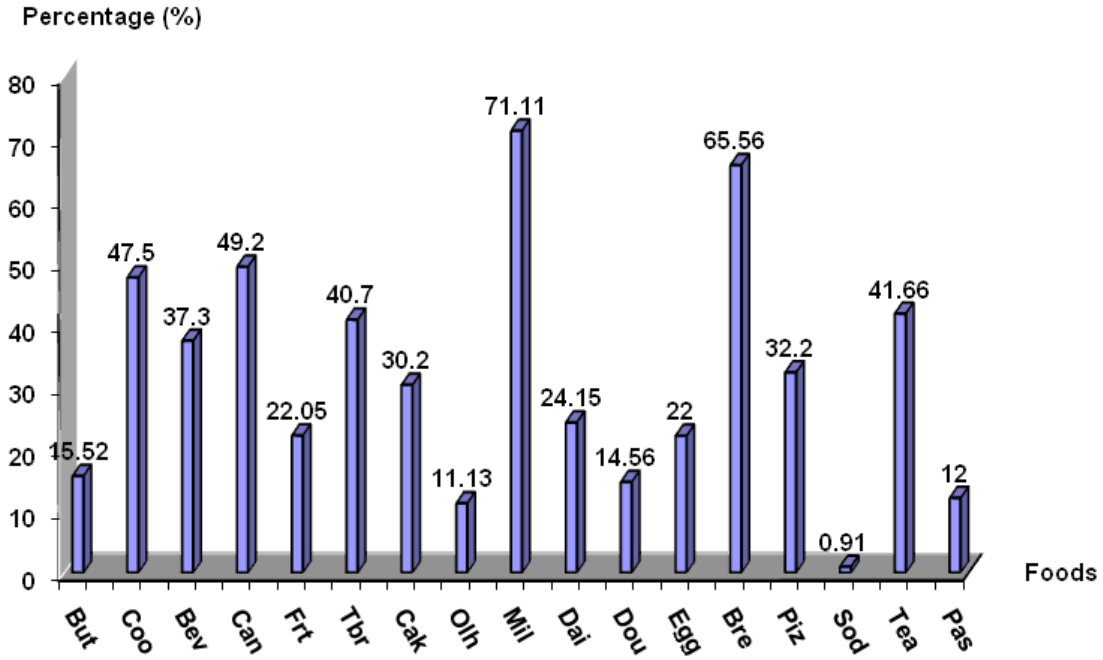

Figure 11. Average frequency of consumption of afternoon snack foods among children 6 to 12 years. But: butter, Coo: cookie, Bev: beverages, Can: candy Chi: chips, Jam: jam, Cho: choocolate, Gro: growings, Frt: fruits, Tbr: traditional bread, Cak: cakes, Olh: olive oil, Mil: milk, Dai: dairy, Dou: dough, Egg: eggs, Bre: bread, Piz: pizza, Sod: sodas, Tea: tea, Pas: pastries.

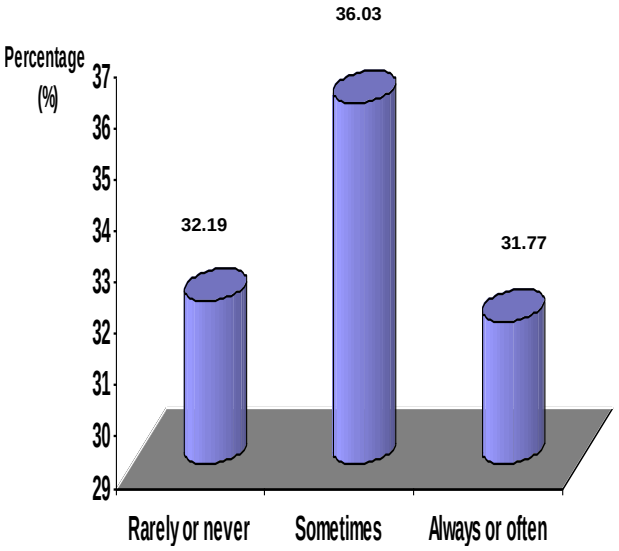

Figure 12. Average frequency per week for taking snacks among children 6 to 12 years.

energy intake. Its failure causes an imbalance in significant daily energy intake.

Composition of the morning and afternoon snack

As for the food consumed, the composition of the meals according to the Directorate of School Education in France (2004) should serve a diversified by focusing on water, fruit, pure fruit juices, milk and dairy products and avoiding energy-dense products rich in sugars and fat. What we observe in our children population is exactly the opposite.

Taken in snacking

Outside of main meals, children eat snack foods (Louis, 2000) such as sweets, chocolate, cupcakes; these products are then input result in sugar and fat that challenges the balance of meals daily, the combination fat and simple sugar is especially harmful to health. Suggested that snacking means the catch in the state of "no psychological hunger" or "satiety", that is to say, taken arising from boredom, stress or just because a little added pleasure is not neglected. A study among young Japanese reported that snacking is a factor in development of obesity (Takahashi et al., 1999).

Conclusion

Our goal is to maintain health and prevent the onset of chronic diseases and syndromes by correcting wrong attitudes practiced by children in their regular diet with a guide to healthy and balanced diet.

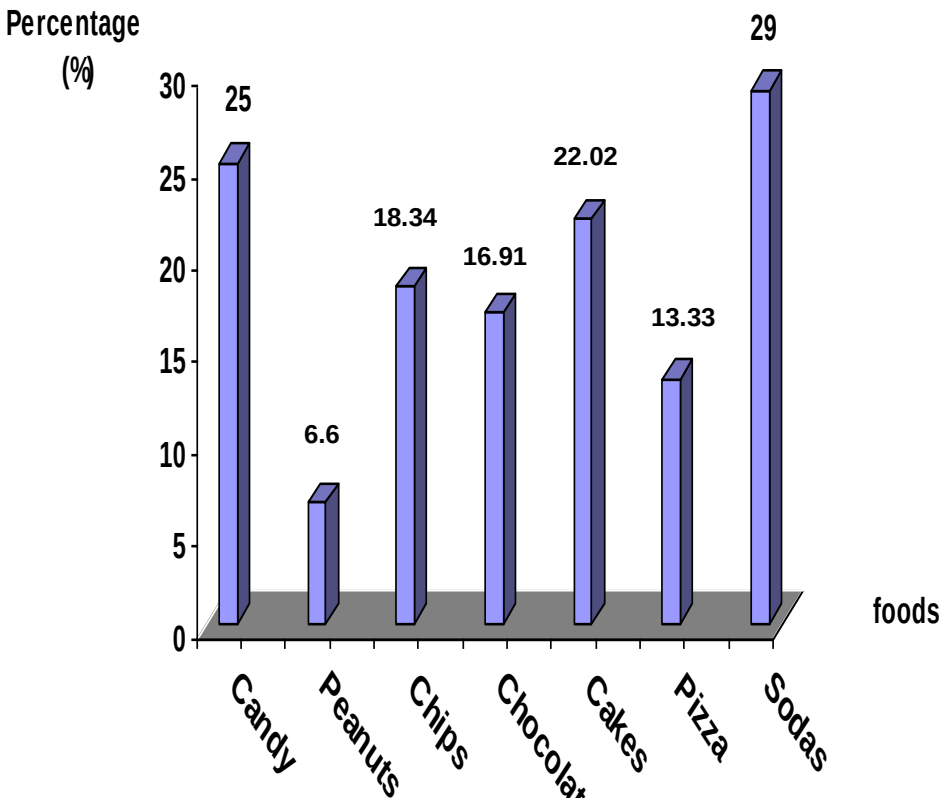

Figure 13. Frequency of consumption of foods like junk food among children 6 to 12 years.

RECOMMENDATIONS

1) The total daily energy intake is based mainly on macronutrient whose distribution is as follows:

i) Carbohydrates represent 50 to 55 %of total energy intake with one fifth of the total carbohydrate in the form of simple sugars and four fifths are complex sugars.
ii) Proteins represent 11 to 15% of total energy intake which half of the total protein is animal and half is of plantorigin.
iii) Lipids represent 30 to 35% of total energy intake with third saturated fatty acids and two thirds are unsaturated fatty acids.

2) Dairy products (milk, yogurt, a small Swiss cheese) the best source of calcium done both their richness in calcium and vitamin D plays an essential role in bone mineralization by increasing the intestinal absorption of calcium as they provide the protein necessary for bone health. Vegetables, fruits, grains, water beverage suppléments calcium intakes.
3) The coverage of iron requirements is ensured by proper intake of meat and fish that are rich in heme iron better assimilated and well absorbed (15 to 35%) than non-heme iron (2 to 20%) found in vegetables such as spinach, dairy products, eggs, chocolate ... Some elements can trap iron as fiber plants such as tea tannin, conversely, vitamin C in plants, the presence of meat, the increases.
4) Follow the regular rhythms of eating meals scheduled four main meals:

i) Morning: breakfast which is a 25% of total energy.
ii) At noon: lunch which represents a 35% of the daily diet.
iii) Afternoon to 16 pm: a snack that is 10% of the daily diet.
iv) Evening: Dinner representing 30% of the daily diet.
v) If breakfast missed or incomplete, a morning snack at 10 am to 5 p. percent of the daily diet.

5) Do not skip the four main meal because it encourages snacking.
6) Avoid repetitive food taken outside the main meals (snacks), especially watching TV or playing video games.
7) Limit consumption of foods such as junk food cakes, pizza, chocolate, soda, peanuts, and chips, except occasionally.
8) Promoting regular physical activity at least equivalent to half an hour of brisk walking per day.
9) During leisure time promote active play and sports, and entertainment not regularly active (video games, television).

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Full Length Research Paper

α-Glucosidase inhibitory activities of some Oxovanadium(IV) complexes: Examples of low IC_{50} values

Sristicheta Misra[1], Krishna Bihari Pandeya[1]*, Ashok K. Tiwari[2], Amtul Z. Ali[2], Thimmapatruni Saradamani[2], Sachin B. Agawane[2] and Kuncha Madhusudana[2]

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[2]Division of Pharmacology, Indian Institute of Chemical Technology, Hyderabad-500007, India.

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α-Glucosidase inhibition measurements have been made on oxovanadium(IV) complexes of eight Schiff base ligands. Three of the complexes show exceptionally low IC_{50} values: $VO(sal\text{-}anl)_2$ = 2.105 μM, $VO(sal\text{-}mxyanl)_2$ = 1.26 μM and $VO(sal\text{-}ntranl)_2$ = 1.26 μM. *In-vivo* experiments for antihyperglycemic activity carried out on one of these complexes, viz., $VO(sal\text{-}mxyanl)_2$, reveal that the complex decreases blood glucose level by ~ 12% .

Key words: α-Glucosidase inhibition, oxovanadium (IV) complexes, Schiff bases, antihyperglycemic activity.

INTRODUCTION

Several metal ions and their complexes exhibit antidiabetic effects (Schwarz and Mertz, 1959; Rubenstein et al., 1962; Coulston and Dandona, 1980; Heyliger et al., 1985; Sakurai et al., 1990; Yoshikawa et al., 2000; 2010; Ueda et al., 2005). A large variety of oxovanadium(IV) complexes have earlier been studied for their antidiabetic activity (Thompson et al., 1999; Rehder et al., 2002; Sakurai, 2002; Sakurai et al., 2003a, b; Crans, 2005; Katoh et al., 2009). [Bis(maltolato)oxovanadium(IV)], BMOV and [bis(ethylmaltolato)oxovanadium(IV)], BEOV, have been studied most exhaustively (McNeill et al., 1992; Yuen et al., 1993; Dai et al., 1993; Caravan et al., 1995; Thompson et al., 2003; Saatchi et al., 2005); BEOV having completed phase I clinical trials in humans (Thompson et al., 2003). Search for newer and newer such drugs has become an important area of current biochemical research. Slowing down of digestion and absorption of dietary carbohydrate using α-glucosidase inhibitors has proved to be a promising therapeutic strategy for reducing risk of diabetes and other carbohydrate mediated diseases (Robinson et al., 1991; Braun et al., 1995; Dwek et al., 2002; Humphries et al., 1986; Mehta et al., 1998; Karpas et al., 1988; Zitzmann et al., 1999). Acarbose is the first α-glucosidase inhibitor approved for the treatment of diabetes (Yee et al., 1996). A variety of α-glucosidase inhibitors, extensively described in a recent review (de Melo et al., 2006), are all organic compounds, both synthetic and natural. There is paucity of data on α-glucosidase inhibition by oxovanadium (IV) complexes (Ashiq et al., 2008; 2009). We have, therefore, worked on rat intestinal α-glucosidase inhibition by oxovanadium(IV) complexes of a series of Schiff bases, derived from salicylaldehyde (and 5-bromosalicylaldehyde) and anilines. These Schiff bases act as ON-type bidentate ligands to form 5-coordinated square pyramidal oxovanadium (IV) complexes (structures and abbreviated names given in Figure 1).

MATERIALS AND METHODS

Preparation of the Schiff bases (Pandeya and Khare, 1992)

All the Schiff bases were prepared by refluxing, for 1-2 h, a mixture

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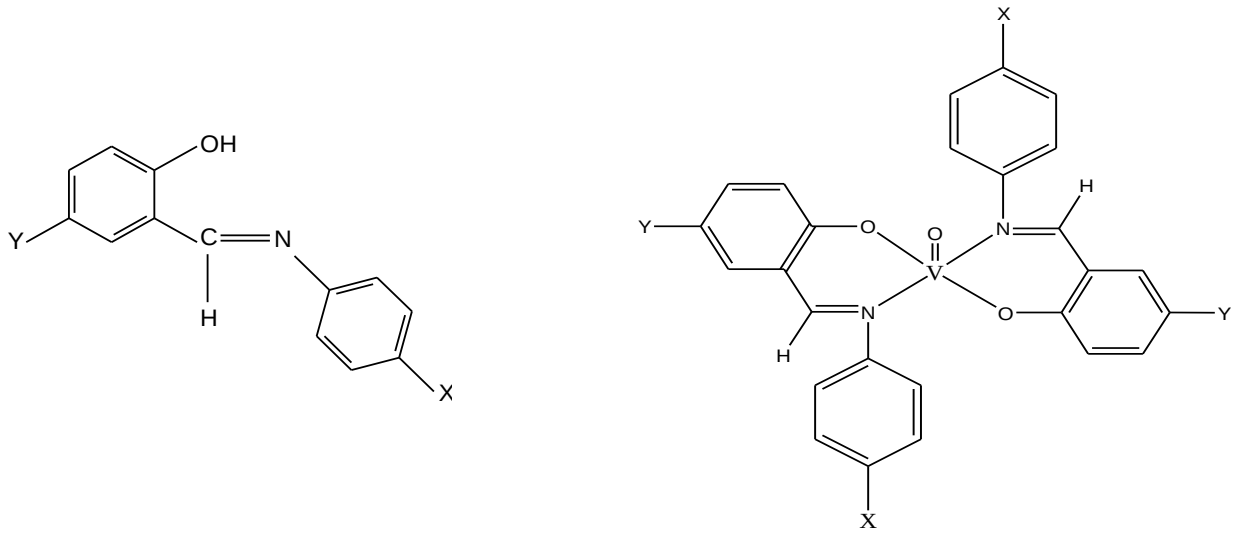

(1) **(2)**

Figure 1. Chemical structures of the Schiff base ligands and their oxovanadium (IV) complexes.
X=H, Y=H (sal-anl 1a; VO (sal-anl)$_2$ 2a), X=Cl, Y=H (sal-clanl 1b; VO (sal-clanl)$_2$ 2b), X=OCH_3,Y=H (sal-mxyanl 1c; VO(sal-mxyanl)$_2$ 2c), X=NO_2, Y=H (sal-ntranl 1d; VO(sal-ntranl)$_2$ 2d), X=H, Y= Br (brsal-anl 1e; VO(brsal-anl)$_2$ 2e), X=Cl, Y= Br (brsal-clanl 1f; VO(brsal-clanl)$_2$ 2f), X=OCH_3,Y= Br (brsal-mxyanl 1g; VO(brsal-mxyanl)$_2$ 2g), X=NO_2, Y= Br (brsal-ntranl 1h; VO(brsal-ntranl)$_2$ 2h).

Table 1. Elemental analyses, IR and electronic spectra.

Complex	Elemental analyses Calculated (Found)			IR band (cm^{-1})	Electronic spectra (cm^{-1})
	%C	%H	%N	$\nu_{v=o}$	$^2B_2 \rightarrow {}^2E$
VO(sal-anl)$_2$ 2a	67.97 (67.64)	4.36 (4.06)	6.10 (6.02)	980	12000
VO(sal-clanl)$_2$ 2b	59.09 (59.47)	3.41 (3.63)	5.30 (5.14)	983	12950
VO(sal-mxyanl)$_2$ 2c	64.74 (64.46)	4.62 (4.20)	5.39 (5.19)	971	11840
VO(sal-ntranl)$_2$ 2d	56.83 (56.33)	3.28 (3.08)	10.20 (10.02)	987	10845
VO(brsal-anl)$_2$2e	50.57 (50.17)	2.92 (3.00)	4.57 (4.50)	986	12500
VO(brsal-clanl)$_2$ 2f	45.48 (45.31)	2.33 (2.39)	4.08 (4.12)	980	13040
VO(brsal-mxyanl)$_2$ 2g	49.63 (49.28)	3.25 (3.55)	4.14 (4.05)	980	NR*
VO(brsal-ntranl)$_2$ 2h	44.13 (44.00)	2.26 (2.60)	7.92 (7.61)	985	11765

*Not resolved (see text).

of the salicylaldehyde/5-bromo-salicylaldehyde and aniline/ substituted aniline in ethanol. In most cases the Schiff bases precipitated out on cooling. Wherever necessary, the excess solvent was evaporated to make the Schiff base precipitate out on cooling. The same was filtered, washed with small amount of ethanol and dried.

Preparation of the complexes (Pandeya and Khare, 1992)

A mixture of 2 mmol Schiff base and 1 mmol of NaOH was dissolved in methanol followed by addition of 1 mmol of vanadyl sulphate (trichurated in minimum amount of methanol). The solution was refluxed for nearly one hour to obtain clear solution. On cooling, the complex precipitated out in most cases. Wherever necessary, the excess solvent was removed by evaporation to get the complex precipitate out on cooling. The same was filtered out, washed with methanol and dried.

Characterization of complexes

The complexes, so obtained, show satisfactory elemental analyses (Table 1). Their infrared spectra (in KBr) show sharp, symmetric $\nu_{v=o}$ bands (Table 2). Electronic spectra of oxovanadium(IV) complexes should exhibit three d-d bands corresponding to transitions $^2B_2 \rightarrow {}^2E$ (ν_1), $^2B_2 \rightarrow {}^2B_1$ (ν_2) and $^2B_2 \rightarrow {}^2A_1$ (ν_3). For the present complexes ν_2 and ν_3 have been obscured by strong charge-transfer absorptions, so that only ν_1 has been observed in the range 10845 to 13040 cm^{-1}. For VO (brsal-mxyanl)$_2$ even ν_1 has been obscured (Table 1). Solution epr spectra (in DMSO at room temperature) of all the complexes show eight hyperfine lines characteristic of oxovanadium(IV). One representative spectrum, VO (sal-anl)$_2$, has been shown in Figure 2.

Table 2. α-Glucosidase inhibition by the oxovanadium(IV) complexes and the Schiff base ligands.

Oxovanadium(IV) complexes	% inhibition at 200 µM	IC_{50} (µM)	Schiff Base Ligands	% inhibition at 200 µM	I C_{50} (µM)
$VO(sal\text{-}anl)_2$	82.06	2.105	sal-anl	36.6	n/d*
$VO(sal\text{-}clanl)_2$	68.89	13.12	sal-clanl	6.63	n/d*
$VO(sal\text{-}mxyanl)_2$	84.57	1.26	sal-mxyanl	11.45	n/d*
$VO(sal\text{-}ntranl)_2$	83.51	1.26	sal-ntranl	98.6	22.72
$VO(brsal\text{-}anl)_2$	79.44	16.92	brsal-anl	33.06	n/d*
$VO(brsal\text{-}clanl)_2$	43.46	n/d*	brsal-clanl	0.00	n/d*
$VO(brsal\text{-}mxyanl)_2$	66.11	92.43	brsal-mxyanl	67.21	74.11
$VO(brsal\text{-}ntranl)_2$	83.15	31.10	brsal-ntranl	43.38	245
$VOSO_4$	11.75	----			
Acarbose	----	18.59			

n/d*= Not determined.

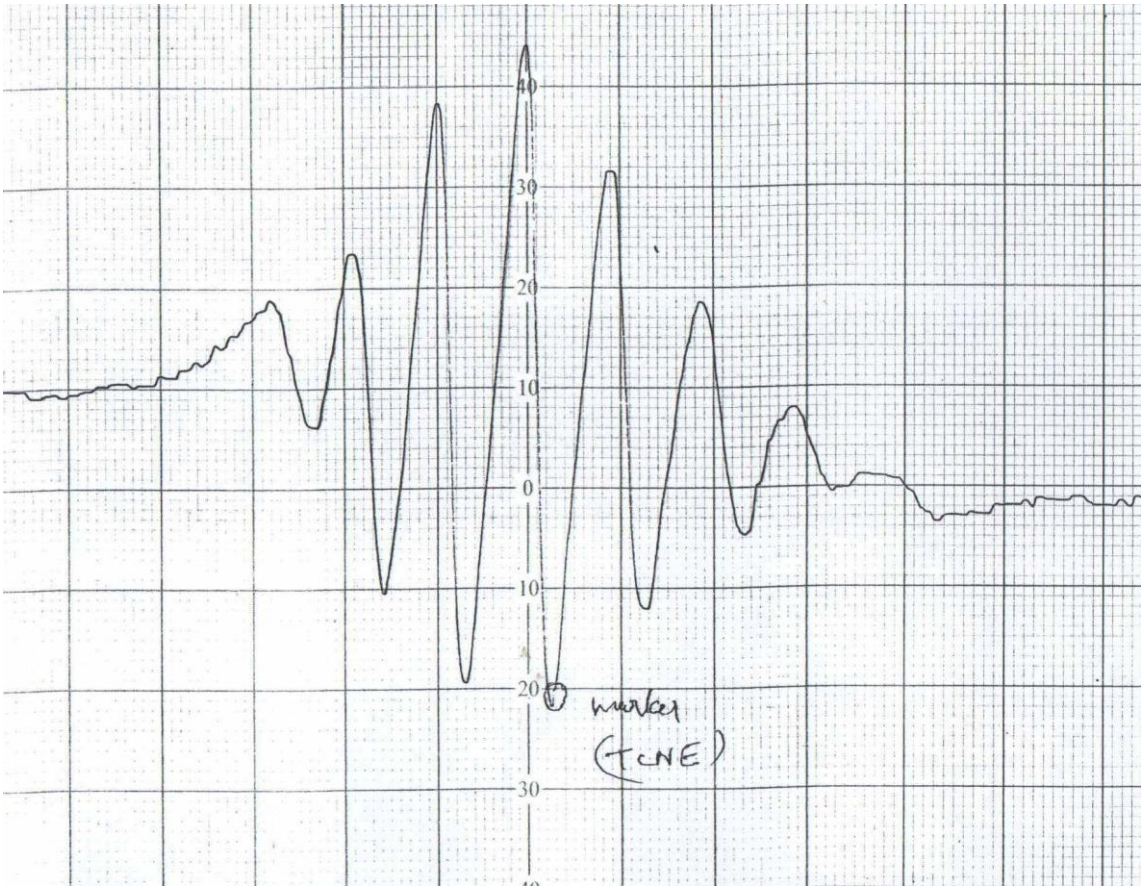

Figure 2. Solution epr spectrum (in DMSO at Room temperature) of VO(sal-anl).

***In-vitro* α-glucosidase inhibition**

Rat intestinal acetone powder (Sigma chemicals, USA) was sonicated properly in normal saline (100:1 w/v) and after centrifugation at 3000 rpm × 30 mins the supernatant was treated as crude intestinal α-glucosidase. Ten microliters of test samples dissolved in DMSO (5 mg/ml solution) were mixed and incubated with 50 µl of enzyme in a 96-well microplate for 5mins. Reaction mixture was further incubated for another 10 mins with 50 µl substrate (5 mM, p-nitrophenyl-α-D- glucopyranoside) prepared in 100 mM phosphate buffer (PH~6.8) and release of nitrophenol was read at, 405 nm spectrophotometrically ($SPECTRA_{max}$ PLUS ^{384}, Molecular devices, USA). Percent α-Glucosidase inhibition was calculated as (1-B/A) ×100, where A was the absorbance of

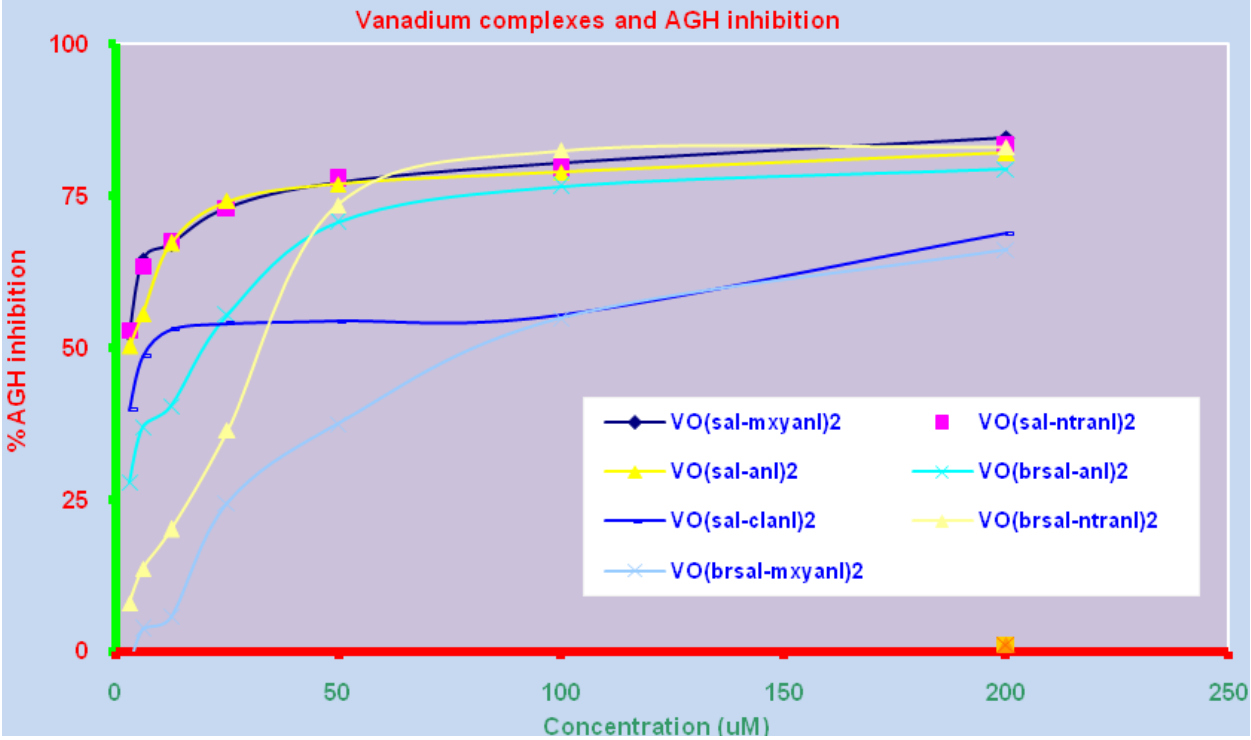

Figure 3. Concentration dependent rat intestinal α-glucosidase inhibitory activities of test compounds. Data represents mean ± SD. N=3.

reactants without test samples and B was absorbance of reactants with test samples. All the samples were run in triplicate and acarbose was taken as standard reference compound. Several dilutions of primary solution (5 mg/ml DMSO) were made and assayed accordingly to obtain concentration of the test sample required to inhibit 50% activity (IC_{50}) of the enzyme applying suitable regression analysis. The results of inhibition of α-glucosidase by oxovanadium (IV) complexes at various concentrations are presented graphically in Figure 3. Percentage inhibition at 200 µM for various compounds and IC_{50} values are collected in Table 2.

In-vivo experiment

In-vivo experiments for blood glucose lowering measurement were carried out on only one complex, viz., $VO(sal\text{-}mxyanl)_2$ 2c. Experiments were performed in Pharmacology Division of IICT Hyderabad, as per the animal ethical committee rules. Male Wistar rats (weight 195±10) were used for the *in-vivo* experiments. Animals were allowed for alternating 12 h light dark cycle at 22±1°C room temperature. Animals were kept for overnight fasting and next morning basal glucose value was measured by auto blood analyzer (Bayer Express Plus). The results of *in-vivo* experiments are shown in Figure 4 and the results of computation of AUC level have been presented in Figure 5.

RESULTS AND DISCUSSION

In-vitro α-glucosidase inhibition

α-Glucosidase inhibition data (percentage inhibition at 200 µM and IC_{50} values) for oxovanadium (IV) complexes are presented in Table 2. Table 2 also gives the data for uncoordinated (free) ligands and for vanadyl sulphate for the sake of comparison. The results of inhibition of α-glucosidase by oxovanadium (IV) complexes at various concentrations are also presented graphically in Figure 3. A perusal of the data reveals the following points:

i. Vanadyl sulphate itself is a poor α-glucosidase inhibitor; all the complexes show much higher inhibition.
ii. All oxovanadium (IV) complexes show much stronger α-glucosidase inhibition compared to the corresponding Schiff base ligands, except $VO(brsal\text{-}mxyanl)_2$ that shows almost same inhibitory potential as brsal-mxyanl.
iii. All complexes of the salicylaldehyde based Schiff bases are stronger α-glucosidase inhibitors compared to the corresponding complexes of bromosalicylaldehyde based Schiff bases.
iv. Among the complexes of salicylaldehyde based Schiff bases $VO(sal\text{-}mxyanl)_2$ and $VO(sal\text{-}ntranl)_2$ show nearly similar inhibition behaviour (IC_{50}=1.26 µM) and stand on top as α-glucosidase inhibitors.$VO(sal\text{-}anl)_2$ follows these two inhibitors and shows only slightly lower inhibition potential (IC_{50} =2.105 µM). Weakest inhibitor among these complexes is $VO(sal\text{-}clanl)_2$ with IC_{50} value of 13.12 µM. Among the complexes of bromosalicylaldehyde based Schiff base ligands $VO(brsal\text{-}clanl)_2$ is the weakest inhibitor and also weakest of all the complexes studied.

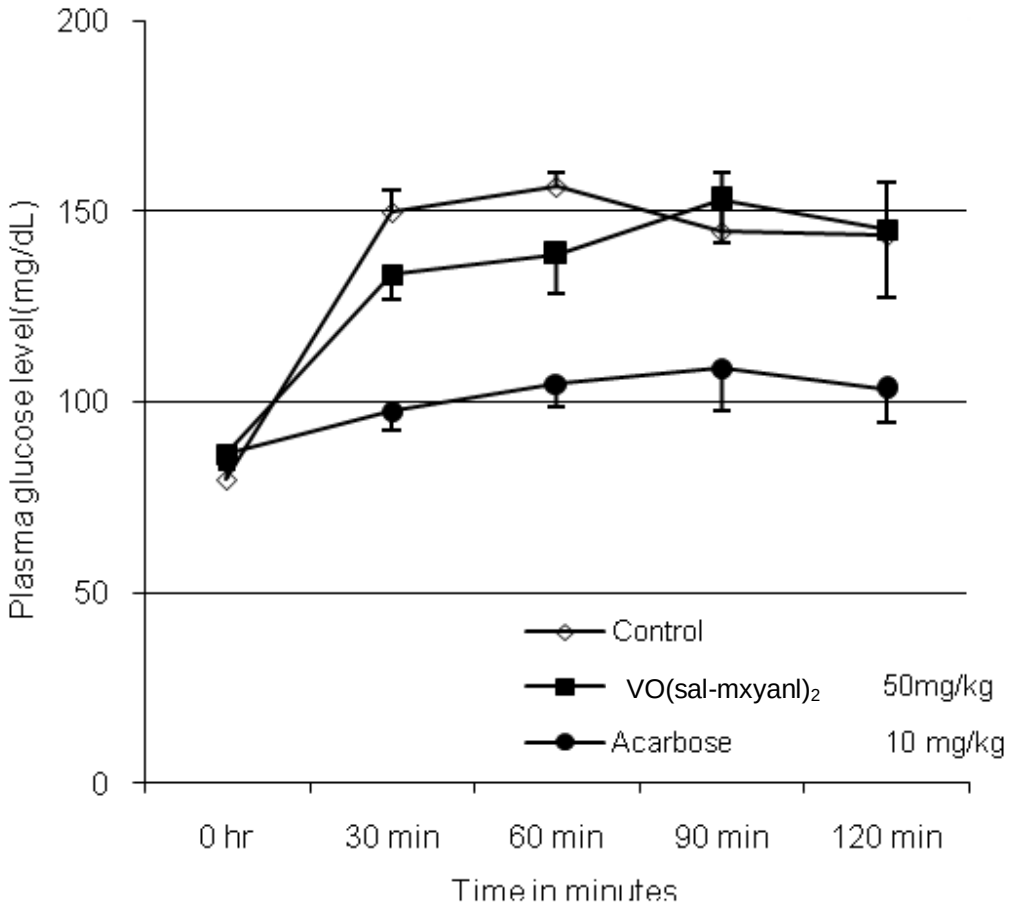

Figure 4. Plasma glucose level under the influence of VO(sal-mxyanl)$_2$ after starch feeding. VO(sal-mxyanl)$_2$ (50 mg/kg body weight) and standard α-glucosidase inhibitory antihyperglycemic drug acarbose (10 mg/kg body weight) were administered 15 min before starch (2 g/kg body weight) feeding to respective group of animals. Plasma glucose levels were measured at different time points post starch feeding. One-way ANOVA followed by Bonferroni's multiple comparison tests was applied to find differences between the groups. Values represent mean ± SD, n=5.

At 200 µM inhibitor concentration this complex shows percentage inhibition value of 43.5 only. Other three complexes of this series viz., VO(brsal-anl)$_2$ (IC_{50}=16.92 µM), VO(brsal-mxyanl)$_2$ (IC_{50} =92.43 µM) and VO(brsal-ntranl)$_2$ (IC_{50} =31.10 µM), show only moderate α-glucosidase inhibitory potential.

In Table 2, % inhibition at 200 µM of sal-ntranl has higher value compared with VO(sal-ntranl)$_2$, but the IC_{50} values of VO(sal-ntranl)$_2$ is much lower compared with sal-ntranl. One would normally expect that if the value of % inhibition at 200 µM was high, the IC_{50} value should be low. Our data in case of sal-ntranl and VO (sal-ntranl)$_2$ is reverse. Higher value of % inhibition, however, does not necessarily lead to lower value of IC_{50}. If the inhibitor concentration vs. % inhibition curves have different nature and cross each other, it may be reverse. Same is the case of sal-ntranl vs. VO(sal-ntranl)$_2$, presumably because of different modes of inhibitory action.

Lack of structural information about the nature of the interaction between α-glucosidase and the inhibitors has been a big hurdle in the interpretation of the available inhibition data in terms of the structures of the inhibitors. Zhou et al. (2006) and more recently Park et al. (2008) have carried out computational calculations yielding some important conclusions about the structural insight into the inhibitory mechanisms. Zhou et al. (2006) have established the importance of the capability of the inhibitor to form hydrogen bond with the catalytic residue of α-glucosidase in the inhibition action. Such hydrogen bonds may be formed between a hydrogen bond donor on the inhibitor and a hydrogen bond acceptor on the enzyme residue or between an acceptor on the inhibitor and a donor on the enzyme residue.

All the Schiff base ligands of the present study fall into the ‘non-glycosidic derivative’ category of de Melo et al. (de Melo et al., 2006) and contain phenyl hydroxyl group. Studies on structure activity relationship on such compounds (Niwa et al., 2003) have shown that this

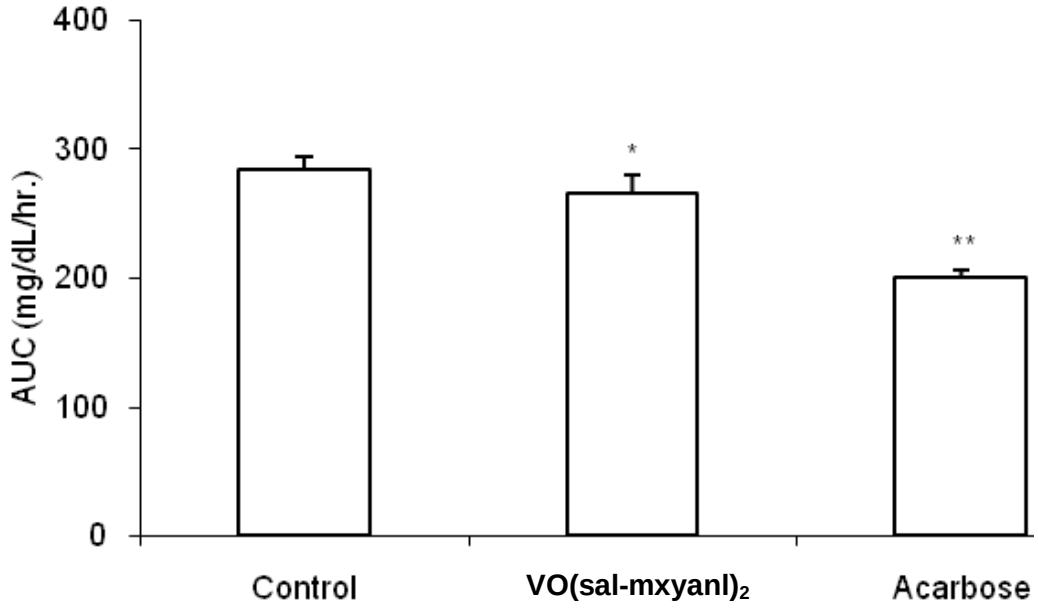

Figure 5. Area under the curve (AUC) represents over all plasma glucose load per hour in respective group of animals after starch feeding under the influence of VO(sal-mxyanl)$_2$ (50 mg/kg body weight) and standard α-glucosidase inhibitory antihyperglycemic drug acarbose (10 mg/kg body weight) administered 15 minutes before starch (2 g/kg body weight) feeding to respective group of animals. Plasma glucose levels were measured at different time points post starch feeding. AUC was calculated applying formula published earlier [39]. One-way ANOVA followed by Bonferroni's multiple comparison tests was applied to find differences between the groups. Values represent mean ± SD, n=5. *p<0.05; **p<0.001 when compared with control group.

phenyl hydroxyl group is fundamental for their inhibition activities. In the present study, variation in the inhibitory potential of the Schiff bases arises due to the substituent groups like bromide on the salicylaldehyde moiety and chloro, methoxy and nitro groups in the para position of the aniline moiety. These groups may influence the hydrogen bond donor capability of the phenyl hydroxyl group and may also act as hydrogen bond acceptors to appropriate hydrogen bond donors of the protein side chains.

Oxovanadium (IV) complexes show much stronger α-glucosidase inhibition compared to the corresponding ligands. For oxovanadium(IV) complexes as inhibitors, α-glucosidase may coordinate to the central vanadium ion at sixth (vacant) coordination position of the five coordinated VO-Schiff base complex (Figure 6). Cornman et al. (1995) have earlier suggested formation of such a bond between the metal ion and protein side chain for inhibition or activation of the enzymes. The inhibitors can be further stabilized in the active site through hydrogen bonds with catalytic residues and the establishment of hydrophobic contacts in a cooperative fashion. For acarbose and acarbose-type molecules as α-glucosidase inhibitors, for example, (Park et al., 2008) have shown that in α-glucosidase, side chain of Thr 215 acts as the hydrogen bond acceptor and the side chain hydroxyl group of Ser244 serves as a hydrogen bond donor.

In-vivo results

A close perusal of the diagram showing time evolution of the serum glucose levels after single oral administration of various compounds (Figure 4) and the AUC level diagram (Figure 5) reveal that, compared to the control, the blood glucose level in VO (sal-mxyanl)$_2$-fed rats is lower by nearly 12% between 30-60 min time interval. For acarbose-fed rats this lowering is nearly 35%. The antihyperglycemic property of VO(sal-mxyanl)$_2$ is, thus, not proportionate to its α-glucosidase inhibition property. At this point it may be mentioned that the α-glucosidase inhibition measurement is an *in-vitro* experiment, while the antihyperglycemic activity measurement is an *in-vivo* experiment. Inside the intestine of the animal the oxovanadium (IV) complex has to face the acidic environment which may cause dissociation of the complex (Kiss et al., 2008).

Conclusion

α-Glucosidase inhibitory potentials of three of the complexes viz., VO(sal-anl)$_2$ 2a, VO(sal-mxyanl)$_2$ 2c, VO(sal-ntranl)$_2$ 2d, are impressively high. Their IC_{50} values are smaller than the IC_{50} value of even acarbose (Table 3). *In-vivo* experiments carried out on one of the

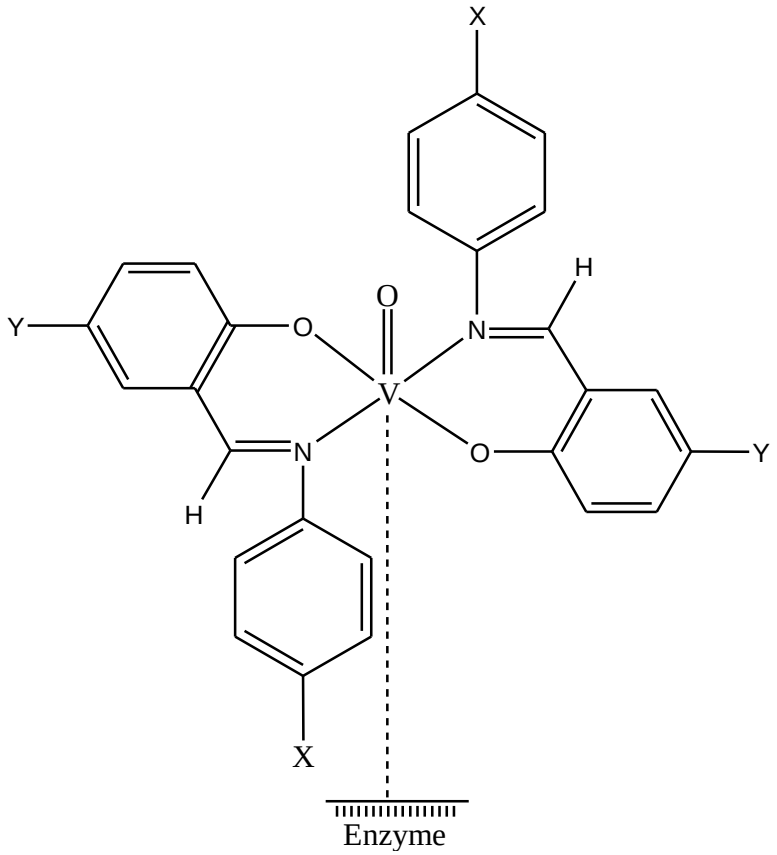

Figure 6. Binding of the α-glucosidase active site residue to the oxovanadium(IV) complex.

Table 3. Inhibitory potentials some complexes.

Inhibitor	IC_{50} (in µM)
Acarbose	18.59
2a, $VO(sal\text{-}anl)_2$	2.11
2c, $VO(sal\text{-}mxyanl)_2$	1.26
2d, $VO(sal\text{-}ntranl)_2$	1.26

complexes viz., $VO(sal\text{-}mxyanl)_2$ 2c, shows lowering in blood glucose level by nearly 12% compared to control, while this lowering for acarbose is nearly 35%.

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Full Length Research Paper

The acute cytotoxicity and lethal concentration (LC_{50}) of *Agaricus sylvaticus* through hemolytic activity on human erythrocyte

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There is limited information regarding acute toxicity and lethal concentration of edible and medicinal mushrooms. The objective of this paper is to estimate the cytotoxicity of the aqueous extract of *Agaricus sylvaticus* mushroom on human erythrocytes by determining the lethal average concentration (LC_{50}). Six concentrations of the mushroom (17, 8.5, 4.25, 2.125, 1.0625 and 0.5312 mg/mL) were submitted for evaluation of hemolytic activity *in vitro,* using a suspension of blood. Through the Prism GraphPad Software, using the Tukey test for statistical analysis ($p < 0.05$), a curve was constructed with values of *A. sylvaticus* mushroom concentrations versus the values determined by absorbance spectrophotometry at 540 nm. Results of hemolytic activity for the aqueous extract were fitted using nonlinear regression and the equation: Yi = axi / (b + Xi). We used values of y as hemolytic activity and x as log of *A. sylvaticus* mushroom concentration. The coefficient for determining the curve (R^2) was 0.95 of the original data. The percentage of haemolysis increased in a concentration-dependent manner of *A. sylvaticus* extract used. The LC_{50} value obtained was 9.213 mg/mL. Results derived from this experiment suggest that this mushroom extract has very low toxicity proving to be safe for human use.

Key words: Lethal concentration, *Agaricus sylvaticus,* hemolytic activity, sun mushroom.

INTRODUCTION

Chemicals used in therapy should be effective and provide safety (Goodman and Gilman, 2007). Unfortunately, any substance can be a toxic agent and cause undesirable effects (Goodman and Gilman, 2007; Oga, 2003), depending on the dose administered or absorbed, time and frequency of exposure and routes of administration (Oga, 2003). Highly toxic substances cause death at concentrations equivalent to a fraction of a microgram. In others, low toxicity may be almost harmless in concentrations of several grams or more (Goodman and Gilman, 2007; Oga, 2003).

The toxicity of a substance to an organism refers to its ability to cause serious injury or death. In therapy, the concentration of a substance should be enough to achieve the desired effect and achieve it well with the lowest concentration, and as much as possible, without producing adverse reactions or side effects (Oga, 2003).

The safety of drugs and foods should be determined through the analysis of several factors related not only to the individual characteristics of the organism, but also considering the physic-chemical, pharmacodynamic and pharmacokinetic of each substance, the various routes of exposure and different methods of administration

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(Silva, 2006).

Depending on the cultivation and composting, mushrooms can have varying levels of toxicity and risk to human health, although preliminary studies suggest that experimental use of *Agaricus sylvaticus* may present low toxicity. The use of this mushroom in folk medicine began in ancient peoples and between indigenous communities (Novaes et al., 2007).

The assessment of exposure can be performed by measuring the concentration of a substance administered to a particular organism (Oga, 2003). The study of concentration-response or concentration-effect in toxicology is essential and is used to determine the median lethal concentration (LC_{50}) of drugs and other chemicals (Goodman and Gilman, 2007).

The concentration-response curve is represented by the Gaussian theory, rarely found in practice. This curve is calculated statistically from observations of mortality after exposure related to concentrations of the substance to be tested, and it is widely used to calculate the 50% lethal concentration (LC_{50}). The LC_{50} is thus a statistical index which indicates the concentration of a chemical agent capable of causing death in 50% of organisms in a population with defined experimental conditions (Oga, 2003).

To know the effects of a toxic substance and classify them according to their potential lethality or toxicity and concentration-response curve, one needs to perform toxicological tests (Oga, 2003).

Mushrooms of the genus *Agaricus* have been widely studied for their nutritional characteristics and many medicinal properties they exhibit. The *A. sylvaticus* mushroom (Sun Mushroom) has been reported to have rich nutritional composition, with high protein content (41.16%), carbohydrates (36.21%), low lipid content (6.60%), considerable amounts of fiber (2.34%) and minerals (7.38%), besides having excellent antioxidant activity (Costa et al., 2011).

A. sylvaticus has been widely used as nutritional supplement for cancer patients, with likely effects of growth inhibition, tumor regression and stimulation of the immune system of patients.[4] According to recent studies there seems to be clear evidence of its immunomodulatory activity and efficacy against carcinogenic activity of the drug pristine (Hi et al., 2008).

There is also indication that dietary supplementation with *Agaricus sylvaticus* may reduce total cholesterol, LDL-C and triglycerides, with favorable outcome on lipid metabolism and, consequently, on the prognosis of patients with colorectal cancer in post-operative phase (Fortes et al., 2008). Furthermore, it has contributed to improve the quality of life of these patients by significantly reducing the harmful effects caused by the disease itself (Fortes et al., 2007).

The safety and effectiveness of medicinal plants and fungi are dependent on various factors, of these the quality of the product commercialized can be highlighted. Effectiveness and low toxicity to humans should be verified as well (Arnous et al., 2005).

In this context, the objective of this study is to evaluate the acute toxicity of *A. sylvaticus* mushroom aqueous extract *in vitro,* from the determination of lethal concentration (LC_{50}) through its hemolytic activity on human erythrocytes so as to refer the determination of toxicity parameters for human use.

METHODS

The experiment, in triplicate, was performed at the Nanotechnology Institute Laboratory of Biological Sciences, University of Brasilia, Brazil, in January and February 2011.

Obtaining the sample

The sample of dried *A. sylvaticus* mushroom (Sun Mushroom) was obtained from a producer in Minas Gerais State, Brazil.

Preparation of the solution containing the *A. sylvaticus* mushroom

We weighed 9.0 g of dehydrated *A. sylvaticus* mushroom and added to the sample 105 mL of distilled water. The solution was stirred for 20 min at room temperature, filtered through paper filter, and then 1000 µL of the solution was distributed into previously weighed Eppendorf tubes. The solution was lyophilized and the Eppendorf tubes were then weighed again, in order to obtain the average weight of the mushroom dissolved in water (17 mg/mL).

Serial dilutions were performed resulting in six concentrations for study: 17, 8.5, 4.25, 2.125, 1.0625 and 0.5312 mg/mL.

Preparation of erythrocyte suspension at 2% (human blood A-)

Erythrocytes were obtained from fresh A Negative type human blood. For erythrocyte suspension, 1 mL of blood was centrifuged for five minutes at 14000 rpm. Next 9.8 mL of saline solution (NaCl 150 mm) and 200 µL of the erythrocytes precipitate were added to the tube. The tube was then centrifuged for ten minutes at 2000 rpm. The supernatant was discarded and the process repeated three more times. Finally, the tube was shaken with the erythrocyte suspension ready for use.

Testing of hemolytic activity - Dose relation/hemolytic activity

Samples with 3 mL of saline solution + 500 µL of erythrocyte suspension + 500 µL of *Agaricus sylvaticus* extract were prepared in six different concentrations. The tubes were stirred manually and incubated at 35°C/60 min. After this interval, the tubes were centrifuged at 2500 rpm for ten minutes. The absorbance of the supernatant was read at 540 nm. The negative control (no haemolysis) was prepared only with saline solution and erythrocyte suspension, and the positive control (100% haemolysis) with 3 mL of distilled water + 500 µL of mushroom extract and a reading taken after 60 min.

We built graphics were built of the kinetics and of the dose-response relationship with mean values and standard deviation (SD). Data were expressed as percentage of viability in control wells, through the GraphPad Prism software, using the Tukey test for statistical analysis ($p < 0.05$).

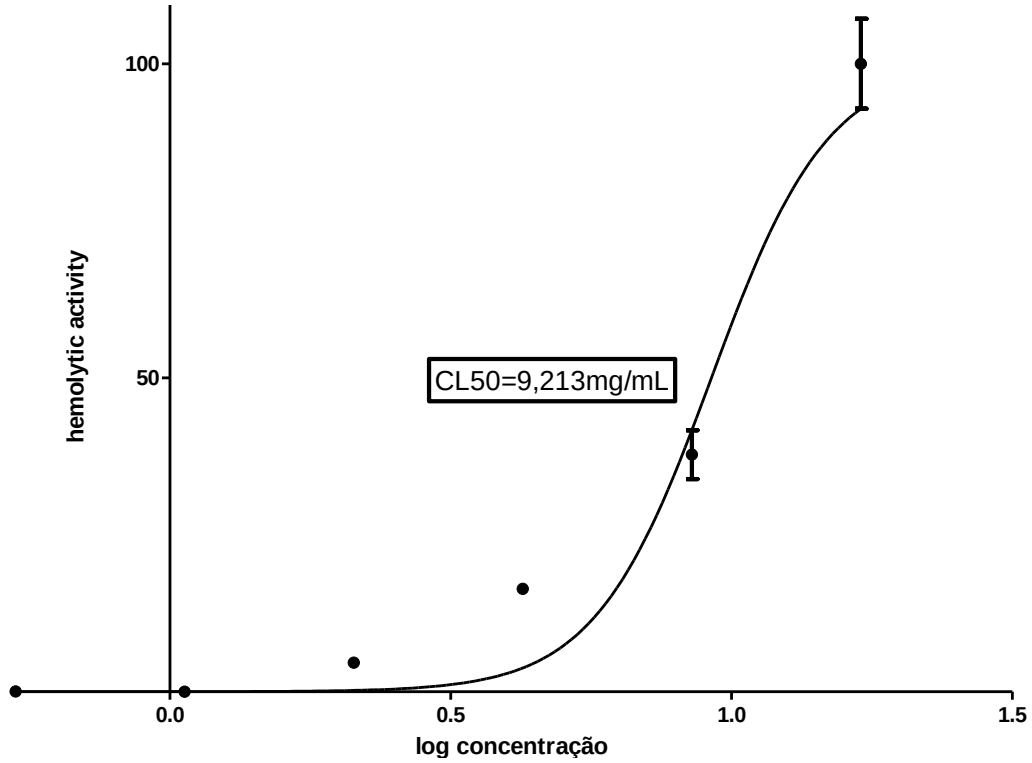

Figure 1. *In vitro* hemolytic activity presented by the aqueous extract of the mushroom *A. sylvaticus* at a 2% suspension of human erythrocytes incubated at 35°C for 60 minutes. The results presented correspond to the average of a test in triplicate.

The assessment of cytotoxicity through hemolytic activity tests has proved to be an alternative screening method for simple toxicity. It is fast, reproducible and inexpensive to evaluate erythrocyte hemolytic activity against concentrations of aqueous extract of *A. sylvaticus,* a fact making it possible to reduce the use of laboratory animals for *in vivo* tests, helping reach the goal to decrease, refine and replace studies conducted with animals.

The intent of reducing animals in the research and development of new methodologies in Brazil is timid and will require further discussion with participation of educational institutions and research laboratories together with the industry and regulatory agencies, since this reality affects all those involved in research, registration and approval of new substances.

As the focus of this article is to observe the acute cytotoxicity of mushroom extract, further studies are still necessary to investigate the mechanism of action of this extract and the possible organs or systems sensitive to the same, as well as additional studies on sub-acute and chronic toxicity, mutagenic and teratogenic activity, embriotoxicity and special studies particularly regarding the choice of concentrations of the extract, so as to validate its safety.

RESULTS

Evaluation of toxicity is paramount when considering a safe treatment. Haemolysis is characterized by erythrocytes rupturing with the release of hemoglobin. The *in vitro* haemolysis test is used as a method for substance toxicity screening, estimating any likely *in vivo* damage (Aparício et al., 2005).

Different aqueous extract concentrations of the *A. sylvaticus* mushroom were tested on a suspension of human erythrocytes at 2% and hemolytic activity determined as haemolysis percentage. We built a curve of concentration (µg of *A. sylvaticus* mushroom) versus percentage of haemolysis and concentration of the mushroom aqueous extract required to produce 50% haemolysis, known as 50% hemolytic concentration or 50% effective concentration (EC_{50}).

Test results of the hemolytic activity in tubes for the aqueous extract of *A. sylvaticus* mushroom were then adjusted using nonlinear regression, through the equation:

$$Yi = axi/(b + Xi).$$

The statistical analysis (Tukey test) was defined according to nonlinear fitting model using the Prism Software. To determine the curve we used the values of y as the hemolytic activity and x as the log of *A. sylvaticus* mushroom concentration. The coefficient for determining the curve (R^2) was 0.95 of the original data.

The percentage of haemolysis increased in a dependent-concentration manner of the extract of *A. sylvaticus* used. The LC_{50} value obtained in this experiment was 9.213 mg/mL.

The curve obtained (Figure 1) represents the hemolytic

activity of aqueous extract of the *A. sylvaticus* mushroom on the solution of human erythrocytes at 2%.

DISCUSSION

Several authors suggest that the exact calculation of LC_{50} is valid only for substances that pose a lethal concentration of 1 and 5000 mg/kg. However, regulatory international institutions of chemical composition toxicity recommend a limit of 2000 mg/kg for the LC_{50} test (Larini, 1997).

By determining the LC_{50} of aqueous extract from the *A. sylvaticus* mushroom, it was observed that this extract has low toxicity, since many grams are needed to cause cellular damage.

No study has been found in the literature using methods of cytotoxicity *in vitro* so that the extracts of this mushroom could be evaluated and compared. Nevertheless, the present results corroborate the results found by Novaes et al. (2007), where the effects of acute toxicity of the aqueous extract of this mushroom were assessed by clinical, biochemical and histopathological parameters in healthy mice, showing very low toxicity.

The low toxicity of this aqueous extract on erythrocytes may be related to the low toxicity of this extract found in animals, suggesting its potential for therapeutic purposes. But there are few studies in the literature regarding comparative sensitivity between these two methods (Cruz et al., 1998).

In 1927, Trevan suggested that lethal concentration should be considered when it kills 50% of the animals (LC_{50}) since the LC_{50} values vary less than those of LD_1 and LD_{99} (dosage required to kill 1 or 99% respectively of the test population) (Silva, 2006). Many toxicity tests currently used for assessment of toxic agents still employ laboratory animals (Harbell et al., 1997). However, the LC_{50} tests advocated by Trevan have been the subject of several reviews and discussions, especially of ethical nature, owing to the large number of animals sacrificed, the suffering caused during some tests, the imprecision of values obtained and the information it fails to provide (Silva, 2006; Cazarin et al., 2004).

Therefore, the completion of toxicological studies in animals with *in vitro* tests is a global trend (Cazarin et al., 2004). The development of new methods for *in vitro* toxicity testing and its recognition by international organizations such as the FDA (Food and Drug Administration) in 1983 and the OECD (Organization for Economic Cooperation and Development) in 1987 has fostered the replacement of tests using laboratory animals (Cruz et al., 1998; Cazarin et al., 2004).

These two organizations, further to promoting the improvement of toxicity tests, have been engaged in reducing costs and time spent in studies, decreasing and replacing animal use (Cazarin et al., 2004).

In this sense, there has been growing demand for *in vitro* tests, which do not sacrifice animals (13). The evaluation of *in vitro* hemolytic action has been used as screening methodology for various toxic agents (Kublik et al., 1996; Mehta et al., 1984). *In vitro* haemolysis tests have also been employed by several authors for the toxicological evaluation of different plants (Gandhi et al., 2000).

According to Queiroz (2009), laboratory experiments with cells reproduce the conditions and even reactions similar to those occurring in the body, and are thus able to observe and quantify changes undergone by cells from a particular product or medicament, as well as the behavior of each cell component separately, restricting the number of variables.

Ralph et al. (2009) through testing for hemolytic activity rated the degree of *in vitro* toxicity according to the observed mortality rate: 0 to 9% = non-toxic, 10 to 49% = slightly toxic, 50 to 89% = toxic; 90 to 100% = highly toxic. Therefore, for new studies to be conducted, the use of non-toxic concentrations (LC0-9) is suggested.

Arguing that the chemical and the pharmaceutical industry perform the LC_{50} test simply because it is required by authorities, in which case without any scientific justification, some authors propose replacing the LC_{50} with maximum non-lethal concentration (MNLC). The MNLC of a substance is defined as the maximum concentration which does not cause any mortality in a number of animals.

This indicator has been proposed as being more useful than the LC_{50} for evaluating the risk/safety of a product by the fact that it uses the non-occurrence of deaths (most severe of toxic effects) as analytical criterion (Larini, 1997). The maximum concentration is defined as the highest dose tolerated without toxic symptoms. The maximum lethal concentration refers to the smallest amount of drug capable of producing death. The therapeutic dose or effective dose is between the minimum and maximum therapeutic dose (Silva, 2006).

Silva et al. (2009) considering that a safe drug cannot cause injury to the plasma membrane of healthy cells, either by forming pores or breaking down the cell, evaluated the cytotoxic activity of triazoles on human erythrocytes. On the other hand, Ralph et al. (2009) evaluated the cytotoxicity of synthetic naphthoquinones on human erythrocytes, demonstrating the possibility of its use for therapeutic purposes, since it had no cytotoxicity on the human erythrocyte membrane.

The hemolytic activity test was also used by Maia et al. (2009), who evaluated the hemolytic activity of dry extract from the bark of *Maytenus guianensis*, verifying that this species did not cause haemolysis on human erythrocytes and may be used for pharmacological purposes.

Furthermore, Schulz et al. (2005) found positive values of the cytotoxic effect from crude extract of *Bacillus amyloliquefaciens* against sheep erythrocytes.

Vieira et al. (2002) in turn, using the hemolytic activity test to investigate the cytotoxic outcome of chloroform on

human lymphocytes, found results that do not prove the cytotoxic action of chloroform, but its genotoxic consequences, since it is capable of causing DNA damage without affecting the normal activity of cells.

Laranjeira et al. (2010) with the purpose of evaluating the hemolytic activity of ethanol extract from *Croton grewioides* leaves on erythrocytes from mice, found results that prove the absence of hemolytic activity on erythrocytes from these animals, suggesting that the cytotoxicity of the extract under analysis was not related to membrane damage, but rather related to apoptosis.

A study by Pita (2010) evaluated the cytotoxicity of natural products utilized in therapy against cancer, obtained from essential oil of *X. langsdorffiana* leaves (trachylobano-360 and OEX) on erythrocytes from mice. The author found values that show the reduced cytotoxic activity of these products.

Cazarini et al. (2004) points out that the *in vitro* alternative tests validated and accepted with regulatory purposes in substitution to methods performed on animals, are still much more a goal than a reality.

The scarcity of literature data to discuss the results and evaluation of acute cytotoxicity *in vitro,* reasserts the need for scientific research of this nature considering that they contribute greatly towards the safe use of such substances by humans.

Results derived from this experiment suggest that this mushroom extract has very low toxicity proving to be safe for human use.

Further study on the safety of using mushroom are needed, since *A. sylvaticus* has now been used for several diseases, including in therapy against cancer.

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