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Combined Cytotoxic Predictors of Morbidity

Highlights

Antioxidant Capacity Electrochemical Sensor

Discovering Thoughts, Inventing Future

VOLUME 14 ISSUE 2 VERSION 1.0

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A Study of Single and Combined Cytotoxic Effects of Fumonisin B1, Aflatoxin B1 and Ochratoxin a on Human Mononuclear Blood Cells using Different Cytotoxic Methods

By Mwanza Mulunda & Michael F Dutton
North West University , South Africa

Abstract - Several studies on cereals destined for animal or human consumption in South Africa and Africa have shown co-contaminations of mycotoxins. There is evidence to suggest that the simultaneous action of different mycotoxins at various concentrations might have synergistic, inhibitive or additive effects on human and animal cells. The aims of this study were to identify combined cytotoxic effects of three of the more commonly occurring mycotoxins (aflatoxin B1 (AFB1) fumonisin B1 (FB1) and ochratoxin A (OTA)) found in South African and other feeds using the methyl thiazoltetrazolium (MTT) cytotoxicity assay, the Comet assay and Flow Cytometry to detect apoptotic and necrotic cells after exposure to the three toxins. Human mononuclear blood cells (mononucleocytes) were exposed to the three mycotoxins both singularly and in combinations at two concentrations (5 and 40ng/ml for OTA and AFB1 and 5 and 40µg/ml for FB1) and at different exposure time of 12, 24 and 48Hours. Results obtained showed cell viability variations dependent on mycotoxin concentrations and time of exposure. In addition, synergistic effects were also observed at the higher doses of 40ng for OTA and AFB1) and 40µg/ml for FB1 of the three mycotoxins compared to combinations of lower doses.

Keywords: cytotoxicity, synergism, additive, mononucleocytes, mycototoxins, carcinogenic, aflatoxin b1, fumonisin b1, ochratoxin a.

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A Study of Single and Combined Cytotoxic Effects of Fumonisin B1, Aflatoxin B1 and Ochratoxin a on Human Mononuclear Blood Cells using Different Cytotoxic Methods

Mwanza Mulunda [α] & Michael F Dutton [σ]

Abstract- Several studies on cereals destined for animal or human consumption in South Africa and Africa have shown co-contaminations of mycotoxins. There is evidence to suggest that the simultaneous action of different mycotoxins at various concentrations might have synergistic, inhibitive or additive effects on human and animal cells. The aims of this study were to identify combined cytotoxic effects of three of the more commonly occurring mycotoxins (aflatoxin B1 (AFB1) fumonisin B1 (FB1) and ochratoxin A (OTA)) found in South African and other feeds using the methyl thiazoltetrazolium (MTT) cytotoxicity assay, the Comet assay and Flow Cytometry to detect apoptotic and necrotic cells after exposure to the three toxins. Human mononuclear blood cells (mononucleocytes) were exposed to the three mycotoxins both singularly and in combinations at two concentrations (5 and 40ng/ml for OTA and AFB1 and 5 and 40μg/ml for FB1) and at different exposure time of 12, 24 and 48Hours. Results obtained showed cell viability variations dependent on mycotoxin concentrations and time of exposure. In addition, synergistic effects were also observed at the higher doses of 40ng for OTA and AFB1) and 40μg/ml for FB1 of the three mycotoxins compared to combinations of lower doses. However, FB1 showed low cytotoxicity effect inducing inhibitive effect when combined with the other two mycotoxins. These results confirms the hypothesis of possibility of the three mycotoxins when combined induced with synergistic effect and imply that exposure to more than one mycotoxin might change the symptomatology and severity of effects observed during single intoxications by mycotoxins.

Keywords: cytotoxicity, synergism, additive, mononucleocytes, mycototoxins, carcinogenic, aflatoxin b1, fumonisin b1, ochratoxin a.

I. Introduction

Mycotoxins are toxic fungal metabolites that when ingested (main route of exposure), inhaled or absorbed through the skin, may pose varying negative health effects [1]. Mycotoxin effects and symptoms vary from one case to another because of their high variable structural chemistries and different toxicological properties [2]. Toxicological studies in vitro and in vivo conducted to establish the role of mycotoxins in causing diseases showed that there is prima facia evidence that a number of these fungal metabolites are involved in the aetiology of certain diseases and may be synergistic in action [1]. As such, a wide range of mycotoxin effects on animals and humans include: cytotoxic, nephrotoxic and neurotoxic, carcinogenic, mutagenic, immunosuppressive and oestrogenic effects [2]. Diseases caused by such exposures are generally referred to as mycotoxicoses, which can be acute and/or chronic depending on the level and period of exposure, although the precise effects may vary among species and to some extent, individuals. Disease conditions caused by mycotoxin actions are: impaired or retarded growth, immuno-suppression, general organ damage, various cancers and death [4]. In addition, synergistic effect between mycotoxins might affect the occurrence of diseases such as tuberculosis, malaria, kwashiorkor and HIV [4,5; 6] which are prevalent in Africa where exposure to dietary mycotoxins is common [7; 8]. This is because staple diets in many African households are based on cereals such as maize which is highly susceptible to AFs and FB contamination [8].

Structurally, fumonisin B1 (FB1) resembles sphingosine; an essential component of phospholipids found in cell membranes, responsible for cell signal transduction pathways, cell growth, differentiation and cell death [9] and hence may interfere with these functions. A specific toxic action of FB1 appears to result from its competition with sphingosine and sphinganine in sphingolipid metabolism, which results in blocking the synthesis of the sphingolipids, causing elevated sphingoid bases and depleting sphingolipids [10; 11]. A concern with FB1 exposure in humans is because of its carcinogenic properties demonstrated in rats [12]. Although there is evidence to suggest a close association between increased levels of FB1 in maize and high prevalence of human oesophageal cancer [13], the hypothesis that is involved in the aetiology of this disease has not been demonstrated in any animal

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spp. including primates and rats [14]. In addition, FB1 has been implicated in human liver cancer in Haimen, Jiangsu Province, China [15] and cardio-vascular disorders [16; 17]. Fumonisin B1 has also been implicated in the reduction of the uptake of folate in different cell lines and hence, been implicated in neural tube defects in human babies [18; 19; 20].

Aflatoxin B1 (AFB1) has been associated with liver cancer as well as kidney damage [21] and has been classified in Group 1 of carcinogens [21]. Aflatoxin B1 (AFB1) has been proven to be a cancer inducer via metabolic activation by cytochrome p540 specifically CYP3A4, CYP3A5 and/or CYP1A2 [22]. Approximately 55% of the hepato-carcinomas from areas where food is contaminated with AFB1 contain an AGG - AGT mutation at code 249 of the p53 tumour suppressor gene [24]. Aflatoxin B1 enters the cell and is metabolised either in the endoplasmic reticulum to hydroxylated metabolites that are further metabolised to glucuronide and sulphate conjugates or oxidised to the reactive epoxide that undergoes hydrolysis and can bind to proteins resulting in cytotoxicity [24]. Aflatoxin B1 is also immuno-suppressive and has been implicated in Reye's syndrome characterised by cerebral oedema and accompanied by fatty acid degeneration of the liver, kidneys, myocardium and fibres of the striated muscles [4]. Aflatoxin B1 affects the liver and is linked with kwashiorkor [7]. Studies have also shown the formation of aflatoxin-albumin adducts levels in children exposed to AFB1 contaminated milk [25].

Ochratoxin A is known as a potential serious carcinogen [26]. The primary toxic effects of OTA seems to be protein synthesis inhibition by the inhibition of enzymes acting on phenylalanine metabolism, lipid peroxidation and mitochondrial function [27] and/or may cause DNA single-strand breakages with a later stage genotoxicity and carcinogenicity [28]. Ochratoxin A immunotoxicity has also been demonstrated [28]. Possibilities of increasing mutagenicity in case of simultaneous occurrence of OTA with AFB1 in the same commodity have been reported [8]. Ochratoxin A has been implicated in the Balkan Endemic Nephropathy, a chronic progressive kidney disease, which is characterised by progressive hypercreatinaemia, uraemia, hypertension, oedema [29; 30]; acute renal failure and acute tubular necrosis [31; 32]. The rural populations in the Balkan states have a high incidence of chronic kidney problems and tumours of the excretory organs [33]. Despite the seriousness of the problem, studies have not completely elucidated the mechanism of action or extent of the carcinogenic potential of OTA in humans [34; 35].

Several studies conducted in South Africa, Nigeria, Bulgaria and other countries [2] have shown mycotoxins co-occurrence in food and feed. This co-occurrence may lead, in addition to their individual cytotoxicity effects, to completely modified clinical findings of the mycotoxicosis in consumers [1]. This can affect the body response in case of the treatment of chronic diseases such as diabetics, tuberculosis or other viral diseases and also in immune suppressed patients such as HIV/AIDS affected populations. It is important to mention that there are some variances with the manifestation of various mycotoxicoses, especially with the clinical or morphological pictures, which in many cases are influenced by the secondary bacterial infections as a result of the pronounced immunosuppression in the affected individuals [36].

II. Materials and Methods

a) Materials

All chemical and mycotoxin standards used in this study were of analytical grade unless otherwise stated and were obtained from Sigma/Hungary and South Africa; BD Bioscience and Sigma South Africa. A BD FACS Calibur automated multicolour Flow Cytometer was obtained from BD Biosciences, San Jose, USA. FB1 was obtained from the Medical Research Council (MRC) South Africa (PROMEC Division).

b) Methodology

In order to determine the cytotoxicity effects of the three mycotoxins (FB1, AFB1 and OTA), human mononuclear cells obtained from healthy male volunteers were exposed to different concentrations, i.e., dose 5 (dose 5 = 5 ng AFB1 and OTA and 5 μg/ml FB1) and dose 40 (dose 40 = 40 ng AFB1 and OTA and 40 μg/ml FB1) singly and in combination over 12, 24 and 48 hrs. Three methods were applied in this study to achieve the objectives:

c) Isolation and purification of mononuclear cells

For all experiments coducted in this study, cells (mononuclear) used were isolated and cultured for 24 hrs and then exposed to single and combined mycotoxins (FB1, AFB1 and OTA) according to the following protocol: Venous blood from a healthy human donor was put into 3x5 ml heparin tubes using a 15 ml sterile syringe with immediate transference. The collected blood was then mixed with an equal volume of tissue culture medium consisting of RPMI-1640 supplemented with 10% foetal calf serum (FCS), 100 U/ml Penicillin and 100μg/ml Streptomycin. The mixture was then overlaid on Histopaque 1077 and centrifuged at 800 g for 30 min and the interface layer consisting of mononuclear cells was carefully removed with a sterile pipette. The mononuclear cells (lymphocytes) were washed 3 times with 5 ml RPMI-1640 at 370C and each time centrifuged at 800 RPM for 10 min. The pelleted cells were re-suspended in 10 ml of complete culture media (CCM), transferred to plastic tissue culture bottles and were cultured at 370C in 5% CO2 humidified incubator for 24 hrs. The paleness of the CCM during

the incubation period confirms the growth of cells. In order to be certain to ascertain the presence and viability of cells, a cell count was done according to the following protocol and the experiment would be continuous only when the cell count was ≥ 95%. Cell suspension (100 µl) was mixed with 100 µl of 0.2% Trypan Blue solution in an Eppendorftube and incubated for 5-10 min at room temperature. A small amount of the trypan blue- cell suspension mixture was transferred to both chambers of Neubauer counting chamber (Haemocytometer) with a cover glass in place using a sterile Pasteur pipette. A cell count was done and% viability was determined as:

% Viability = (viable cell counted (dye excluded)/total no. of cells) x 100

d) Methyl Tetrazolium (MTT) assay

The exposed cells were examined by application of the Methyl Tetrazolium (MTT) assay for cell viability; Comet assay for DNA damage and Flow cytometry after treatment with the Annexin V and APO-BRDU reagents for apoptosis, necrosis induction and DNA cleavage. For all the experiments conducted in this study, cells (PBMC) used were isolated from blood samples obtained from volunteer healthy male donors (within 30 minutes after collection) cultured for 24 hrs and then exposed to single and combined mycotoxins (FB1, AFB1 and OTA) and placed in a sterile 5% CO2 incubator for (12, 24 and 48hrs) according to Mwanza [36] and Meky [37]. Isolated PBMCs were counted and placed in a 96-well culture plates containing 100µl of culture medium. The MTT assay was also done according to Mwanza [36] and Meky [37].

e) Comet Assay

The DNA damage assessment carried out using the comet assay technique was done according to Singh [38] and Collins [39]. The following solutions and methods were used to achieve the objectives.1% NMP was prepared by dissolving 0.5 g in 50 ml PBS in a beaker and heating it in a microwave until at boiling point with occasional mixing until it completely dissolved. New slides were coated with warm 1% NMP Agarose gel prepared by dipping the slide vertically into the melted warm 1% NMP gel in a beaker. Excess Agarose was drained off the slide and the back cleaned and dried in a warm oven overnight. About 140 µl of 1% warm LMP Agarose gel (370C) in PBS prepared as above was added to the cells obtained after centrifugation following mutagenic treatments (Section 5.2.2 above). The suspension was mixed gently and quickly and 70 µl transferred to two spots on the previously coated slides above. The suspensions were covered with cover slips (20X20 mm) and allowed to set for least 10 min in the cold room (40C). The cover slips were removed and mononuclear cellular membranes were lysed with lysing buffer solution (pH 10) for 1 hour at 40C. The resulting nucleoids were unwound in alkaline electrophoresis buffer (pH13) in an electrophoresis tank (Amersham Pharmacia Biotech) for 40 min at 40C and followed by electrophoresis at 25 V (300 mA) for 30 min at 40C. The slides were placed in neutralizing buffer (pH 7.5) with 3 washes of 5 min each and finally washed in distilled water for 5 min. The gels were allowed to dry overnight, stained with 30 µl of working solution of DAPI, covered with a slipped and examined by an Olympus BH-RFCA Epifluorescent Microscope (Wirsam Scientific).

Manual scoring was done following the methods of Singh [38] and Collins [39] in which comets were classified into '0', '1', '2', '3' and '4' according to the DNA damage and head/tail migration using a fluorescent microscope. A minimum of 100 cells per samples were scored according to the movement or shape of the comet formed [40]. A positive response was considered as one in which, there was a dose-related change in the defined measurement between the control and test groups at single sampling time [40]. A total score of damage for each gel was calculated by multiplying the number of comets assigned to each class of damage by its own value to give a summative total overall score and expressed in arbitrary units. In order to present the rationalised total toxicity score, the arbitrary score was recalculated using the formula below:

((((100-mean % viability)*5) + 5mean% viability/100)*raw arbitrary score)))

A study of levels of apoptosis and DNA cleavage induction by AFB1, FB1 and OTA singularly and in combination was done using the FITC Annexin V apoptosis detection kit II, Lot. 35856 and the APO-BRDU™ Kit obtained from BD Biosciences, San Diego, USA and supplied by Merck was used for staining of already treated cells and analysed on Flow Cytometry.

f) Study of Cell death on flow cytometry

The Flow cytometer used in this study was a BD FACS Calibur automated multicolour flow cytometer (BD Biosciences, San Jose, USA) at excitation at 488 nm, using a 639 nm band pass filter to collect the red propidium iodide fluorescence. Cells used in this study were extracted and exposed to mycotoxins according to the description mentioned for the MTT and Comet assays. Prepared and already exposed cells were then subjected to following steps before the analysis on flow Cytometry. Cell fixation using Para-formaldehyde consisted of 7 steps; cells were suspended in 1% (w/v) paraformaldehyde in PBS (pH 7.4) at a concentration of

1-2 x 106 cells/ml and placed on ice for 30-60 min. Cells were then centrifuged for 5 min at 300 x g and supernatant discarded. Cells were washed by centrifugation in 5 ml of PBS twice. The cell pellet was re-suspended in the residual PBS in the tube by gently vortexing. The cell concentration was then adjusted to 1-2 x 106 cells/ml in 70% (v/v) ice cold ethanol and the cells were left to stand for a minimum of 30 min on ice or in a freezer. These were then stored in 70% (v/v) ethanol at -20°C until use. Cells can be stored at -20°C several days before use. Eppendorf tubes containing cells exposed to mycotoxins and cells with positive and negative controls were centrifuged for 5 min at 300 x g and the 70% (v/v) ethanol was removed by aspiration without disturbing the cell pellets. The cell pellet wasthen re-suspended with 1.0 ml of Wash Buffer and centrifuged as before and the supernatant was removed by aspiration. This was repeated twice. Each tube was re-suspended in 50µl of the DNA labelling solution prepared earlier as described in the kit instructions and incubated for 60 min at 37°C in a temperature-controlled bath. Every 15 min the tubes were shaken to re-suspend the cells. At the end of the incubation time, 1.0 ml of the rinse buffer was added to each tube and centrifuged at 300 x g for 5 min and the supernatant removed by aspiration. This was repeated a second time. The cell pellets were re-suspended in 0.1 ml of the Antibody Staining Solution prepared before as described in the kit instructions and incubated with the FITC-labelled anti-BrdU Antibody Solution (50*µ*l) in the dark for 30 min at room temperature. Finally, 0.5 ml of the PI/RNase Staining Buffer was added to the tube containing the 0.1 ml Antibody Staining Solution and the cells incubated in the dark for 30 min at room temperature. The assay was run on the flow cytometer equipped with a 488 nm Argon laser as the light source. Propidium I fluoresces at about 623 nm and FITC at 520 nm when excited at 488 nm. No fluorescence compensation was required. Two dual parameter and two single parameter displays were created with the flow cytometer data acquisition software. The gating display was the standard dual parameter DNA doublet discrimination display with the DNA Area signal on the Y-axis and the DNA Width on the X-axis. From the display, a gate was drawn around the non-clumped cells and the second gated dual parameter display was generated. The DNA (Linear Red Fluorescence) was displayed on the X-axis and the FITC-BrdU (Log Green Fluorescence) on the Y-axis. Two single parameters gated histograms, DNA and FITC-BrdUwere also added to determine apoptotic cells and their cell cycle stages. In all, three studies positive and negative control cells were included to each experiment with negative control being cells treated in the same conditions the rest of the experiment but not exposed to any of the toxins and the positive control cells treated in similar conditions as but treated with hydrogen peroxide known to induce cell death.The percentage of cells in each stage of the cell cycle was automatically calculated and generated on Flow cytometry BD FACS Comp™ in conjunction with BD Calibrite™ beads, software.

g) Regression analysis

To conclude this study, regression analysis were done in order to studythe relationship between different methods used in this study and predict cytotoxicity levels of mycotoxins by using results obtained fromeach study as dependent while data obtained from other methods were used as independent variables.

h) Statistical analysis

The results obtained were analysed using the Sigma Stat11.0 software package. A one-way and multiple comparison analysis variances and the comparison of results between groups were done using the Holm-Sidak method.

i) Ethical clearance

The ethical clearance number 09/08 was obtained from the ethical committee of the Faculty of Health Sciences, University of Johannesburg to undertake this study.

III. Results

MTT assay results as shown in Tables 1 and 2 and illustrated in Figure 1 show the effect of exposure time versus mycotoxin concentration as it influences the curves representing mononuclear cell viability after exposure to single or combined mycotoxins (AFB1, OTA and FB1) are shown. The exposure of untreated cells with mycotoxins induced cell viability decreased from 99-97 % for untreated cells. In this study, the untreated cells were considered as control and constituted 100%. The exposure of cells with FB1 gave 90-84% and 87-81% of cell viability; 91-80% and 85-73% for AFB1; 85-78% and 83-69% for OTA respectively after dose 5 (dose 5 = 5 ng AFB1 and OTA and 5 *µ*/ml FB1) and 40 (dose 40 = 40 ng AFB1 and OTA and 40 *µ*g/ml FB1) of mycotoxins concentration were exposed to and this between 12 and 48hrs of exposure. The combination of the three mycotoxins show a great decrease in viability as compared respectively to the combined two and single exposure with cell viability decrease up reaching 85-82% and 42-31% for OTA-FB1; 82-73% and 42-32% for OTA-AFB1 and 85-78% and 43-33% between 12 and 48hrs of exposure at respectively 5 and 40*µ*g/ml of mycotoxins concentration (Table 1). The exposure to all three mycotoxins show a decrease in cell viability reaching up to 77-73% and 39-26 % of viable cells respectively at doses 5 and 40 of each toxin added of mycotoxins concentration between the same times of exposure. It was observed that singly, OTA induces the biggest decrease of cell viability, followed by AFB1 and then FB1 (Table 1 and Fig. 1).The results on cell inhibition (Table 2) obtained from the MTT assay are

comparable to calculated results expected by adding individual results of each mycotoxin (FB1, OTA and AFB1) (Table 1). In addition, these results have shown that there is a dose dependentsynergetic effect depending on concentrations with the of the reaction being when the three mycotoxins combined at the dose 40 compared to low dose 5 when combined in twos (Table 1). It is important to mention the increased cell viability noted at dose 5 of all 3 mycotoxins at 24 hrs before a drop at 48 hrs (Figure 1). This increase could be explained by the fact that MTT assay is based on NDH cell activity reading, suspicion that at low dose an antagonistic effect could have been induced between the 3 mycotoxins that led to the NDH reduction potential redox leading to the production of NDH2-FAD causing antagonistic effects to mycotoxins activity at 24 hrs and then was reversed after a much longer exposure. Statistically, there were significant differences ($P<0.050$) found among data of three mycotoxins when exposed singularly over time (12, 24and 48hrs) and between data obtained at different concentrations of exposure. Significant differences ($P<0.001$) were obtained among data from combined mycotoxins and among data from all three mycotoxins combined, combination of two mycotoxins and single mycotoxins over time and concentrations.

Results obtained with Comet assay revealed a time and dose dependent response after mononuclear cells are exposed to different mycotoxins (AFB1, OTA, and FB1) singularly or in combination and are summarised in Table3 and illustrated in Fig. 3. In this study, the score of 400 and above means the toxicity induced the death of all cells. Scores obtained with cells exposed to single mycotoxins indicated little effect on DNA as evidenced by the low scores. Cells exposed to FB1 alone showed lower scores of 37-80 and 175-231 respectively at 5μg/ml and 40 μg/ml between 12 and 48 hrs exposure; this is in comparison with AFB1 with scores of 50-90 and 169-253 and OTA with scores of 64-107 and 169-253 respectively at 5μg/ml and 40 μg/ml between 12 and 48 hrs of exposure. Similarly to the results obtained with the MTT cytotoxicity test, results obtained with mycotoxins combinations in twos were higher compared to single ones. the OTA-FB1 combination scores were 70-114 and 211-263, FB1-AFB1 were 75-120 and 206-265; OTA-AFB1 with 82-130 and 219-279 while the combination of all three mycotoxins gave scores of 110-132 and 217-284 respectively at doses 5 and 40 between 12 and 48 hrs of exposure. Statistical analysis revealed thatcomet assay results showed significant differences ($P<0.001$) found between data obtained with the three mycotoxins when exposed singularly over time at 12, 24 and 48hrs and between data obtained at the two different concentrations of exposure, as would be expected. Significant differences ($P<0.050$) were obtained among data from two combined mycotoxins and among data from all three mycotoxins combined, combination of two mycotoxins and single mycotoxins over time and concentrations. In addition, significant differences were obtained between data obtained from single mycotoxins and all three mycotoxins exposure ($P<0.001$). Among mixture data, significant differences ($P<0.050$) were between all data obtained after 12 and 48hrs and between 12 and 24hrs of cell exposure.

However, no differences were obtained between 24 and 48hrs with both concentrations (5 and 40). The absence of significant differences between 24 and 48hrs of exposure confirms as well the observations made with MTT assay and this finding shows as well that after 24hrs of exposure, there is a decrease of cytotoxicity induction after 24hrs reaching saturation between 24 and 48 hrs of cell exposure. Results obtained from the investigation into mycotoxins induction of apoptosis Table 4 and DNA cleavage Table 5 on mononuclear cells using a flow cytometer revealed a time-dose dependent increase of cell apoptosis after exposure to different mycotoxins (AFB1, OTA, and FB1) singularly or in combination. It was observed that FB1 single exposure induced apoptosis on mononuclear cells at 6-15% and 19-46% respectively at dose 5 and 40 between 12 to 48 hrs; AFB1 induced apoptosis at 8-23% and 23-58%, while OTA cells apoptotic induction varied between 11-16% and 26-69% of exposed cells respectively at 5 and 40 μg/ml for FB1 and for the rest mycotoxins at 5 or 40 ng/ml between 12 and 48 hrs of exposure.

These results correlate with those obtained with MTT trail and comet assay, results obtained with mycotoxins combinations in twos were higher compared to single ones. The combination of OTA-FB1 showed results varying between 13-45% and 39-79% of apoptotic cells; while FB1-AFB1 combination produced 12-46% and 41-71% of apoptosis and OTA-AFB1 combination results were 15-53% and 41-87%. The three mycotoxins combined induced apoptosis at 17-56% and 44-95% respectively at dose 5 and 40 between 12 and 48 hrs of exposure.Similar to the results obtained with the comet assay analysis, the apoptosis data analysis showed significant differences ($P<0.050$) between MTT assay data obtained from the exposure of cells with the three mycotoxins and each mycotoxin singularly over time (12, 24 and 48hrs) and between data obtained at different concentrations of exposure. Significant differences ($P<0.050$) were obtained among data from combined mycotoxins and data from all three mycotoxins combined, between combination of two mycotoxins and single mycotoxins over time and concentrations. Significant differences were obtained between data obtained from single mycotoxins and all three mycotoxins exposure ($P<0.001$). Among mixture data, significant differences ($P<0.050$) were among data obtained after 12 and 48hrs and between 12 and

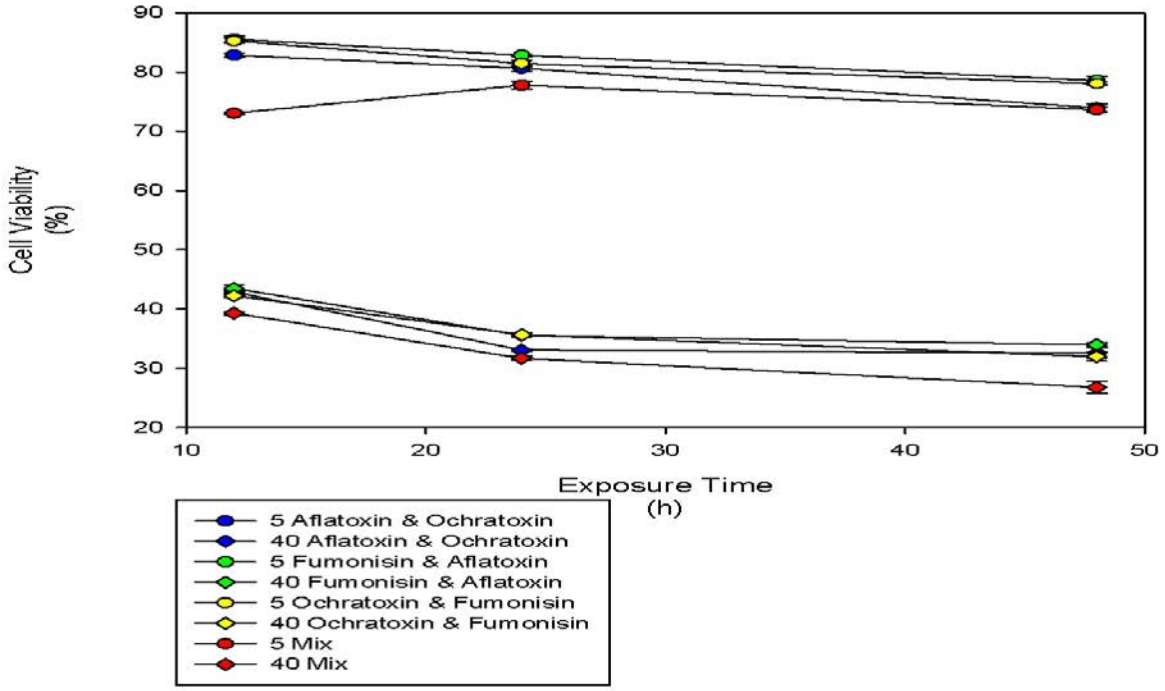

Figure 1 : comparative study of cell viability between cells exposed to mycotoxins combined in twos and cells exposed to all three mycotoxins combined by the Methyl Tetrazolium assay.

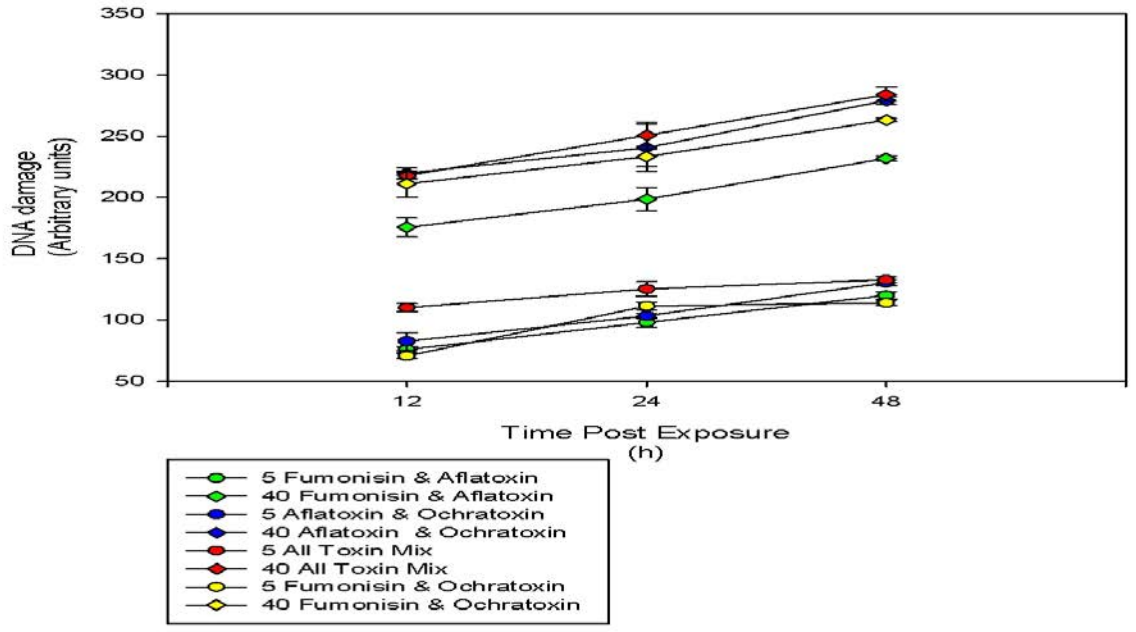

Figure 2 : A comparative study of DNA damage between cells exposed to mycotoxins combined in twos and cells exposed to all three mycotoxins combined by the Comet assay.

24hrs of cell exposure. However, no differences were obtained between 24 and 48hrs with both concentrations (5 and 40). The absence of significant differences between 24 and 48hrs of exposure confirms as well the observations made with MTT assay and this finding shows well that after 24hrs of exposure there is a decrease of cytotoxicity induction after 24hrs reaching saturation between 24 and 48 hrs of cell exposure. Figures 7-10 show the apoptosis induction on mononuclear cell layout illustrations by flow cytometry for FB1, AFB1 and OTA combined in twos and all three together after 24hrs of incubation.One of the later steps in apoptosis is DNA fragmentation, a process which results from the activation of endonucleases during the apoptotic programme. The comet assay scoring method being subjective, as this scoring of DNA damage was done arbitrarily based on visual judgement. The flow cytometer was then used to evaluate and confirm the DNA damage (DNA cleavage) of mononuclear cells previously done on comet assay and the mean percentage of apoptotic cells undergoing DNA cleavage due to mycotoxins exposure was obtained by flow cytometry. The results obtained from this study are summarized in Table 3 and illustrated in Figure 11 and Figure 12. Single and combined mycotoxins (AFB1, OTA, and FB1) exposure to mononuclear cells showed an induction of DNA cleavage which was time and dose dependant. Single mycotoxin exposed with cells showed lower cytotoxicity effects compared to combined mycotoxins.

Table 1 : Summary results of mononuclear % cell viability after mycotoxins exposures at 12, 24 and 48 hours exposure to aflatoxin B1, ochratoxin A and Fumonisin B1 by methyl tetrazolium assay (MTT).

Mycotoxins + Conc*.	Exposure time (hours)	Mean (% cell viability)	Std Dev	SEM
FB1 5	12hrs	90.8	0.3	0.1
FB1 5	24hrs	88.1	0.7	0.4
FB1 5	48hrs	84.1	0.3	0.1
FB1 40	12hrs	87.0	0.3	0.2
FB1 40	24hrs	80.0	0.6	0.3
FB1 40	48hrs	81.7	1.0	0.6
AFB 5	12hrs	91.1	0.8	0.4
AFB 5	24hrs	86.5	0.8	0.5
AFB 5	48hrs	90.2	0.6	0.3
AFB 40	12hrs	85.8	0.5	0.3
AFB 40	24hrs	74.8	0.7	0.4
AFB 40	48hrs	73.1	0.4	0.2
OTA 5	12hrs	91.1	0.9	0.5
OTA 5	48hrs	83.3	1.0	0.5
OTA 5	24hrs	88.1	1.4	0.8
OTA 40	12hrs	83.1	0.6	0.3
OTA 40	24hrs	72.9	0.5	0.2
OTA 40	48hrs	69.9	0.4	0.2
OTA-FB1 5	12hrs	85.2	0.6	0.3
OTA-FB1 5	24hrs	81.4	0.9	0.5
OTA-FB1 5	48hrs	78.0	0.5	0.3
OTA-AFB1 5	12hrs	82.8	0.6	0.3
OTA-AFB1 5	24hrs	80.6	0.9	0.5
OTA-AFB1 5	48hrs	73.9	1.0	0.6
FB1-AFB1 5	12hrs	85.5	0.9	0.5
FB1-AFB1 5	24hrs	82.8	0.3	0.2
FB1-AFB1 5	48hrs	78.6	1.1	0.6
OTA-FB1 40	12hrs	42.2	0.5	0.2
OTA-FB1 40	24hrs	35.6	0.5	0.3
OTA-FB1 40	48hrs	31.9	1.3	0.7
OTA-AFB1 40	12hrs	42.8	0.6	0.4

OTA-AFB1	40	24hrs	33.0	0.3	0.2
OTA-AFB1	40	48hrs	32.5	1.5	0.8
FB1-AFB1	40	12hrs	43.4	1.1	0.6
FB1-AFB1	40	24hrs	35.5	0.4	0.2
FB1-AFB1	40	48hrs	33.9	0.6	0.3
MIXTURE	5	12hrs	79.0	0.3	0.1
MIXTURE	5	24hrs	77.8	1.1	0.6
MIXTURE	5	48hrs	73.6	0.7	0.4
MIXTURE	40	12hrs	39.3	0.4	0.2
MIXTURE	40	24hrs	31.6	0.5	0.3
MIXTURE	40	48hrs	26.7	1.7	0.9

*Individual and mixtures concentrations of aflatoxins and ochratoxins concentrations are in (ng/ml) and fumonisin B1 concentration is in (µg/ml).

Table 2 : Inhibition (%) of isolated peripheral blood mononuclear cells by mycotoxins at various times and concentrations of exposure by Methyl Tetrazolium assay.

Time (hr)	Aflatoxin B_1		Fumonisin B_1		Ochratoxin A	
	5µg/ml	40µg/ml	5µg/ml	40µg/ml	5µg/ml	40µg/ml
12	9	14	10	13	9	17
24	13	25	12	20	17	28
48	10	27	16	19	12	30

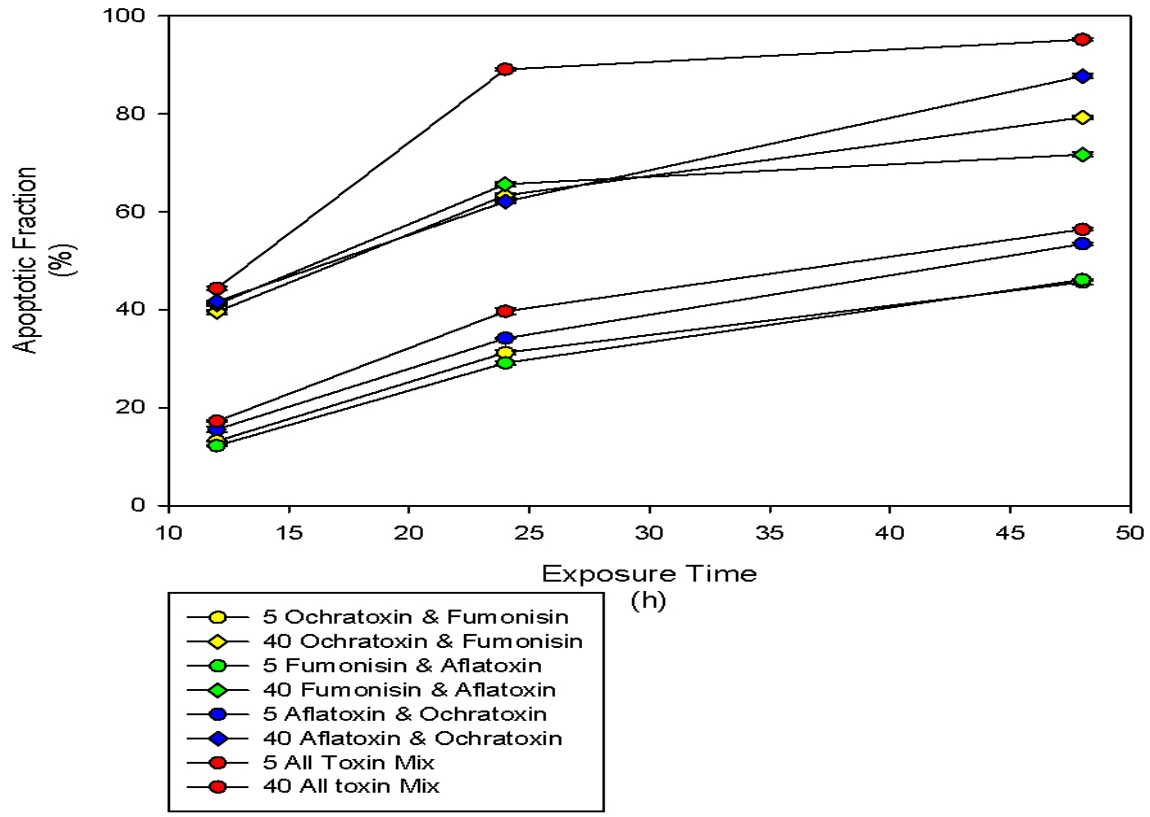

Figure 3 : comparison study of cell apoptosis induction between cells exposed to mycotoxins combined in twos and cells exposed to all three mycotoxins combined by flow cytometry.

Fumonisin B1, when exposed singularly, induced mononuclear cell's DNA cleavage in 4-13 % and 18-38% respectively at dose 5 and 40 between 12 to 48 hrs; AFB1 induced apoptosis at 5-21% and 21-55%, while OTA DNA cleavage inductions were 8-19% and 19-58% of exposed cells respectively at dose 5 and 40 between 12 and 48 hrs of exposure. These results correlate with those obtained with MTT cytotoxicity assay and comet assay, results obtained with mycotoxins combinations in twos were higher compared to single ones.

The combination of OTA-FB1 showed DNA cleavage induction in 12-43% and 37-71% of apoptotic cells; while FB1-AFB1 combination produced 10-41% and 39-69% of cleaved DNA and OTA-AFB1 combination results were of 13-49% and 37-86%. The three mycotoxins combined revealed DNA cleavage at 15-59% and 42-92% respectively at dose 5 and 40 at 12, 24 and 48 hrs of exposure. In this study, significant differences ($P<0.050$) were obtained among single mycotoxins, combined in twos, single and combined in twos, all groups at dose 5 and 40, all groups at 12, 24 and 48hrs of exposure. In addition, significant differences ($P<0.001$) were seen in all studied groups at dose 5 and 40. Figure 2 illustrates a layout of the DNA cleavage (%) of apoptotic mononuclear cells by Flow Cytometer for FB1, AFB1 and OTA combined in twos and all three together after 24hours of incubation.

Results obtained with linear regression studies showed correlations between all three techniques. Two linear regression analyses were plotted and the first one (Figure 4) used the apoptosis data as the dependent variable while MTT data was considered as the independent one. A linear regression equation was obtained as well as a regression coefficient ($R = 0.810$). Although not perfect, it follows the linear regression equation: This shows that

$$\text{APOPTOSIS} = 101.645 - (0.897 * \text{MTT \% CELL VIABILITY})$$

Finally a multiple linear regression analysis was done (Figure 5) to assess if the toxicity can be predicted using DNA cleavage data as a dependant variable and data from the other three test (Comet assay; Apoptosis and MTT % cell viability) as independent variable.

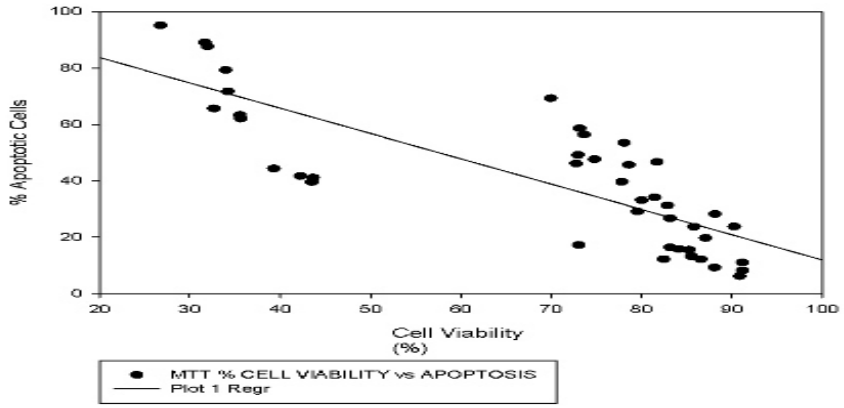

Figure 5 : Illustration of a linear regression curve of the tythe Methyl Tetrazolium (MTT) cytotoxicity assay vs apoptosis by Flow Cytometry

The second regression study was the one in which DNA cleavage data was considered as dependent variable while comet assay data was considered as the independent one. As in the first study, the regression ($R = 0.853$) coefficient was lower than the ideal which needed to be next to 1. This low regression coefficient can be explained by data variability within different methods.

$$\text{DNA cleavage} = -6.434 + (0.280 * \text{COMET ASSAY})$$

This study shows a positive regression coefficient ($R = 0.994$) and a regression equation was obtained. The regression coefficient R obtained here was of about ± 1 revealing that in this study, one can predict toxicity induced by AFB1, OTA and FB1 using one of the four methods used in this study but in addition, confirmed the correlation between the three methods. Statistically, all independent variables appear to contribute to predicting DNA cleavage ($P < 0.050$).

DNA CLEAVAGE = 10.373 + (1.002 * APOPTOSIS) - (0.0305 * COMET ASSAY) - (0.114 * MTT % CELL VIABILITY)

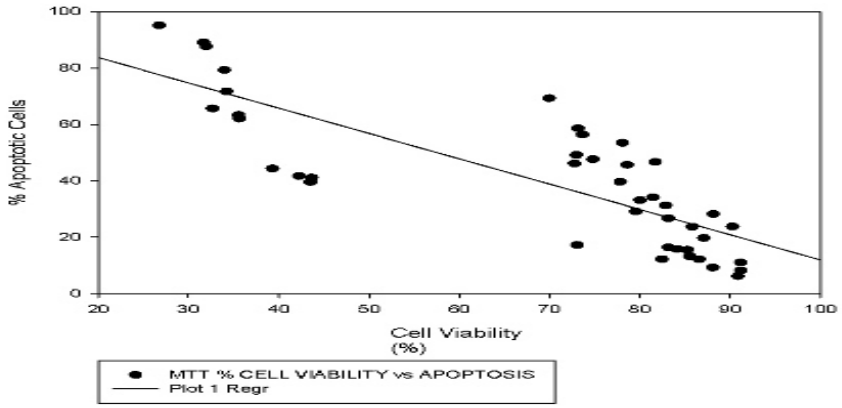

Figure 5 : Illustration of a linear regression curve of the tythe Methyl Tetrazolium (MTT) cytotoxicity assay vs apoptosis by Flow Cytometry.

Statistical analysis showed that the dependent variable of the DNA cleavage can be predicted from a linear combination of the independent variables: Apoptosis, Comet Assay, MTT % cell viability ($P<0.001$).

IV. Discussion

The choice of peripheral blood mononuclear cell (PBMC) in this study is justified by their composition including mainly lymphocytes consisting of T cells (CD4 and CD8 positive ~75%), B cells and NK cells (~25% combined) and monocytes and macrophage because of their critical role in the immune system to fight infection and adapt to infections in the first place[41].

It was noted in this study that at dose 5 of exposure in all combinations, no synergistic effect was observed. However, at low doses, only chronic exposure (24hrs) of cells to mycotoxins was needed in order to be able to observe significant toxicity. These confirm results obtained by Timbrell[46]who also found similar results in his studies on cell toxicity.

The absence of additive effects when two or three mycotoxins were combined as observed in this study and confirmed in all three methods can be explained by a possible inhibition or competition among these mycotoxins. There are evidences that FB1 when combined with OTA or AF1 induced low cytotoxicity in comparison to the OTA and AFB1 combination. This inhibitive effect of FB1 might be explained by the mode of action and low toxicity. These results are similar to those obtained by Mwanza et al [36] on MTT cells. Theinhibition of FB1 on the two other mycotoxins used in this study might explain also the absence of additive rather than synergistic effect when the three mycotoxins were combined [36].

The methyl thiazoltetrazolium (MTT) assay is based on the action of living cells to convert a soluble yellow tetrazolium salt [3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl-tetrazolium bromide] into insoluble purple formazan crystals. The reaction is catalysed by mitochondrial succinyl dehydrogenase and requires NADH, which has to be supplied by the living cells, thus providing an indication about mitochondrial or respiratory activity [37; 42; 43; 44; 45] hence providing information on cell viability and cell proliferation, after their exposure to xenobiotic agents. Significant decrease of cell viability was observed when all three mycotoxins were combined as compared to single or two combinations.

In addition, higher cytotoxicity induction characterised by rapid decrease in cell viability was observed between 0 and 24hrs in comparison to the effects observed between 24 and 48hrs of exposure (Fig. 1). This could mean that the cytotoxicity induced was high between 12 and 24hrs and reached saturation between 24 and 48 hrs in both single and combined mycotoxins between 12 and 24 hrs exposure. The inhibition results (Table 2) obtained from the MTT assay in comparison to calculated results expected by adding individual results of each mycotoxin (FB1, OTA and AFB1) (Table 3) have shown that there is synergistic effect between the three mycotoxins at the dose 40 ng/ml and 5ng/ml 40 μg/ml. while at dose 5μg/ml the effects were additive rather than synergistic. The absence of synergism effects at the dose 5 in all combinations can be explained by low doses of mycotoxins exposed as well as by the time of cell exposure to mycotoxins. The probability is that at low doses longer exposure periods of cells to mycotoxins

are needed to induce measurable toxicity [46]. Mycotoxins were mixed and exposed to mononuclear cells. Result obtained in this study showed dose dependent synergistic effect was observed when mycotoxins were combined as compared to single mycotoxins.

Statistically, comparison showed that there were significant differences ($P<0.050$) found among data of three mycotoxins when exposed singularly over time (12, 24 and 48hrs) and between data obtained at different concentrations of exposure. Significant differences ($P<0.001$) were obtained among data from combined mycotoxins and between all data from all three mycotoxins combined over time and concentrations.

Obtained results on flow cytometry (Table 4) have shown that in contrast to necrosis, which is a form of traumatic cell death that results from acute cellular injury, apoptosis is a process of programmed cell death that remove individual cells that are no longer needed or that function abnormally without inducing inflammatory reaction noxious to surrounding cells [48] and hence it plays a major role during development and homeostasis. The difference between results obtained in Table 3 on comet assay as compared to DNA cleavage (Table 5) obtained on flow cytometry mostly concerned the methodology. The DNA damage observed with the comet assay is the DNAwhich converts lesions to DNA breaks, increasing the amount of DNA in the comet tail due to mycotoxins effects (46). Results obtained with the flow cytometry (Table 5) are the effects of the DNA strands cleavage dueto a reaction of the covalent sugar-phosphate linkages between nucleotides that compose the sugar phosphate backbone of DNA. It is catalysed enzymatically, chemically or by radiation in which the cleavage may be exonucleolyticremoving the end nucleotide, or endonucleolytic splitting the strand into two (46).

Apoptosis occurs when a cell is damaged beyond repair, infected with a virus, or undergoing stressful conditions such as starvation. Damage to DNA from ionizing radiation or toxic chemicals can also induce apoptosis via the actions of the tumour-suppressing gene p53 [48]. In general, any substance that causes DNA damage or anything that produces necrosis by direct cell destruction can induce apoptosis if the cell initially survives [48] which invariably suggest that mycotoxins with genotoxic properties at non acute toxic concentration would shift the balance between necrosis and apoptosis to the latter as observed in this study. Therefore, understanding the mechanism of mycotoxin actions at bio molecular levels, particularly with regards to alterations of DNA in the nucleus and mitochondria may explain the dose-dependent apoptotic pattern of cell death induced by the studied mycotoxins. These results are in line with those obtained by Domijan [49]. In their study on comet assay exposed rats kidney cells with ochratoxin A and fumonisin B1 also observed synergistic increase in the tail intensity when OTA and FB1 were combined. The aim of this study was to assess the single and combined cytotoxic effects of mycotoxins on mononuclear cells and to predict their possible impact on the human's immunity. Observations made in the three experiments revealed that similar results were obtained with higher cytotoxic potent activity of OTA and AFB1 compared to FB1. The OTA-AFB1 showed a slightly higher toxicity as compared to FB1-OTA or FB1- AFB. The mixture of the three toxins showed a significant higher toxicity as compared to the single and combined mycotoxins effects in the three studies. Another important observation made in the three experiments was that in general low doses of mycotoxins (5ng/ml and 5μg/ml exposures) between 24 and 48 hrs of exposure showed slow recoveries orplateaus also called "adaptive response" [46; 50].

It is likely that these represent a most dangerous status, as this could lead up to mutations and cellular stimulations or cancer [46; 50; 51]. The slightly low and inhibitive action observed with FB1 in all four studies correlate with the findings of Minervini [52] who in their studies on the toxicity of the nivalenol (NIV), deoxynivalenol (DON) and FB1 in the K562 human erythroleukemia cell line using the Trypan Blue, MTT and BrdU (uptake for cytotoxicity analysis, found that cellular metabolism and proliferation, respectively on flow cytometry were for both NIV and DON significantly more toxic than FB1. In addition they correlate are in correlation with the results obtained by Theumer [53] in an in vivo study on immuno-biological effects of FB1 and AFB1 in experimental sub-chronic mycotoxicoses in rats in which FB1 induced low effects compared to AFB1.

The cytotoxicity of OTA can be explained by its ability to directly inhibit enzymes involved in phenylalanine metabolism as mentioned in the Introduction). In return, this has an effect on DNA, RNA, and protein synthesis, presumably due to an effect by the phenylalanine moiety of the molecule [27]. The cytotoxic potential shown by AFB1 is explained by its inhibiting capacity of both DNA and RNA synthesis [54] and the activated AFB1 metabolite formation of a covalent bond with the N7 of guanine [55] and AFB1-n7-guanine adduct in the target cells resulting in transversions of G-T bond, DNA repair, lesions, mutations and subsequently tumour formation [56], or the possibility of hydrolysing to AFB1-8, 9-dihydrodiol that ionizes to form a Schiff's base with primary amine groups in the proteins [57]. During in vitro studies of rat liver mitochondria it was observed that OTA inhibited the respiration of whole mitochondria, by acting as a competitive inhibitor of carrier proteins located in the inner mitochondrial membrane [58].

The understanding of the cellular effects of the three toxins used in the three experiments might also be residing at the genomic level. Aflatoxin B1 is metabolized, mainly in the liver, into AFB1-8,9-exo-epoxide and 8,9-endo-epoxide, but it is the exo-epoxide that binds to DNA to form the predominant 8,9-dihydro-8-(N7-guanyl) 9-hydroxy AFB1 (AFB1-N7-Gua) adduct [59]. AFB1-N7-Gua can result in two secondary lesions, an apurinic site and a more stable ring opened AFB1-formamidopyrimidine (AFB1–FAPY) adduct; the latter is far more persistent in vivo than AFB1-N7-Gua [59;60]. Under this pathological condition, oxidative stress is elicited which activates the caspase-3 cysteine proteases that mediate the apoptotic cascade [61].

Table 3 : Comet assay summary results of mononuclear cells DNA damage scoring after 12, 24 and 48 hours exposure to aflatoxin B1, ochratoxin A and fumonisin B1

Mycotoxins + Conc*.	Exposure time (hours)	Mean (% DNA damage)	Std Dev	SEM
FB1 5	12hrs	37.5	4.4	2.2
FB1 5	24hrs	62.2	11.5	5.7
FB1 5	48hrs	80.7	2.2	1.1
FB1 40	12hrs	175.5	40.0	20.0
FB1 40	24hrs	198.5	23.3	11.6
FB1 40	48hrs	231.7	3.9	1.9
AFB1 5	12 hrs	50.5	3.6	1.8
AFB1 5	24 hrs	66.5	6.3	3.1
AFB1 5	48 hrs	93.7	8.6	4.3
AFB1 40	12 hrs	169.5	20. 0	10.0
AFB1 40	24 hrs	201.5	9.6	4.8
AFB1 40	48 hrs	253.5	5.7	2.8
OTA 5	12hrs	64.5	6.6	3.3
OTA 5	24hrs	88.7	9.0	4.5
OTA 5	48hrs	107.2	10.1	5.0
OTA 40	12hrs	169.5	20.0	10.0
OTA 40	24hrs	201.5	9.6	4.8
OTA 40	48hrs	253.5	5.7	2.8
OTA -FB1 5	12hrs	70.5	3.6	1.8
OTA -FB1 5	24hrs	111.2	6.3	3.1
OTA -FB1 5	48hrs	114.0	4.0	2.0
FB1-AFB1 5	12hrs	75.7	4.7	2.3
FB1-AFB1 5	24hrs	98.0	8.0	4.0
FB1-AFB1 5	48hrs	120.0	5.8	2.9
OTA-AFB1 5	12hrs	82.5	13.9	6.9
OTA-AFB1 5	24hrs	103.2	4.0	2.0
OTA-AFB1 5	48hrs	130.2	4.8	2.4
OTA -FB1 40	12hrs	211.0	21.8	10.9
OTA -FB1 40	24hrs	233.2	16.6	8.3
OTA -FB1 40	48hrs	263.0	2.5	1.2
OTA-AFB140	12hrs	219.7	8.1	4.0
OTA-AFB140	24hrs	240.5	38.2	19.1
OTA-AFB140	48hrs	279.0	6.3	3.1
FB1-AFB1 40	12hrs	206.2	15.5	7.7
FB1-AFB1 40	24hrs	221.2	19.2	9.6
FB1-AFB1 40	48hrs	265.0	4.0	2.0
MIXTURE 5	12hrs	110.0	6.7	3.3
MIXTURE 5	24hrs	125.2	11.9	5.9
MIXTURE 5	48hrs	132.7	4.7	2.3
MIXTURE 40	12hrs	217.7	6.1	3.0
MIXTURE 40	24hrs	250.5	21.7	10.8
MIXTURE 40	48hrs	284.0	11.5	5.7

*Individual and mixtures concentrations of aflatoxins and ochratoxins concentrations are in (ng/ml) and fumonisin B1 concentration is in (µg/ml).

It has also shown by Golli-Bennour [62] that AFB1 and OTA separately and in combination, are involved in apoptotic processes in cultured monkey kidney Vero cells by causing increased DNA fragmentation with consequent activation of p53 tumour suppressor protein and suppression of production of anti-apoptotic factor bcl-2. Ochratoxin A has also been established to facilitate apoptosis by causing the reduction of protein synthesis [15] and increasing caspase-3 activity, DNA fragmentation and chromatin condensation [63]. Caspase-dependent mitochondrial alterations and triggering of the activity of p53 are other mechanisms by which OTA induces apoptosis [61].

The relative concentration of apoptotic mediators will essentially, determine the outcome of TNF receptor stimulation. The TNFR1 may activate apoptosis JNK activation which in return,inhibits the anti-apoptotic protein Bcl-2. Once Bcl-2 is inhibited, cytochrome C is released from mitochondria into the cytosol where it activates Apaf-1, which may associate with caspases and thereby initiate apoptosis. Alternatively, the TNFR2 may, together with TNFR1, activate NF-κβ which may inhibit apoptosis. Unlike the other mycotoxins, fumonisins elicitation of apoptosis seems to be mainly non genotoxic, as FB1 does not interact with DNA but inhibits the enzyme ceramide synthase, thereby disrupting de novo sphingolipid biosynthesis, overall sphingolipid metabolism and, consequently, the accumulation of sphingoid bases whith sphingolipid-mediated regulation of important cell functions including apoptosis and mitosis [64, 65]. Fumonisin B1-induced apoptosis is also known to be mediated by the cytokine tumour necrosis factor (αTNF) pathway [64]. Tumour necrosis factor (TNF) is involved in the regulation of apoptosis and cell replication just as like sphingoid bases, sphinganine and sphingosine sphingolipids. It must be pointed out here that the AFB1 and OTA can also induce apoptosis via non genotoxic route by inhibiting macromolecular synthesis, which disrupts many lipids/protein/DNA-mediated cell function regulations with consequent deregulation of processes including apoptosis [65]. The finding that tested toxins induced cell death mainly via apoptosis is in excellent consistency with many reports [1; 43; 67].The mechanism of FB1 effect in the presence of other mycotoxins, such as OTA and AFB1 on the immune system, remains unknown to date. It has been shown in the four experiments that, when combined with one of the two mycotoxins used here it induces a low inhibitive effect. The FB1 low toxicity which was observed throughout in the three experiments (MTT, comet assay, flow cytometer) is similar to results obtained in studies done by Bondy and Pestka [67] on the effects of FB1 on immune system in chicken. In addition, Mwanza [36] on cytotoxic effects of OTA and FB1 on pigs and human mononuclear cells, confirmed the relative low and inhibitive effect of FB1 when combined together. However, these toxins were also found to cause both stimulation and suppression of responses to foreign antigen. The immuno-modulatory properties of FB1, mostly depend on its effect on lipid metabolism, antioxidant/pro-oxidant balance and interactions with other factors such as CD3 receptors expression, decrease in the thymus seen both in vivo and in vitro studies [68]. In addition, this FB1 immunologic effect is confirmed by another study on bovine lymphocytes cells, which caused significant micronucleus formation [69]. Fumonisin B1 also inhibits other intracellular enzymes including protein phosphatase and arginosuccinate synthase [70]. Therefore, the cytotoxicity of FB1 exerts its toxicity through its ability to inhibit sphingolipid metabolism, protein metabolism and the urea cycle.

Table 4 : Flow Cytometry summary results of mononuclear cells undergoing apoptosis after 12, 24 and 48 hours exposure to aflatoxin B1, ochratoxin A and Fumonisin B1

Mycotoxins + Conc*.		Exposure time (hours)	Mean (% apoptotic cells)	Std Dev	SEM
FB1	5	12hrs	6.1	0.6	0.3
FB1	5	24hrs	9.2	0.4	0.2
FB1	5	48hrs	15.8	0.5	0.3
FB1	40	12hrs	19.7	0.5	0.3
FB1	40	24hrs	33.1	0.6	0.4
FB1	40	48hrs	46.6	0.1	0.0
AFB	5	12hrs	8.2	0.7	0.4
AFB	5	24hrs	12.1	0.2	0.1
AFB	5	48hrs	23.7	0.4	0.2
AFB	40	12hrs	23.6	0.7	0.4
AFB	40	24hrs	47.6	0.6	0.3
AFB	40	48hrs	58.6	0.5	0.3
OTA	5	12hrs	11.0	0.7	0.4

OTA	5	48hrs	28.2	0.2	0.1
OTA	5	24hrs	16.4	0.2	0.1
OTA	40	12hrs	26.7	0.6	0.3
OTA	40	24hrs	49.2	0.3	0.2
OTA	40	48hrs	69.2	0.3	0.2
OTA-FB1	5	12hrs	13.1	0.2	0.1
OTA-FB1	5	24hrs	31.2	0.8	0.4
OTA-FB1	5	48hrs	45.6	1.0	0.6
OTA-AFB1	5	12hrs	15.5	1.0	0.6
OTA-AFB1	5	24hrs	34.1	0.2	0.1
OTA-AFB1	5	48hrs	53.4	0.4	0.2
FB1-AFB1	5	12hrs	12.2	0.2	0.1
FB1-AFB1	5	24hrs	29.1	0.6	0.4
FB1-AFB1	5	48hrs	46.1	0.2	0.1
OTA-FB1	40	12hrs	39.5	0.8	0.5
OTA-FB1	40	24hrs	63.3	0.7	0.4
OTA-FB1	40	48hrs	79.2	0.7	0.4
OTA-AFB1	40	12hrs	41.6	0.4	0.2
OTA-AFB1	40	24hrs	62.0	0.5	0.3
OTA-AFB1	40	48hrs	87.7	0.8	0.5
FB1-AFB1	40	12hrs	41.0	0.6	0.3
FB1-AFB1	40	24hrs	65.6	0.6	0.3
MIXTURE	5	12hrs	17.2	0.3	0.1
MIXTURE	5	24hrs	39.6	1.0	0.6
MIXTURE	5	48hrs	56.4	0.5	0.2
MIXTURE	40	12hrs	44.3	0.5	0.3
MIXTURE	40	24hrs	89.0	0.3	0.2
MIXTURE	40	48hrs	95.1	0.4	0.2

*Individual and mixtures concentrations of aflatoxins and ochratoxins concentrations are in (ng/ml) and fumonisin B1 concentration is in (µg/ml).

The FB1 apoptosis induction and DNA damage seen in this study have been confirmed by Domijan [49].In their study of oxidative status and DNA damage in rats they observed DNA lesions in the kidney cells of experimental animals. The FB1 carcinogenic role, however, has been linked to the accumulation of sphingoid bases that cause unscheduled DNA synthesis [71] alteration of signalling by cAMP [72] and disruption of normal cell cycling [73].

Aflatoxin B1 effects observed on mononuclear cells can be explained by its immuno-suppressive ability to act primarily on cell mediated and phagocytic function [74]. Aflatoxin B1 has been shown to act on mononuclear cells activity as well as macrophages [74]. Thymus is also a target organ for aflatoxin in which thymic involution results with the loss of cortical thymocytes. It is primarily the cell-mediated immune responses that are affected by aflatoxin; prominent among these are diminished responses in delayed cutaneous hypersensitivity, graft-versus-host reaction, leukocyte migration and lymphoblastogenesis [75]. Aflatoxin also reduces phagocyte activity in a dose-related manner. Some humoral components are diminished by aflatoxin, including complement (C4), interferon, IgG and IgA, but not IgM, which is not affected. However, high levels of aflatoxin will affect antibody titres and gut-associated lymph tissue or the bursa of Fabricius in poultry [75]. The immunosuppressive effect of AFB1 was also observed by Marin [76] who found that AFB1 reduces the pro-inflammatory cytokine and increased anti-inflammatory cytokine mRNA expression in weanling piglets.

Although Creppy [1] in their study on cells revealed the potential synergistic effects of mycotoxins combination it was anticipated that comparable effects on human lymphocytes would induce immunosuppression and poor responses to immunisation and treatment when exposed to single or combined toxins. The high decrease in cell viability observed when FB1 and OTA are combined indicates a synergistic activity of both toxins action, which induces a stronger metabolic suppression activity on lymphocytes at low concentrations, which increases also with concentration and this effect has been reported before in others studies [1]. Thus, it poses a problem for feeds derived from crop grown in temperate areas where the possibility of both mycotoxins occurring in feeds and foods is possible.

V. Conclusion

This study has shown that a synergistic effect of FB1, OTA and AFB1 may be induced when exposed to mononuclear cell dependent of concentration and time of exposure. However, it was observed that the FB1 induces an inhibitive effect when combined with OTA and AFB1. These results agree with the hypothesis that the combination of the three mycotoxins currently considered as the most important contaminant in both animal feed and human food, can induce increased immuno-suppression and increase cases of immunisation and treatment failure currently observed in treated patients suffering from chronic diseases such as tuberculosis, malaria, cancers and HIV-AID. The novelty of this work is that, this is a first report done with the three mycotoxins combined and analysed using three different methods confirming their cytotoxicity.

Table 5 : A summary of flow cytometry results of mononuclear cell's DNA cleavage after 12, 24 and 48 hours exposure to aflatoxin B1, ochratoxin A and fumonisin B1

Mycotoxins + Conc*.		Exposure time (hours)	Mean (% DNA cleaved cells)	Std Dev	SEM
FB1	5	12hrs	4.3	0.7	0.4
FB1	5	24hrs	8.8	0.2	0.1
FB1	5	48hrs	14.6	0.6	0.3
FB1	40	12hrs	18.2	0.5	0.3
FB1	40	24hrs	30.1	0.5	0.3
FB1	40	48hrs	38.7	5.4	3.1
AFB1	5	12hrs	5.9	0.5	0.3
AFB1	5	24hrs	11.0	0.4	0.2
AFB1	5	48hrs	21.6	0.9	0.5
AFB1	40	12hrs	21.5	0.9	0.5
AFB1	40	24hrs	45.7	0.6	0.3
AFB1	40	48hrs	55.0	0.3	0.2
OTA	5	12hrs	8.9	0.5	0.3
OTA	5	24hrs	12.8	0.3	0.2
OTA	5	48hrs	19.4	0.4	0.2
OTA	40	12hrs	19.3	0.4	0.2
OTA	40	24hrs	48.2	0.5	0.3
OTA	40	48hrs	58.1	0.3	0.2
OTA-FB1	5	12hrs	12.1	0.5	0.3
OTA-FB1	5	24hrs	32.8	0.4	0.2
OTA-FB1	5	48hrs	44.4	0.6	0.3
OTA-AFB1	5	12hrs	13.3	0.9	0.5
OTA-AFB1	5	24hrs	33.1	0.5	0.3
OTA-AFB1	5	48hrs	49.7	0.6	0.3
FB1-AFB1	5	12hrs	10.4	0.2	0.1
FB1-AFB1	5	24hrs	25.1	0.5	0.3
FB1-AFB1	5	48hrs	41.8	0.4	0.2
OTA-FB1	40	12hrs	37.8	0.6	0.3
OTA-FB1	40	24hrs	71.1	0.7	0.4
OTA-FB1	40	48hrs	77.2	0.5	0.3
OTA-AFB1	40	12hrs	37.8	0.6	0.3
OTA-AFB1	40	24hrs	64.2	0.4	0.2
OTA-AFB1	40	48hrs	86.4	1.1	0.6
FB1-AFB1	40	12hrs	39.6	0.8	0.4
FB1-AFB1	40	24hrs	62.9	0.4	0.2
FB1-AFB1	40	48hrs	69.3	0.5	0.3
MIXTURE	5	12hrs	15.7	0.2	0.1
MIXTURE	5	24hrs	37.6	0.6	0.3
MIXTURE	5	48hrs	59.9	0.5	0.3
MIXTURE	40	12hrs	42.4	1.1	0.6
MIXTURE	40	24hrs	87.1	0.3	0.1
MIXTURE	40	48hrs	92.5	1.0	0.6

*Individual and mixtures concentrations of aflatoxins and ochratoxins concentrations are in (ng/ml) and fumonisin B1 concentration is in (µg/ml).

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Conflicts of Interest

"The authors declare no conflict of interest".

References Références Referencias

1. Creppy, E.E.; Chiarappa, P.; Baudrimont, I.; Borracci, P.; Moukha, S.; Carratu, M.R.. Synergistic effects of fumonisin B1 and ochratoxin A: are in vitro cytotoxicity data predictive of in vivo acute toxicity. Toxicol.2004, 201, 15-123.
2. Njobeh, P.B.; Dutton, M.F.; Makun, A. H. Mycotoxins and human health: Significance, prevention and control. In: Mishra AK, Tiwari A, Mishra SB and Kobayashi H (Eds), Smart Biomolecules in Medicine. Research Signpost/Transworld Research Network.VBRI Press, India.2010, pp132-177.
3. Hussein, S.H.; Brasel, M.J. Toxicity, metabolism, and impact of mycotoxins on humans and animals. Toxicol.67, 2001, 101-134.
4. William, P.; Jolly, E.; Nii-Ayi A.; Ellis, W.O.; Afriyie-Gyawu, E.; Johnson, N.M.; Robinson, AG.; Phillips, T.D. HIV and hepatocellular and oesophageal carcinomas related to consumption of mycotoxin prone foods in sub-Saharan Africa. Am. J. Clin. Nutr,2010, 92, 154-160.
5. Turner, P.C.; Moore, S.E.; Hall, A.J.; Prentice, A.M.; Wild, C.P. Modification of immune function through exposure to dietary aflatoxin in Gambian children. Environ. Health Perspect, 2003,111, 217-220.
6. Gong, Y.Y.; Hounsa, A.; Egal, S.; Turner, P.C.; Sutcliffe, A.E.; Hall A.J.; Cardwell, K.; Wild, C.P. Post-weaning exposure to aflatoxin results in impaired child growth: a longitudinal study in Benin, West Africa. Environ. Health Perspect.2004,112, 1334-8.
7. Williams J.H.; Phillips, T.D.; Jolly, P.E.; Stiles, J.K.; Jolly, C.M.; Aggarwal, D. Human aflatoxicosis in developing countries: a review of toxicology, exposure, potential health consequences and interventions. Am. J. Clin. Nutr.2004, 80, 1106-1122.
8. Wagacha, J.M.; Muthomi, J.W. Mycotoxin problem in Africa current status implications to food safety and health and possible management strategies. Inter. J. Food Microbiol, 2008, 124, 1-12.
9. Riley, R.T. Mechanistic interactions of mycotoxins: theoretical consideration. In: Sinha, K.K., Bhatnagar, D. (Eds.), Mycotoxins in Agriculture and Food Safety. Marcel Dekker, Inc, Basel, New York, ,1998, 227-254.
10. Yoo, H.S.; Norred, W.P.; Showker, Riley. R.T.Elevated sphingoid bases and complex sphingolipid depletion as contributing factors in fumonisin-induced cytotoxicity.Toxicol& Appl. Pharmacol.1996;138, 211-218.
11. Merrill, A.H. Jr.; Schmelz, E.M.; Dillehay, D.L.; Spiegel, S.; Shayman, J.A.; Schroeder, J.J.; Riley, R.T.; Voss, K.A.; Wang, E. Sphingolipids: The enigmatic lipid class: Biochemistry, Physiology and Pathophysiology. Toxicol Appl. Pharmacol., 1997, 142, 208-225.
12. Shephard, G.S.;Van der Westhuizen, L. Liquid chromatographic determination of the sphinganine/sphingosine ratio in serum. J. Chromat B, 1998, 710, 219-22.
13. Marasas, W.F.O.; Jaskiewics, K.; Venter, F.S.;VanSchalkwyk, D.J. Fusarium moniliforme contamination of maize in oesophageal cancer areas in Transkei. S. A. Med. J. 1988, 74, 110-114.
14. Diaz, G.J. and Boermans, H.J., Fumonisin toxicosis in domestic animals: a review. Vet. Human Toxicol.1994, 36, 548-555.
15. Ueno, Y.; Lijima, K.; D-Wang, S.D.; Sugiura, Y.; Sekijima, T.; Tanaka, T.; Chen, C.; Yu, S.Z. Fumonisins as a possible contributory risk factor for primary liver cancer: A 3-year study of corn harvested in Haimen, China, by HPLC and ELISA. Food Chem. Toxicol.1997, 35, 1143-1150.
16. Fincham, J.E.; Marasas, W.F.O.; Taljaard, J.J.F.; Kriek, N.P.J.; Badenhorst, C.J.; Gelderblom, W.C.A.; Seier, J.V.; Smuts, C.M.; Faber, M.; Weight, M.J.; Slazus, W.; Woodroof, C.W.; Van Wyk, M.J.; Kruger, M.; Thiel, P.G. Atherogenic effects in a non-human primate of Fusarium moniliformecultures added to a carbohydrate diet. Atherosclerosis1992, 94, 13-25.
17. Dutton, M.F. The African Fusarium/maize disease.Mycotox. Res. 2009, 25:29-39.
18. Marasas, W.F.O.; Riley,R.T.; Hendricks, K.; Stevens, V.L.; Sadler, T.W.; Gelineau-van Waes, J.; Missmer, S.; Cabrera, J.; Torres, O.; Gelderblom, W.C.A.; Allegood, A.; Martinez, C.; Maddox, J.; Miller, J.D.; Starr, J.; Sullards, M.C.; Roman, V.A.; Voss, K.A.; Wang, E.; Merrill, A.H. Jr. Fumonisins disrupt sphingolipid metabolism, folate transport, and neural tube development in embryo culture and in vivo: A potential risk factor for human neural tube defects among populations consuming fumonisin-contaminated maize. J. Nutr.2004, 134, 711-716.
19. Gelineau-van Waes, J.; Starr, L.; Maddox, J.; Aleman, F.; Voss, K.A.; Wilberding, J.; Riley, R.T. Maternal fumonisin exposure and risk for neural tube defects: Mechanisms in an in vivo mouse model. Birth Defects Research A: Clin. Mol. Teratol.2005, 73, 487-497.

20. Missmer, S.A.; Suarez, L.; Felkner, M.; Wang, E.; Merrill, A.H.; Jr., Rothman, K.; Hendricks, K.A. Exposure to fumonisins and the occurrence of neural tube defects along the Texas–Mexico border. Environ. Health Perspect.2006, 114, 237-241.
21. Devegowda, G.; Radu, M.V.L.; Nazar, A.; Swamy, H.V.L.M. Mycotoxin picture worldwide: Novel solutions for their counteraction. In: Proceedings of Alltech's 14th Annual Symposium, Biotechnology in Feed industry. Passport of the year 2000. Nottingham University Press, 2000
22. International Agency for Research on Cancer (IARC), Some Traditional Herbal Medicines, Some Mycotoxins, Naphthalene and Styrene.Monograph2002, 82, http:// monographs.
23. Lillerberg, S.L.; Cabonce, M.A.; Raju, N.R.; Wagner, L.M.; Kier, L.D. Alterations in the p53 tumour suppressor gene in rat liver tumours induced by aflatoxin B1. Program.Clin. Biol. Res.1992, 376, 203-222.
24. Aguilar, F.; Hussdain, S.P.; Cerutti, P.. Aflatoxin B1 induces transversion of GT in codon 249 of the p53 tumour suppressor gene in human hepatocytes, Proc. Nation. Acad. Sci., 1993, 8586-8590.
25. Gong, Y.Y.; Egal, S.; Hounsa, A.; Turner, P.C.; Hall, A.J.; Cardwell, K.F.; Wild, C.P. Determinants of aflatoxin exposure in young children from Benin and Togo, West Africa: the critical role of weaning. Food Res. Intern.2003, 37, 985-1000.
26. International Agency for Research on Cancer (IARC). Monographs on the evaluation of the carcinogenic risk of chemicals to human: Some naturally occurring substances. Food items and constituents, heterocyclic aromatic amines and mycotoxins.IARC, Lyon, 1993, 397-444.
27. Creppy, E.E. Comparative study of the effect of ochratoxin A analogues on yeast aminoacyl-tRNAsynthetases and on the growth and protein synthesis of hepatoma cells. Toxicol. Letters 1983, 19, 217-224.
28. Pfohl-Leszkowicz, A. Manderville R.A. Ochratoxin A: an overview on toxicity and carcinogenicity in animals and humans. Mol. Nutrit. Food Res.2007, 51, 61-99.
29. Joint Expert Committee on Food Additives (JECFA). Evaluation of Certain Mycotoxins in Food.(56th Report of the Joint FAO/WHO Expert Committee on Food Additives). Technical Reports Series No. 906, JECFA2002.
30. Mantle, P.G., Faucer-Marquis, V., Manderville, R.A., Squillaci, B. and Pfohl-Leszkowicz, A., Structures of covalent adducts between DNA and ochratoxin A: a new factor in debate about genotoxicity and human risk assessment. Chem. Res.Toxicol. 2010, 23, 89-98.
31. Stoev, S.D.Complex etiology, prophylaxis and hygiene control in mycotoxic nephropathies in farm animals and humans. Intern. J. Mol. Sc.2008b, 9, 578-605.
32. Stoev, S.D.; Denev, S.; Dutton M.F.; Njobeh, P.B.; Mosonik, J.S.; Steenkamp, P.A.; Petkov. I. Complex aetiology and pathology of mycotoxic nephropathy in South African pigs. Mycotox. Res.2010, 10, 31-46.
33. Stoev, S.D. Mycotoxic nephropathies in farm animals' diagnostics, risk assessment and preventive measures. Mycotox. Farm Anim. J.2008a 155-195.
34. Fink-Gremmels, J.; Leistner, L. Toxicological evaluation of moulds. Food Biotech.1990, 4, 579-584.
35. Stoev, S.; Denev, S.; Dutton, M.; Nkosi, B. Cytotoxic Effect of Some Mycotoxins and their Combinations on Human Peripheral Blood Mononuclear Cells as Measured by the MTT Assay. Toxinol. J. 2009, 2, 1-8.
36. Mwanza, M.; Kametler, L.; Bonais, A.; Rajli, V.; Kovacs, M.; and Dutton, M.F. The cytotoxic effects of fumonisin B1, aflatoxin B1 and ochratoxin A on human and pig lymphocytes using Methyl thiazoltetrazolium (MTT) assay. Mycotox. Res.2009,25, 233-238.
Meky, F.A.; Hardie, L.J.; Evans, S.W.; Wild, C.P. Deoxynivalenol-induced immunomodulation of human lymphocyte proliferation and cytokine production. Food Chem. Toxicol. 2001, 39, 827-836.
37. Singh, N.P.; McCoy, M.T.; Tice, R.R.; Schneider, E.L. A simple technique for quantitation of low levels of DNA damage in individual cells.Experiment. Cell Res. , 1988, 175, 184-191.
38. Collins, A.R.; Oscoz, A.A.; Brunborg, G.; Gaivao, I.; Giovannelli, L.; Kruszewski K. The comet assay: topical issues. Mutagenesis1997, 23, 143-151.
39. Hartmann, A., Agurell, E., Beevers, C., Brendler-Schwaab, S., Burlinson, B. and Clay, P., 2003: Recommendations for conducting the in vivo alkaline Comet assay. Mutagenesis 18:45-51.
40. Delves, P.; Seamus M.; Burton, D.; Roitt, Y. Roitt's essential immunology, Wiley-Blackwell; 11th Edition Place.2006, 496.
41. Charoenpornsook, K., Fitzpatrick, J. L., and Smith, J.E., 1998. The effects of four mycotoxins on the mitogen stimulated proliferation of bovine peripheral blood mononuclear cells in vitro. Mycopathologia 143, 105-111.
42. Dombrink-Kurtzman, M. A.; Javed, T.; Bennett, G.A.; Richard, J.L.; Cote, L.M.; Buck, W.B. Lymphocyte cytotoxicity and erythrocytic abnormalities induced in broiler chicks by fumonisins B1 and B2 and moniliformin from Fusarium proliferatum, Mycopathol.1993, 124, 47-54.

43. Grabsch, G.; Wichmann, G.; Loffhagen, H.; Herbarth, O.; Muller, A. Cytotoxicity assessment of gliotoxin and penicillic acid in Tetrahymenapyriformis, Environ. Toxicol.2006, 2, 111-7.
44. Lewis, C.W.; Smith J.E.; Anderson J.G.; Freshney R.I. Increased cytotoxicity of food-borne mycotoxins toward human cell lines “in vitro” via enhanced cytochrome p450 expression using the MTT bioassay. Mycopathol.1999, 148, 97-102.
45. Timbrell, J. Toxic responses to foreign compounds. In Principles of Biochemical Toxicology, 3rd ed. Taylor and Francis.2001, 240-247.
46. Kiechle, F.L. and Zhang, The omics era and its impact. Arch. Pathol. Lab Med. 2004,128, 1337-1345.
47. Vinatier, D.; Dufour, P.; Subtil, D. Apoptosis: a programmed cell death involved in ovarian and uterine physiology. European J. Obstetric Gynaecol Repro. Biol. 1996, 67, 85-102.
48. Domijan, A.; Zeljezic, D.; Kopjar, N.; Peraica, M. Standard and Fpg-modifed comet assay in kidney cells of 14 S.D. ochratoxin A- and fumonisin B1-treated rats. Toxicol.2006, 222, 53-59.
49. Lutz, P.L.; Manuel, L. Maintenance of adenosine A1 receptor functions during long term anoxia in the turtle brain. Am. J. Physiol. 1999; 276, 633-636.
50. Mantovani, M.S.; Bellini, M.F.; Angeli, J.F.; Oliveira, R.J.; Silva, A.F.; Ribeiro, L.R. β-Glucans in promoting health: Prevention against mutation and cancer. Mutation Res. 2008, 658, 154-161.
51. Minervini, F.; Fornelli, F.; Flynn, K.M. Toxicity and apoptosis induced by the mycotoxins nivalenol, deoxynivalenol and fumonisin B1 in a human erythroleukemia cell line. Toxicology in Vitro, 2004, 18, 21-28.
52. Theumer, M.G. Immunobiological effects of AFB1 and AFB1–FB1 mixture in experimental subchronic mycotoxicoses in rats. Toxicol.2003,186, 159-170.
53. Butler, W.H.; Neal, G.E., Mode of action and human health aspects of aflatoxin Purchase, I.F.H. (Editor). Elsevier Scientific Publishing Co., New York.1977.
54. Lillehoj, E.B. Aflatoxin: an ecologically elicited activation signal. In, J.E., Anderson, R.A. (Eds), Mycotoxins and Animal Foods.CRC Press, Boca Raton, FL,1991, 119-139.
55. Foster, L.M., Kozack, K.R., Loftus, M.G. and Steven, J., The polymerase chain reaction and its application to filamentous fungi. Mycolecular Res.1993, 97, 769-781.
56. Raney, K.D.; Meyer, D.J.; Ketterer, B.; Harris, T.M.; Guengerish, F.P. Glutathione conjugation of aflatoxin B-1 exo- and endo-epoxides by rats and human glutathione-S-transferases. Chem. Res. Toxicol.1992, 5, 470-478.
57. Wei, Y.H.; Lu, C.Y.; Lin, Wei, R.D. Effects of ochratoxin A on rat liver mitochondrial respiration and oxidative phosphorylation.Toxicol.1985, 36, 119-130.
58. Wild, C.P. and Turner, P.C., The toxicology of aflatoxins as a basis for public health decisions. Mutagenesis 2002, 17, 471-481.
59. 60Wild, C.P.; Gong, Y.Y. Mycotoxins and human disease: a largely ignored global health issue. Carcinogen.2010, 31, 71-82.
60. Bouaziz, C.; Sharaf el Deinb, O.; El Golli E.; Abid-Essefia, S.; Brennerb, C.; Lemaireb, C.; Bacha, H. Different apoptotic pathways induced by zearalenone, T-2 toxin and ochratoxin A in human hepatoma cells. Toxicol.2008, 254, 19-28.
61. Golli-Bennour, E.; Kouidhi, B.; Bouslimi, A.; Abidd-Essefi, I.; Hassen, W.; Bacha, H. Cytotoxicity and genotoxicity induced by aflatoxin B1, ochratoxin A and their combination in cultured Vero cells. J. Biochem. Mol.Toxicol.2010. 24, 42-50.
62. Ringot, D.; Chango, A.; Schneider, Y-J. Larondelle, Y. Toxicokinetics and toxicodynamics of ochratoxin A, an update.Chemico-Biol. Interact.2006.159, 18-46.
63. Voss, K.A.; Howard, P.C.; Riley, R.T.; Bucci, T.J.; Lorentzen. R.J. Carcinogenicity and mechanism of action of fumonisin B1: a mycotoxin produced by Fusarium moniliforme (=F. verticillioides). Cancer Detection Prev. 2002, 26, 1-9.
64. Seefelder,W. Induction of apoptosis in cultured human proximal tubule cells by fumonisins and fumonisin metabolites. Toxicol. Appl. Pharmacol.2003, 192, 146-153.
65. CAST. Mycotoxins: Risks in Plant, Animal, and Human Systems. Ames Council for Agricultural Science and Technology. Iowa, USA. Task Force Report No. 139 January 2003. 199pp
66. Wang, E.; Norred, W.P.; Bacon, C.W.; Riley, P.F. Merrill, A.H. Jr. Inhibition of sphingolipids biosynthesis by fumonisin. J. Biol. Chem.1998, 273, 11025-11031.
67. Bondy, G.S.; Pestka, J.J. Immunomodulation by fungal toxins. Environ. Health 3B, 2000, 109-143.
68. Martynova, Fumonisin B1 modulates sphingomyelin cycle product levels and the expression of CD3 receptors in immunocompetent organs. Biochem. 1995, 60, 461-465.
69. De Lorenzo, C.; Cozzolino, R.; Carpentieri, A.; Pucci, P.; Laccetti, P.; D’Alessio; G. Biological properties of a human compact anti-ErbB2 antibody. Carcinogen.2005, 26, 1890-1895.
70. Jenkins, G.R.; Tplleson, W.H.; Newkirk, D.K.; Robert, D.W.; Rowland, K.L.; Seheki, T.; Koyabashi, K.; Howard, P.C., Melchior, W.B. Identification of

fumonisin B1 as an inhibitor of argininosuccinatesynthetase using fumonisin affinity chromatography and in vitro Kinetic studies. J. Biochem. Mol. Toxicol.2000, 14, 320-328.

71. Schroeder, J.J.; Crane, H.M.; Xia, J.; Liotta, D.C.; Merrill, A.H. Disruption of sphingolipid metabolism and stimulation of DNA synthesis by fumonisin B1. A molecular mechanism for carcinogenesis associated with Fusarium moniliforme. J. Biol. Chem.1994, 269, 3475-3481.
72. Huang, C., Dieckeman, M., Henderson, G., Jones, C., Repression of protein kinase C and stimulation of cyclic AMP response elements by Fumonisins, a fungal encoded toxin which is carcinogen. Cancer Res. 1995, 55,1655-1659.
73. Ramljak, D.; Calvert, R.J.; Wiensenfeld, P.W.; Diwan, B.A.; Catipovic, B.; Marasas, W.F.; Victor, T.C.; Anderson, L.M.; Gelderblom, W.C. A potential mechanism for fumonisin B1 mediated hepatocarcinogenesis: cyclin D1 stabilization associated with activation of AKT and inhibition of GSK-3beta activity. Carcinogen.2000, 21, 1537-1546.
74. Surai, P.F.; Dvorska, J.E. Effects of mycotoxins on antioxidant status and immunity; InThe Mycotoxin Blue Book. D. Diaz, ed. Nottingham Univ. Press, UK, 2005, 93-137.
75. Pier, A.C. Major biological consequences of aflatoxicosis in animal production. J. Anim. Sci. 1992, 70, 8860-8867.
76. Marin, D.E.; Taranu, I.; Bunaciu, R.P.; Pascale, F.; Tudor, D.S.; Avram, N.; Sarca, M.; Cureu, I.; Criste, R.D.; Suta, V.; Oswald, I.P. Changes in performance, blood parameters, humoral and cellular immune responses in weanling piglets exposed to low doses of aflatoxin. J. Anim. Sci. 2002, 80, 250-1257.

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Renal Parameters as Predictors of Morbidity and Mortality in Snake Bite Patients in a Tertiary Care Hospital in Southern India

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Abstract - *Background:* Snake bite is predominantly an occupational hazard and a common cause of morbidity and mortality. Acute renal failure has been associated with venomous viper and sea snake bites.

AIMS: This study was carried out to study the incidence and clinical profile of the snake bite patients who develop acute renal failure;and to identify the predictors of morbidity and mortality in these patients.

Material and methodology: We carried out prospective study on fifty (50) cases of definitive snake bite admitted to Department of Medicine/Emergency medicine, Kempegowda institute of medical sciences, Bangalore from May 2012 to November 2013.

Statistical analysis: SPSS for Windows version 17.0 (SPSS, Inc, Chicago, Ill) was used for statistical analysis. The Pearson Chi-Square Test was used to analyze parametric variables. A P value of 0.05 or less was considered statistically significant.

Keywords: *snake bite, acute renal failure, oliguria, morbidity, mortality.*

GJMR-B Classification: *NLMC Code: WB 330, QV 34*

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Results: Prevalence of ARF in snake bite patients was 40%. The other common symptoms were swelling/inflammation of bite area (90%), muscle pain/tenderness (60%), oliguria (50%), fever(26%) and vomiting(26%), hematuria(40%), proteinuria(52%) and bleeding from site was present in 40% patients. Common findings on examination were tachycardia (38%), breathlessness (34%), and hypotension (36%).

Laboratory findings were Anaemia (26%); Leucocytosis (64%); Thrombocytopenia (48%); Coagulopathy (36%). The incidence of acute renal failure was 40% out of which 13(26%) underwent hemodialysis and 1 patient progressed to chronic kidney disease. The mortality rate was 4%

Conclusions: Snake bite induced ARF has mortality of 4%. The factors associated with morbidity and mortality were presence of coagulopathy and increase in the number hospitilsation days

Keywords: snake bite, acute renal failure, oliguria, morbidity, mortality.

I. Introduction

Snake bite is predominantly an occupational hazard in the rural tropics and a common cause of morbidity and mortality[1,2].The majority of victims initially are treated by professional snakebite healers, snake charmers, and religious men, who use herbal remedies, chant divine "mantras," and apply "snake stone," all of which are supposed to magically draw out the venom from the victim[3]. Death often occurs even before the patient can be brought to the hospital.

Globally, at least 421,000 envenomations and 20,000 deaths occur each year due to snake bite. These figures may be as high as 1,841,000 envenomations and 94,000 deaths. Based on the fact that en¬venoming occurs in about one in every four snakebites, be¬tween 1.2 million and 5.5 million snakebites could occur an¬nually[4]. In many parts of Southeast Asian region, snake bite is a familiar occupational hazard of farmers, plantation workers and others, resulting in tens of thousands of deaths each year and innumerable cases of chronic physical handi¬cap[5]. India accounts for about 30,000 deaths per year due to snake bite[6].

More than 2,700 species of snakes are recognized the world over, but only about 450 of these have front fangs that make them capable of injecting venom during the bite7. The venomous snakes belong to four families: Elapidae, Viperidae, Hydrophiidae, and Colubridae.

Elapids are land snakes, the venom of which contains a high concentration of neurotoxins. The elapids, encountered in Africa and Asia[7] include cobras, kraits, mambas, and coral snakes. Renal involvement is uncommon in victims of bites from members of this family.

Vipers include the Russell's viper, Echis carinatus (sawscaled viper), puff adder, pit vipers, and rattlesnakes. The vipers are the most widely distributed species. Russell's viper is found in India, Burma, Pakistan, Thailand, and other areas of Asia; Echis carinatus in Africa, India, Pakistan, Sri Lanka, and the middle east; and the puff adder (Bitis arietans) in Africa [1, 2, 8, 9, 10]

The carpet or saw-scaled viper, Echis carinatus, justifiably can be labeled the most dangerous snake in the world. The factors contributing to its deadliness are its widespread distribution, abundance in farming areas, diurnal habits, good camouflage, and its highly toxic venom.

Hydrophid or sea snake bites are reported mainly among fishing folk of Malaysia, Thailand, and western pacific coastal areas[7,11,12,13,14]. Sea snake venom is primarily myotoxic.

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Colubrids include the boomsiang (Dispholidus typus), and the bird snake (Thelotornis kirtlandi), which are back-fanged African species. Back-fanged colubrids are usually harmless to humans but are occasionally known to cause serious and fatal poisoning[2].

Renal lesions have been associated with bites from members of the last 3 families, including the Russell's viper[15-19,] Echis carinatus[10,16,18,] puff adder [20,21] ,pit viper [22,23,24,25] and sea snake[11,12].

Acute renal failure (ARF), the most significant of all the renal manifestations, has been reported with varying frequency in different studies. . In India, the most widely distributed vipers are Echis carinatus and Russell's viper 3. Most Indian patients are victims of Russell's viper or Echis Carinatus bites15-21

In India, the incidence of ARF is 13% to 32% following Echis carinatus or Russell's viper bite[16,28,29].

A variety of histopathological findings have been described in snake bite patients. The most common of them have Acute tubular necrosis [3,7-9,30,] Acute cortical necrosis[31],Acute diffuse interstitial nephritis[32,33,]Proliferative glomerulonephritis.[34].

The aim of this study were to describe the incidence and clinical profile of the snake bite patients who develop acute renal failure; and to identify the predictors of morbidity and mortality in these patients.

II. Materials and Methods

Fifty (50) cases of definitive snake bite consecutively admitted to Department of Medicine/Emergency medicine, Kempegowda institute of medical sciences, Bangalore from May 2012 to November 2013 were taken up in this prospective obsvervational study.

a) *Inclusion Criteria*

1. Definitive history of snake bite
2. Clinical picture consistent with snake bite, as presence of fang marks or cellulitis or coagulopathy or neuroparaly¬sis
3. Presence of Acute Renal Failure, defined as an abrupt (within 48 hours) absolute increase in the serum creatinine concentration of ≥ 0.3 mg/dL from baseline value measured after admission to our hospital or elsewhere after snake bite, before referral to our hospital, or a percentage increase in the serum creatinine concentration of ≥ 50 percent above base¬line, or oliguria of less than 0.5 mL/kg per hour for more than six hours .Serum creatinine more than 1.5 mg/dL or oli¬guria (urine output less than 400 mL/day)35.

b) *Exclusion Criteria*

1. Patients with pre-existent renal disease (Serum creatinine > 1.5 mg/dL prior to snake bite or ultrasonography of abdomen suggestive of bilateral small kidneys/loss of corticomedullary differentiation / obstruc¬tive nephropathy/other renal pathology)
2. Diagnosed cases of hypertension/diabetes mellitus
3. Exposure to nephrotoxic drugs/toxins.

Data was collected by using pre-tested proforma meeting the objectives of the study. Purpose of the study was carefully explained to the patients and consent was taken.

All patients were interviewed, detailed history was taken with respect to risk factors and detailed physical examination were carried out. Appropriate investigations were carried out.

Laboratory investigations in¬cluded hemoglobin, total and differential leucocyte counts, platelet counts, red cell counts, bleeding and clotting time, coagulation profile including prothrombin time, activated partial thromboplastin time and international normalised ra¬tio (INR), urine microscopy, urinary protein, kidney and liver function tests and serum electrolytes. Radiological investi¬gations included X-ray chest and ultrasonography of abdo¬men.

Statistical analysis: SPSS for Windows version 17.0 (SPSS, Inc, Chicago, Ill) was used for statistical analysis. The Pearson Chi-Square Test was used to analyze parametric variables. A P value of 0.05 or less was considered statistically significant.

III. Results

During the study period of 18 months a total of 50 patients were admitted for snake bite, out of which 36(72%) were males and the male: female was 2.5:1.A majority were from rural areas 37(74%) and the rest from urban areas 13(26%). The mean age of the male patients was 41.81 years and that of the female patients was 48.29 years.

A majority of the patients were farmers (54 %), labourers (18%) and housewife (16 %).The biting species identified were viper (70%),cobra (6%) and unknown species (14%).

The peak incidence in the snake bite cases occurred during the months of july to September. Most of the patients were bitten during the day time (78%).The most frequently bitten site was the lower extremity (70%).

Definitive fang marks were seen in (66%) of the cases. Tourniquet was applied in (10%) of patients.Cellulitis was seen in patients, out of which patients developed compartment syndrome, requiring fasciotomy.

Only 12% of patients presented to hospital within 2 hours of bite and the mean lapse of time from bite to hospitalisation in males was 17.[39] hours and in females was 19.94 hours.

Table 1 : The clinical characteristics of snakebite patients

Symptoms	Number of patients	%
Swelling/inflammation of the bite area	45	90
Fang marks	33	66
Muscle pain/Tenderness	30	60
Proteinuria	26	52
Reduced urine output	25	50
Bleeding from bite site	20	40
Hematuria	20	40
Breathlessness	17	34
Vomiting	13	26
Bleeding from the gums	7	14
Ptosis	5	10

Table 2 : Hematological parameters

Hematological parameters	Number of patients	%
Hemoglobin (Hb in gms)		
<10.0%	13	26
>10.0%	37	74
Total count(/mm3)		
<11000	18	36
>11000	32	64
Platelet count(/mm3)		
<100000	24	48
>100000	26	52
Prothrombin time (in secs)		
< 15 sec	11	22
>15 sec	39	78
Activated partial thromboplastin time(APTT in secs)		
< 30 sec	9	18
>30 sec	41	82
INR		
< 1.5	26	52
>1.5	24	48
Fibrin degradation products(FDP)		
Positive	22	44
Negative	28	56

Table 3 : Sr.Creatinine of patients

Sr creatinine	No patients	%
<1.5	30	60
>1.5	20	40

Table 4 : WBCT in minutes of patients studied

WBCT in minutes	Number of patients	%
<20 min	14	28
>20 min	36	72
Total	50	100

Table 5 : End results of snake bite patients studied

End results	No of patients	%
Discharged	47	94
Death	2	4
Chronic kidney disease	1	2

Table 6 : Number of hospitalization days

Sr .Creatinine	Number of hospitalization days	%	Mean	SD	P value
<1.5*	32	64	9.88	7.534	0.003
>1.5*	18	36	15.94	4.659	0.001

**p<0.005*

Table 7 : Levels of Blood urea of patients studied

Blood urea	Mean ± SD	P value
Baseline	44.66 ± 32.677	0.180
24hrs	53.71 ± 39.688	0.90
2nd day	51.49 ± 42.390	0.186
3rd day	47.86 ± 45.155	0.105

Table 8 : Levels of Serum creatinine of patients studied

Serum creatinine	Mean ± SD	P value from Baseline
Baseline	1.641± 1.319	0.091
24hrs	1.827 ± 1.362	0.076
48hrs	1.756 ± 1.545	0.188
72hrs	1.780 ± 1.639	0.201

Table 9 : Level of urine output

Urine output	Mean ± SD	P value from Baseline
Baseline	651.37 ± 509.825	0.074
24hrs	1436.59 ± 784.178	0.318
48hrs	1751.46 ± 923.601	0.564
72hrs	1956.22 ± 1331.394	0.913

Table no 10 : INR VS Serum Creatinine

INR<1.5	Serum creatinine							
	Baseline		24hrs		48hrs		72hrs	
	Mean±SD	P value	Mean±SD	P value	Mean±SD	P value	Mean±SD	P value
	1.55±1.48	1.000	1.54±1.17	1.000	1.39±0.87	1.000	1.43±1.17	1.000
INR>1.5	Mean±SD	P value	Mean±SD	P value	Mean±SD	P value	Mean±SD	P value
	1.91±1.5	0.071	2.26±1.69	0.071	2.34±2.1	0.352	2.25±2.09	0.728

Laboratory data showed anemia (hemoglobin<10gm%) in 13(26%), leucocytosis in 32(64%), thrombocytopenia in 24(48%), coagulopathy in 18(36%), hematuria in 20(40%), proteinuria in 26(52%), and hyperkalemia in 9(18%) .

Mean urine output at baseline, 24hrs, 48hrs and 72hrs were 651.37 ml, 1436.59 ml, 1751.46 ml and 1956.22 ml. Mean Blood urea at baseline, 24hrs, 48hrs and 72hrs were 44.66 mgs%, 53.71mgs%,51.49 mgs% and 47.86 mgs% and mean serum creatinine at baseline, 24hrs, 48hrs and 72hrs were 1.641mgs%, 1.827mgs%,1.756mgs% and 1.780 mgs% respectively.

There was no significant between group differences in creatinine between those with INR<1.5 and INR>1.5

Out of these 50 patients, 20(40%) patients developed acute kidney injury and 13(26%) required hemodialysis and 1 patient progressed to chronic kidney disease. The mortality rate was 4%.

IV. Discussion

Snakebite is a common medical emergency and an occupational hazard. Renal manifestations include proteinuria, hematuria, pigmenturia, and acute renal failure.

In India, the incidence of ARF is 13% to 32% following Echis carinatus or Russell's viper bite [16, 28, 29].

A variety of histopathological findings have been described in snake bite patients.The most common of them have Acute tubular necrosis[3,7-9,30,] Acute cortical necrosis[31,] Acute diffuse interstitial nephritis [32, 33,] Proliferative glomerulonephritis.[34].

Most of the patients were found to be men in working age group, especially from rural population. Majority of the snake bites occurred between 6 am to 6 pm, i.e., during working hours in the field. As expected, the snake bites more commonly involving lower limbs. So, this also shows that use of protective footwear can reduce the snake bites.

In our study, out of 50 number of snake bite patients, 20(40%) patients developed acute renal failure. This prevalence is higher compared to the other studies from India[16,28,29]. The higher prevalence probably due to higher number of viper bites and delay in administration of ASV, as there is a delay in taking the patient to hospital after snake bite. 45(90%) of the patients had local cellulitis, indicating the vasculotoxic nature of envenomation.

There was a significant increase in duration of hospitalization in those with Creatinine >1.5 (18 days) compared to those with creatinine<1.5 (32 days).

The other common symptoms were swelling/inflammation of bite area (90%), muscle pain/tenderness (60%), oliguria (50%), fever (26%) and vomiting(26%), hematuria(40%) and bleeding from site

was present in 40% patients. Similar figures have been reported previously also[38].

Common findings on examination were tachycardia (38%), breathlessness (34%), and hypotension (36%) and Proteinuria (52%) patients. Common laboratory findings were Anaemia (26%);, Leucocytosis (64%); Thrombocytopenia(48%), Coagulopathy (36%). Coagulopathy is an important factor contributing to increased mortality. The prevalence of coagulopathy in this study (36%) is comparable to that noted by Athappan et al[39] i.e., 27.7%, whereas it is less as compared to other series (60-80%) [40]. By itself, coagulopathy is a marker of the vasculotoxicity and hemotoxicity of the poison, which means that these patients will have nephrotoxicity due to damage to renal microvasculature. Also coagulopathy leads to bleeding and hypotension which, further leads to renal insufficiency as a result of prerenal insult.

The mortality of snake bite induced acute renal failure is found to be 4% in this study. This is less compared to estimates from other studies from India (22-50%)[39]. Kalantri et al reported an overall mortality of 11% in venomous snake bite patients[41]. The mortality can be prevented by intervention at various levels, which include early transfer of the patient to a primary health care facility, where ASV should be administered at the earliest. The high risk patients should be identified early and referred to higher centre.

The limitations of this study were a smaller sample size and lack of investigations like renal biopsy.

This study concludes that acute renal failure occurs in 40% victims of snake bite and is associated with significant increase in the number of hospilisation days. Common manifestations in¬clude cellulitis, oliguria, proteinuria, coagulopathy and thrombocytopenia. The overall mortality of snake bite in¬duced acute renal failure is 4%. Presence of coagulopa¬thy and increase in the number of hospitisation days are predictors of morbidity and mortality in snake bite patients who develop acute renal failure.

References Références Referencias

1. CHUGH KS, PAL Y, CHAKRAVARTY RN, DATTA BN, MEI-ITA R, SAKHUJA V, MANDAL AK, SOMMERS SC: Acute renal failure following poisonous snake bite. Am J Kidney Dis 4:30—38, 1984.
2. SHASTRY JCM, DATE A, CARMAN RH, JOHNY KV: Renal failure following snake bite. Am J Trop Med Hyg 26:1032—1038, 1977.
3. SITPRI.IA V, BOONPUCKNAVIG V: Snake venoms and nephrotoxicity, in Snake Venoms, edited by LEE CY, Berlin, Springer- Verlag, 1979, pp 997—1018.
4. SARANGI A, PATNAIK BC, DAS GC, TRIPATHY N, MIsIt G, SWAIN AK, DAS GK: Renal involvement in viper snake bite. Indian J Med Res 918—923, 1980.
5. MITTAL BY, KINARE SG, ACHARYA VN: Renal lesions following viper bites. Indian J Med Res 83:642—651, 1986.
6. SANT SM, TEMBE VS. SALGAONKAR DS, PURANDARE NM: Lesions produced by Echis cariantus venom in experimental animals. J Postgrad Med 20:70—79, 1974.
7. SEEDAT YK, REDDY J, EDINGTON DA: Acute renal failure due to proliferative nephritis from snake bite poisoning. Nephron 13:455—463, 1974.
8. WARRELL DA, ORMEROD D, DAVIDSON N MCD: Bites by the puff adder (Bitis arietans) in Nigeria and value of antivenom. BrMedJ 4:697—700, 1975.
9. DASILVA OA, LOPEZ M, GODOY P: Bilateral cortical necrosis and calcification of the kidney following snake bite: a case report. Clin JVephrol 11:136—139, 1979.
10. AMORIM MF, MELLO RF: Intermediate nephron nephrosis from snake poisoning in man. Histopathological study. Am J Pathol 30:479—499, 1954.
11. (Jpn) 30:129—135, 1978.
12. DASILVA OA, LOPEZ M, GODOY P: Intensive care unit treatment of acute renal failure following snake bite. Am J Trop Med Hyg 28:401—407, 1979.
13. VERMA SL, GUPTA V, WADHWA MB, MANHAS AS, BASSI SD, SHARMA M, SUDAN SS: Incidence of renal involvement following snake bite poisoning in Jammu. Proc XII Ann Conf Indian Soc Nephrol, 1982, p 4.
14. MATHEW MT. RAJARATNAM K: Renal papillary necrosis in ophitoxemia (abstract). J Assoc Physicians India 35:20, 1987.
15. CHUGH KS, SAKHUJA V: Renal failure from snake bites. IntfArt,f Organs 3:319—321, 1980
16. SHETH S, REGE U, TALWALKAR NC, ACHARYA VN: Acute renal cortical necrosis. Proc XAnn Conf Indian Soc Nephrol, 1980, p 14.
17. SITPRIJA V. SUVANPHA R, POCHANU000L C, CHUSIL S, TUNa- SANGA K: Acute interstitial nephritis in snake bite. Am J Trap Med Hyg 31:408—410, 1982.
18. INDRAPRASIT S, BOONPUCKNAVIG V: Acute interstitial nephritis after a Russell's viper snake bite. Clin Nephrol 25:III, 1986.
19. SITPRIJA V, BOONPUCKNAVIG V: Extracapillary proliferative gbrnerulonephritis in Russell's viper bite. Br Med J 2:1417, 1980.
20. MEHTA RL, KELLUM TA,SHAH SV,MOLITORIS BA RONCOC,WAMOCK DG,LEVIN A. Acute Kidney Injury Net¬work: report of an initiative to improve outcomes in acute kidney injury. Crit Care. 2007;11(2):R31.
21. KANJANABUCH T, SITPRIJA V. Snakebite nephrotoxicity in Asia. Semin Nephrol. 2008;28(4):363-372.

22. KOHLI HS, SAKHUJA V.Snake bites and acute renal fail¬ure. Saudi J Kidney Dis Transpl. 2003;14(2):165-176.
23. Mahmood K, Naqvi IH, Talib A, Salkeen S, Abbasi B, Akhter T, Iftikhar N, et al. Clinical course and outcome of snake envenomation at a hospital in Karachi. Singapore
24. Med J. 2010;51(4):300-305
25. Athappan G, Balaji MV, Navaneethan U, Thirumalikol¬undusubramanian P. Acute renal failure in snake enven¬omation: a large prospective study. Saudi J Kidney Dis Transpl. 2008;19(3):404-410.
26. Ali G, Kak M, Kumar M, Bali SK, Tak SI, Hassan G, Wadhwa MB. Acute renal failure following echis cari¬natus (saw–scaled viper) envenomation. Indian Journal of Nephrology 2004;14:177-181.
27. Kalantri S, Singh A, Joshi R, Malamba S, Ho C, Ezoua J, Morgan M. Clinical predictors of in-hospital mortality in patients with snake bite: a retrospective study from a rural hospital in central India. Trop Med Int Health. 2006;11(1):22-30.

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Development and Validation of RP-HPLC Method for Simultaneous Determination of Guaifenesin Impurities in Multi Drug Combinations

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Abstract - A High Performance Liquid Chromatographic method was developed and validated for quantitative determination of Guaifenesin impurities including 2-(2- methoxyphenoxy)propane-1,3-diol (β-isomer) and 2-methoxyphenol (guaiacol) in different multi drug components pharmaceutical dosage forms, containing guaifenesin, ambroxol hydrochloride and salbutamol sulfate . The different analytical performance parameters such as linearity, precision, accuracy, limit of detection (LOD), limit of Quantification (LOQ) were determined according to International Conference on Harmonization (ICH) Q2B guidelines. The chromatographic separation was achieved on EC NUCLEODUR-100-3C18 (250x4,6 mm, 5µm packing) column using gradient elution of Solvent A (0.1 M ammonium acetate buffer of pH 6.8) and solvent B (acetonitrile : methanol (80:20)) The Ultra Violet spectrophotometric determination was performed at 275 nm. The Linearity of the calibration curves for the analytes in the desired concentration range is good (r2 = 0.999) by High Performance Liquid Chromatography. The LOQ were 1 and 0.1 µg/ml respectively for guaifenesin β-isomer and guaiacol. The average percentage recovery of guaifenesin impurities was found to be within 98.6 – 101.2% of range. The developed method can be successfully used for identification and quantification of guaifenesin impurities β-isomer and guaiacol in the presence of guaifenesin, ambroxol hydrochloride and salbutamol sulfate in multi drug components pharmaceutical formulations.

Keywords: RP-HPLC, validation, guaifenesin impurities, 2-(2-methoxyphenoxy)-propane-1,3-diol (β-isomer) and 2-methoxyphenol (guaiacol).

GJMR-B Classification: NLMC Code: WA 730, WB 330, WB 340

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Development and Validation of RP-HPLC Method for Simultaneous Determination of Guaifenesin Impurities in Multi Drug Combinations

Levon A. Melikyan [α], Rosa S. Grigoryan [σ] & Tigran K. Davtyan [ρ]

Abstract- A High Performance Liquid Chromatographic method was developed and validated for quantitative determination of Guaifenesin impurities including 2-(2-methoxyphenoxy)propane-1,3-diol (β-isomer) and 2-methoxyphenol (guaiacol) in different multi drug components pharmaceutical dosage forms, containing guaifenesin, ambroxol hydrochloride and salbutamol sulfate . The different analytical performance parameters such as linearity, precision, accuracy, limit of detection (LOD), limit of Quantification (LOQ) were determined according to International Conference on Harmonization (ICH) Q2B guidelines. The chromatographic separation was achieved on EC NUCLEODUR-100-3C18 (250x4,6 mm, 5μm packing) column using gradient elution of Solvent A (0.1 M ammonium acetate buffer of pH 6.8) and solvent B (acetonitrile : methanol (80:20)) The Ultra Violet spectrophotometric determination was performed at 275 nm. The Linearity of the calibration curves for the analytes in the desired concentration range is good (r2 = 0.999) by High Performance Liquid Chromatography. The LOQ were 1 and 0.1 μg/ml respectively for guaifenesin β-isomer and guaiacol. The average percentage recovery of guaifenesin impurities was found to be within 98.6 – 101.2% of range. The developed method can be successfully used for identification and quantification of guaifenesin impurities β-isomer and guaiacol in the presence of guaifenesin, ambroxol hydrochloride and salbutamol sulfate in multi drug components pharmaceutical formulations.

Keywords: RP-HPLC, validation, guaifenesin impurities, 2-(2-methoxyphenoxy)-propane-1,3-diol (β-isomer) and 2-methoxyphenol (guaiacol).

I. Introduction

Increased mucus secretion is a clinical feature of severe respiratory diseases, such as asthma, cystic fibrosis and chronic obstructive pulmonary disease [1]. Pharmacological approaches for relieving mucus hypersecretion currently include several classes of agents, including expectorants, mucoregulators, mucolytics, bronchodilators anti-inflammatory drugs and antioxidants [2]. Classic mucolytic drugs such as N-acetylcysteine decrease the viscoelastic properties of mucus by reducing disulfide bonds. In contrast, expectorants change mucus consistency and make coughing more productive, mucokinetics improve transportability, and mucoregulators suppress mucus secretion. Mucolytics generally decrease mucus viscosity by reducing the dicysteine bridges that contribute to the rigidity of the mucins [3]. Guaifenesin (GFN) is a commonly used expectorant drug for productive cough, which is reported to increase the volume and reduce the viscosity of tenacious sputum [4, 5].

Currently recommend consideration for management of mucus hypersecretion is the combination of expectorants, mucoregulators, mucolytics and even bronchodilators in different multi drug components pharmaceutical formulations [3,6]. Therefore, the simultaneous identification and quantification of active pharmaceutical ingredients (API) and its related impurities along with some other active ingredients and excipients in multicomponent pharmaceutical products is a very intensive activity performed at many levels of the drug discovery pipeline and beyond. Impurities relate to starting materials, by-products, breakdown products or polymorphs are of significant concern as they may carry activity responsible for eventual undesirable side effects or toxicity and may interfere with the drug's activity. Thus monitoring impurities in API which exist as various combinations in cough-cold multicomponent drug products is a prerequisite for insuring drug safety and quality.

A literature survey reveals some HPLC methods that are reported for the simultaneous determination of GFN along with some other active ingredients in a multicomponent tablet and liquid dosage formulation as anticipated with the variation of mobile phase, column and detector. Different HPLC methods for individual assay and related impurities are available for GFN in official pharmacopoeia and several LC-MS/MS methods were used for determination of GFN in Human Plasma [11]. Hence an attempt has been made to develop a simple, efficient and selective method for the determination of guaifenesin impurities (Figure 1), 2-(2-methoxyphenoxy)-propane-1,3-diol (β-isomer) and 2-

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methoxyphenol (guaiacol) in the presence of guaifenesin, ambroxol hydrochloride and salbutamol sulfate in multi drug components pharmaceutical formulations.

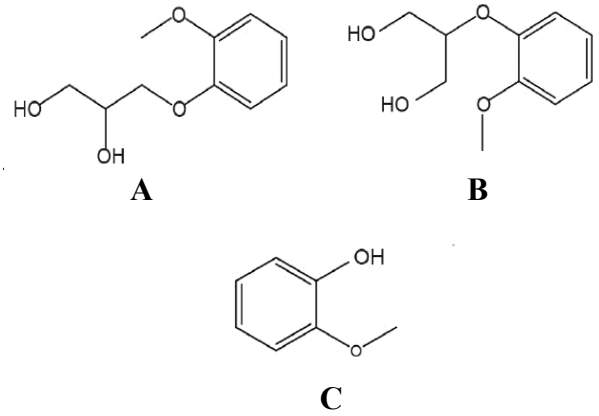

Figure 1 : Structure of guaifenesin (A), guaifenesin β-isomer (B) and guaiacol (C).

II. Materials and Methods

a) Instrumentation

A High Performance Liquid Chromatography (HPLC) method for GFN β-isomer and guaiacol analytical method was developed on PLATIN BLUE UPLC (Knauer, Germany) with diode array detector. NUCLEODUR-100-3C18 (250x4, 6 mm, 3 µm packing, Machery-Nagel, Germany) column was used. The elution was carried by gradient elution method of mobile phases A and B.

b) Chemicals

Ammonium acetate (Sigma-Aldrich, HPLC grade), Millipore water, methanol (HPLC grade, Alpha chemika, purity: 99.9%, batch: A----); acetornitrile (HPLC grade, Alpha chemika; purity: 99.9%, batch: A5982;), impurity A, 2-methoxyphenol (guaiacol) Sigma-Aldrich, purity: 99,9%); impurity B 2-(2-methoxyphenoxy)-propane-1,3-diol (β-isomer) (Sigma-Aldrich, purity: 99,9%); GFN and ambroxol hydrochloride (Sigma-Aldrich, purity: 99,9%); salbutamol sulfate (Sigma-Aldrich, purity: 99,8%); methylparaben (Sigma-Aldrich, purity: 100%); propylparaben (Sigma-Aldrich, purity: 100%) and citric acid monohydrate (Sigma-Aldrich, purity: 99.7%) were used in this study.

c) Preparation of stock solution and working standard solution

Preparation of mobile phase. Solvent A - 7.7 gm of ammonium acetate was weighed and transferred into a 1000 ml beaker, dissolved and diluted with 1000 ml water and pH brought to 6.8 by ammonia or acetic acid. The solvent A was filtered through 0.45 µm membrane filter under vacuum filtration and was degassed before used, then delivered at a flow rate 1.0 ml/min. Solvent B - acetonitrile and methanol (80:20).

d) Preparation of solvent for standards and sample

Solvent C- 750 ml of solvent A and 250 ml of solvent B are mixed together.

e) Preparation of standard solution

10.0 mg of GFN standard was weighed and transferred into 10 ml volumetric flask. 8 ml of solvent C was added sonicated for 5 min, mixed thoroughly to dissolve and make up the volume to 10 ml with mobile phase (1 mg/ml concentration). 5.0 mg of guaiacol reference standard was weighed and transferred into 20 ml volumetric flask and make up the volume to 20 ml with solvent C. 1.0 ml of guaiacol solution was transferred into 50 ml volumetric flask and make up the final volume to 50 ml with solvent C (5 µg /ml concentration). 10.0 mg of GFN β-isomer reference standard was weighed and transferred into 20 ml volumetric flask and make up the volume to 20 ml with solvent C. 1.0 ml of GFN β-isomer solution was transferred into 50 ml volumetric flask and make up the final volume to 50 ml with solvent C (10µg/ml concentration).

f) Preparation of sample solution

Melon® (Aversi, Georgia) which is a combination of ambroxol hydrochloride (15 mg); salbutamol sulfate (2.4 mg); guaiphenesin (100 mg), per 190 mg tablet or a cough mixture of ambroxol hydrochloride (15 mg); salbutamol sulfate (1.2 mg); guaiphenesin (50 mg), per 5 mL syrup were used in this study. 20 tablets were grinded in to a homogenous powder and 190 mg were transferred into 100 ml volumetric flask and make up the final volume to 100 ml with solvent C (1mg/ml concentration). 10.0 ml of the syrup was transferred into 100 ml volumetric flask and make up the final volume to 100ml with solvent C (1mg/ml concentration). The sample solutions were sonicated for 5 min, mixed thoroughly to dissolve and filtered through 0.45 µm membrane filter.

g) Specificity and Robustness

The specificity of the assay method is established by injecting blank, containing 1 mg/ml GFN, ambroxol hydrochloride, salbutamol sulfate methyl-, propylparaben and citric acid monohydrate as well as standard and samples into the HPLC. The identity of GFN impurities, including β-isomer and guaiacol was confirmed by comparison of its retention time (RT) and UV-spectra. Robustness was established by varying the chromatographic condition with respect to specificity of the method in various pH conditions of mobile phase. Standard and sample solutions were injected and the chromatograms were recorded.

h) Quantification Limits

The quantification limit was defined as the lowest fortification level evaluated at which acceptable average recoveries were achieved and analyte peak is

consistently generated at approximately 10 times the baseline noise in the chromatogram. The limit of quantification (LOQ) was defined as LOQ=10 S/K. Where 'S' is the standard deviation of replicate determination values; 'K' is the sensitivity namely the slope of the calibration graph.

i) Calibration curve

The calibration curve was constructed by plotting peak area concentration of GFN impurities standard solutions. Aliquots of guaiacol standard stock solutions in the concentration range 0.1-10 µg/ml and GFN β-isomer reference standard in the concentration range 1.0 - 100 µg/ml were transferred into 25 ml volumetric flask and 10 ml of solvent C was added, sonicated for 5 min, mixed thoroughly to dissolve and make up the volume to 25 ml with solvent C. Each concentration of the standard solutions 10µl was injected and the chromatograms were recorded. The calibration graph was done by external standard calibration and confirmed using back calculation method.

j) Accuracy

Accuracy was determined for standard quality samples (in addition to calibration standard) prepared in triplicates at different concentration levels (5.0, 50, 100 µg/ml for GFN β-isomer and 0.5, 5.0, 10.0 µg/ml for guaiacol standard solutions respectively.) within the range of linearity of GFN impurities. The results of analysis of recovery studies were obtained by method validation by statistical evaluation.

k) Precision

The precision of the instruments was checked by repeatedly (intra day) intermediate (inter day) and reported as % RSD for a statistically significant number of replicate measurements. Repeatability and intermediate precision of the method were determined by analyzing 6 samples of the test concentration 5.0, 50, 100 µg/ml for GFN β-isomer and 0.5, 5.0, 10.0 µg/ml for guaiacol standard solutions respectively.

l) Stress Conditions

The stress conditions employed for degradation study included oxidative hydrolysis and photochemical degradation as it described in [12]. To 10 ml of both GFN standard solution and pharmaceutical formulations 10 ml of 1 % v/v H2O2 was added separately. These mixtures were refluxed separately for 1 hour at room temperature. The forced degradation in oxidative media was performed in the dark in order to exclude possible photo-degradation. For carrying out photolysis studies the samples were treated with UV light for 6 hours at 254 nm and also in sunlight.

III. Results and Discussion

a) Method development

The aim of this study was to develop a simple, efficient and selective method for the determination of GFN impurities 2-(2-methoxyphenoxy)-propane-1,3-diol (β-isomer) and 2-methoxyphenol (guaiacol) in the presence of GFN, ambroxol hydrochloride and salbutamol sulfate in multi drug components pharmaceutical tablet and syrup formulations. Various attempts were made to separate all degradation products with different pH of the mobile phase buffer and composition of methanol in the mobile phase using C-18 and C-8 stationary phase columns. The RP-HPLC method for GFN β-isomer and guaiacol was optimized (Table 1). To ensure great resolution between all known and unknown degradation compounds, the C-18 stationary phase with an end-capping was used. HPLC parameters, such as detection wavelength, ideal mobile phase & their proportions and flow rate were carefully studied (Table 1). After trying different ratios of mixtures of methanol:acetonitrile and ammonium acetate buffer the best results were achieved by using a gradient elution. The mobile phase gradient constituted by ammonium acetate buffer: (solvent A) and acetonitrile: methanol (80:20) (solvent B). At a flow rate of 1.0 ml/min, the retention time were 6, 32 min for guaiacol and 12, 73 min for GFN β-isomer. The analytes peak areas were well defined and free from tailing under the described experimental conditions.

b) System suitability

System suitability test was carried out on freshly prepared solution of GFN β-isomer and guaiacol to ensure the validity of the analytical procedure. Data from six injections were used to confirm system suitability parameters like retention time, UV-spectra and peak area. The results are presented in Table 2. The values obtained demonstrated the suitability of the system for the analysis of GFN impurities. The method gives sharp and well defined peaks with significant RT values which were desired for quantification of GFN related impurities in the presence of blank, containing GFN, ambroxol hydrochloride and salbutamol sulfate (Table 2).

c) Specificity

Specificity is the ability of the method to measure the analytes response in the presence of their potential impurities and degradation products. Blank (placebo) interference was evaluated by analyzing the blank, containing GFN, ambroxol hydrochloride, salbutamol sulfate methyl-, propylparaben and citric acid, prepared as in the test method (Figure 2a). The method showed specificity because GFN β-isomer and guaiacol were well-resolved and no interfering peaks were observed as it appears in Figure 2b. Stress studies were performed either for guaifenesin impurities and tablet to provide an indication of the stability-indicating

property and specificity of proposed method. The stress conditions employed for degradation study included oxidative hydrolysis and photochemical degradation. GFN β-isomer and guaiacol were found stable under oxidative and photolytic stress conditions (Figure 3). The peak purity test was carried out for the guaifenesin peak by using the PDA detector in stress samples. The mass balance (% assay + % sum of all degradants + % sum of all impurities) results were calculated and found to be more than 95%. The purity of GFN β-isomer and guaiacol was unaffected by the presence of GFN, ambroxol hydrochloride, salbutamol sulfate methyl-, propylparaben and citric acid and degradation products, and thus confirms the stability-indicating power of the developed method.

d) Linearity and LOQ

The linearity was determined by constructing calibration curve. The calibration curves in this study were plotted between amount of each of analyte versus peak area and the regression equations with a regression coefficient were obtained. The linear regression data (Table 3) showed good linear relationship over a concentration range of 1-100 μg/ml for GFN β-isomer and 0.1-10.0 μg/ml for guaiacol. Regression equation for GFN β-isomer was Y=7.709X + 0.165 and Y=5.588X + 0.005 for guaiacol with a regression coefficient of 0.9999 for each of analyte. The linearity of estimated RP-HPLC method was found to be over the concentration range of 1-100 μg/ml for GFN β-isomer and 0.1-10.0 μg/ml for guaiacol which furthermore have been confirmed using back calculation method. The RE % of linearity back calculation method requirements for analyte calculated to introduced concentration ration to be less than 15% for at last 6 calibration standards or 75 % of samples, expect LOQ, which should be not less than 20%. As it shown in the Table 3, the GFN β-isomer and guaiacol RP-HPLC assay linearity meets all the validation quality requirements.

In order to determine the quantification limit analytes concentration in the lower part of calibration curve was used. GFN β-isomer and guaiacol solutions of 1μg/ml and 0.1 μg/ml respectively were prepared and analyzed using six replicates and the amount of each analyte peak area was determined. The LOQ values for GFN β-isomer and guaiacol are shown in Table 3.

e) Accuracy and precision

The intra day precision was determined by measurement of analyte concentration using five replicates of GFN impurities solutions at three different concentrations 0.5; 5 and 10 μg/ml for guaiacol and 5,0; 50 and 100 μg/ml for GFN β-isomer two times on the same day and inter day variations were determined similarly on consecutive days. These concentrations have been selected according to the assay quantification low, medium and high limits for each of analyte (QCL, QCM and QCH respectively). The repeatability of sample application was assessed 5 times on HPLC followed by recording of the amount of GFN related impurities solutions. The % RSD for peak values of guaiacol was found to be 2.188% and 2.591% for QCL intra and inter-day precision respectively. The % RSD and results for GFN related impurities QCL, QCM and QCH concentration are depicted in Table 4, which reveal intra and inter day variations of analytes concentration.

f) Recovery studies

The accuracy of the proposed method was also further assessed by performing recovery experiments using the standard addition method. Recovery studies of the different samples were carried out for the accuracy parameter. These studies were carried out at three levels (QCL, QCM and QCH respectively); sample solutions of 5, 50 and 100 μg/ml as well as standard solutions were prepared for the GFN β-isomer and recovery studies were performed using five replicates. For guaiacol accuracy parameter studies three concentration levels either of sample solutions as well as standard solutions QCL, QCM and QCH, corresponding to 0.5, 5.0 and 10.0 μg/ml concentration respectively were used. The repeatability of sample application was assessed 5 times on HPLC followed by recording of the peak area of GFN related impurities solutions. Percentage recovery was found to be within the limits as listed in Table 5.

g) Robustness

To determine the robustness of the developed method, experimental conditions were deliberately altered and the relative retention time of β-isomer and guaiacol with respect to guaifenesin; and system suitability parameters for guaifenesin standard was recorded. The variables evaluated in the study were pH of the mobile phase buffer (±0.2), column temperature (± 5°C). In all the deliberate varied chromatographic conditions, all analytes were adequately resolved and the elution order remained unchanged.

IV. Conclusion

A new, accurate and selective HPLC method were proposed for the determination of guaifenesin impurities, 2-(2-methoxyphenoxy)-propane-1,3-diol (β-isomer) and 2-methoxyphenol (guaiacol) in the presence of guaifenesin, ambroxol hydrochloride , salbutamol sulfate in multi drug components pharmaceutical formulations as per the ICH guidelines. The methods were found to be simple, selective, precise and accurate. Therefore, these methods can be used as routine testing as well as stability analysis of guaifenesin and ambroxol impurities in bulk and in formulations. All statistical results (Percentage, Mean, RSD, Percentage

difference and recovery %) were within the acceptance criteria.

V. Acknowledgements

We thank Professor Hakob V. Topchyan PhD, DSc, Director of Scientific Center of Drug and Medical Technology Expertise JSC, Ministry of Health of Armenia, for his critical reading of the manuscript and support of this study in the Centre.

Table 1 : Optimized chromatographic conditions for GFN impurities, including GFN β-isomer and guaiacol, and ambroxol impurities

Parameter/Condition	Specification				
Column	EC NUCLEODOR-100-3C18 (250x4,6mm, 5μm packing)				
Mobile phase gradient	Solvent A- 0.1 M ammonium acetate buffer of pH 6.8 Solvent B - acetonitrile:methanol (80:20)				
Working wavelength	275 nm				
Column temperature	45°C				
Sample volume	10 uL				
Run time	60 min				
Gradient elution		Time (min)	Flow (ml/min)	Comp. A (%)	Comp. B (%)
	1.	0.0	1.0	25	75
	2.	20.0	1.0	25	75
	3.	40.0	1.0	50	50
	4.	42.0	1.0	50	50
	5.	50.0	1.0	25	25
	6.	60.0	1.0	25	25

Table 2 : Specificity of RP-HPLC method for GFN β-isomer and guaiacol *

Parameters	guaiacol	GFN β-isomer
R_T	12,726 ± 0,0087	6,318 ± 0,060
Peak area	17517,4 ± 417,17	11591.6 ± 180,76
R_T %RSD ¶	0,068	0,95
Peak area %RSD¶	2,381	1,559

**All data represent Mean ± SD for n=6 standard samples for each of mentioned analyte. Grubbs test detects no outliers from normal distribution (α = 0.02). %RSD = 100 × (SD/Mean).*

Table 3 : Regression analysis of the calibration curve for GFN β-isomer and guaiacol for the proposed RP-HPLC method

Parameters	guaiacol	GFN β-isomer
Concentration range	0.1-10 μg/ml	1-100 μg/ml
Slope	5.588	7.709
Intercept	0.005	0.165
Correlation coefficient	0.9999	0.9999
Regression equation	Y=5.588X + 0.005	Y=7.709X + 0.165
RE%*	-2.051	0.175
LOQ (μg/ml)	0.098 ± 0.0029	1.017 ± 0.0109
LOQ %RSD	2.974	1.078

** RE % of linearity back calculation method represented the percentage of ration 100×(E-T)/T, where E is a calculated concentration and T – is a introduced concentration of the analyte. All data represent Mean ± SD for n=6 standard samples for each of mentioned analyte. Grubbs test detects no outliers from normal distribution (α = 0.02). %RSD = 100 × (SD/Mean).*

Table 4 : Intra day and inter day precision of the RP-HPLC method for GFN related impurities solutions

Parameters	guaiacol			GFN β-isomer		
Concentration of analyte added (μg/ml)	0.5 QCL	5.0 QCM	10.0 QCH	5.0 QCL	50.0 QCM	100.0 QCH
Concentration of analyte found (μg/ml) *	0.515 ±0.011	5.219 ±0.043	10.23 4±0.046	4.981 ±0.104	51.516 ±0.536	98.030 ±0.219
Intra day %RSD ¶	2.188	0.842	0.452	2.095	1.041	0.224
Inter day %RSD ¶	2.591	3.681	1.702	1.248	1.694	4.253

**Mean and SD represent for n=5 standard samples for each of mentioned analyte. %RSD = 100 × (SD/Mean).*

Table 5 : Recovery analysis of the RP-HPLC method for GFN related impurities

Parameters	guaiacol			GFN β-isomer		
	QCL 0.5 μg/ml	QCM 5 μg/ml	QCH 10 μg/ml	QCL 5 μg/ml	QCM 50 μg/ml	QCH 100 μg/ml
Peak area of sample*	8697 ± 249.2	87717± 832.2	180311 ± 2290	60141 ± 780.7	624380 ± 18210	1369669 ± 44541

Peak area of standard*	8690 ± 76.7	87091 ± 554.1	174405 ± 820.3	59578 ±1681	609760 ± 30391	1289523 ± 47336
Recovery %¶	101.24	110.7	103.4	100.94	102.49	103.21
Average %	101.77			103.21		
%RSD¶	1.39			2.63		

**Mean and SD represent for n=5 standard samples for each of mentioned analyte. Recovery % is calculated by 1-(sample average peak area/ standard average peak area)×100%. %RSD = 100 × (SD/Mean).*

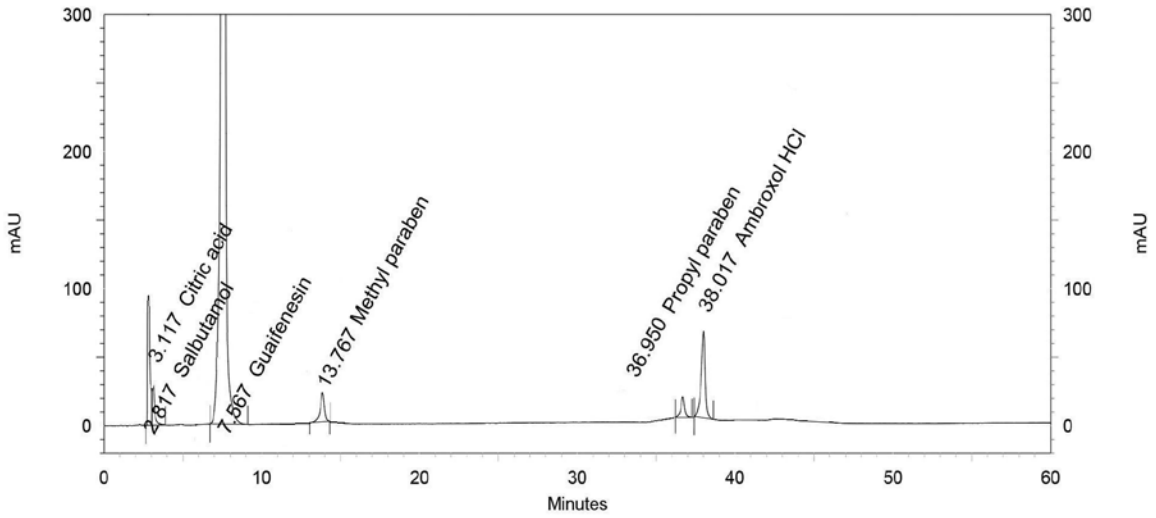

Figure 2a : Typical Chromatogram of blank solution containing GFN, Salbutamol and Ambroxol

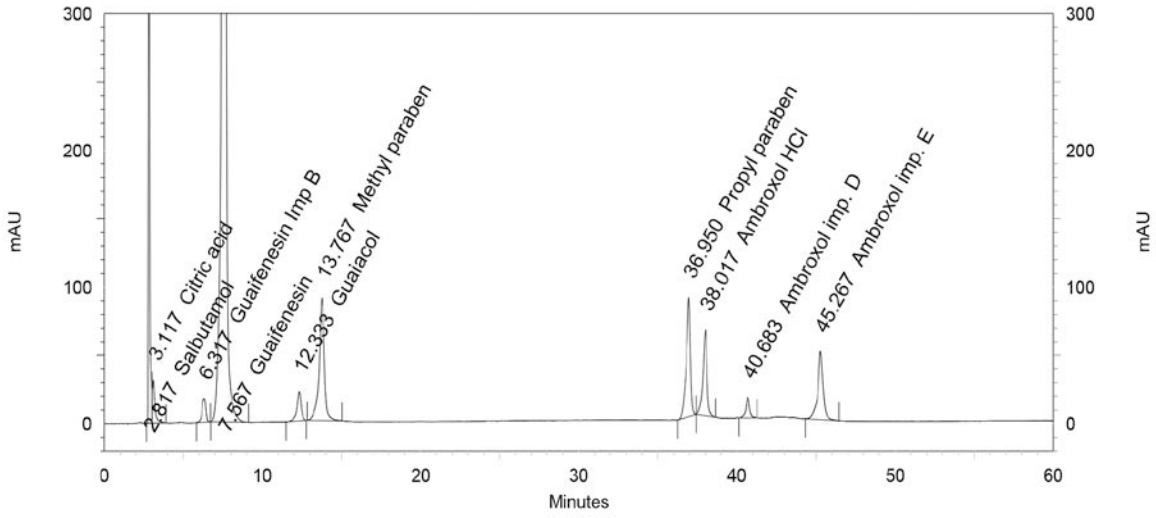

Figure 2b : Chromatographic separation of guaifenesin impurities in mix solution containing salbutamol, ambroxol HCl, GFN, preservatives and Ambroxol HCl impurities

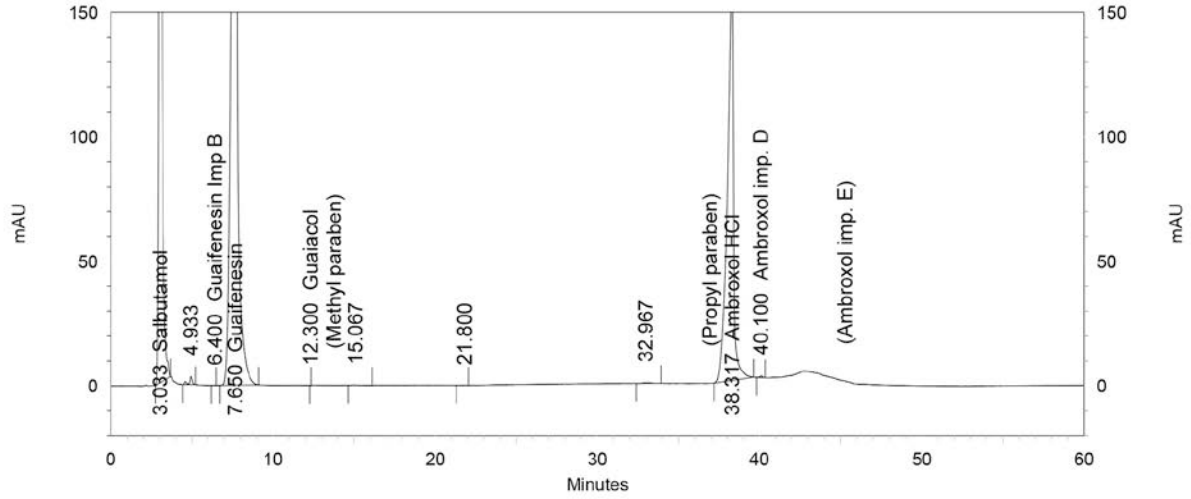

Figure 3 : Typical Chromatogram of sample spike solution after oxidation stress

References Références Referencias

1. Cerveri I, Brusasco V. Revisited role for mucus hypersecretion in the pathogenesis of COPD. Eur Respir Rev 2010; 19: 116, 109–112.
2. Houtmeyers E, Gosselink R, Gayan-Ramirez G, Decramer M. Effects of drugs on mucus clearance. Eur Respir J 1999; 14: 452-467.
3. Balsamo R, Lanata L, Egan CG. Mucoactive drugs. Eur Respir Rev 2010; 19: 116, 127–133.
4. Seagrave JC, Albrecht HH, Hill DB, Rogers DF, Solomon G. Effects of guaifenesin, N-acetylcysteine, and ambroxol on MUC5AC and mucociliary transport in primary differentiated human tracheal-bronchial cells. Respiratory Research 2012, 13:98.
5. Prabhu Shankar S, Chandrashekharan S, Bolmall CS, Baliga V .Efficacy, safety and tolerability of salbutamol + guaiphenesin + bromhexine (Ascoril) expectorant versus expectorants containing salbutamol and either guaiphenesin or bromhexine in productive cough: a randomised controlled comparative study. J Indian Med Assoc. 2010;108: 313-314.
6. Yakoot M, Salem A, Omar AM. Clinical efficacy of farcosolvin syrup (ambroxol–theophylline–guaiphenesin mixture) in the treatment of acute exacerbation of chronic bronchitis. Int. J Chronic Obstructive Pulmonary Disease 2010:5 251–256.
7. Abdelwahab NS. Determination of ambroxol hydrochloride, guaifenesin, and theophylline in ternary mixtures and in the presence of excipients in different pharmaceutical dosage forms. J AOAC Int. 2012 Nov-Dec;95(6):1629-38.
8. Jain JK, Prakash MS, Mishra RK, Khandhar AP. Simultaneous determination of multi drug components Theophylline, Etofylline, Guaiphenesine and Ambroxol Hydrochloride by validated RP-HPLC method in liquid dosage form. Pak J Pharm Sci. 2008 21:151-8.
9. Porel A, Haty S, Kundu A. Stability-indicating HPLC Method for Simultaneous Determination of Terbutaline Sulphate, Bromhexine Hydrochloride and Guaifenesin. Indian J Pharm Sci. 2011 73:4 6-56.
10. Reddy SP, Babu KS, Kumar N, Sekhar SY V V. Development and validation of stability indicating the RP-HPLC method for the estimation of related compounds of guaifenesin in pharmaceutical dosage forms. Pharmaceutical Methods: 2011; 2 229-234.
11. Andrew Asirvatham A, Manikandan K, Mailvelan R., Konam K, Rajavel P. Estimation of guaifenesin in human plasma by liquid chromatography coupled with tandem mass spectroscopy. Int J. Biol. & Pharm. Res. 2012; 3(3): 463-468.
12. Bhattacharyya I, Bhattacharyya SP, Kyal C, Choudhury P, Dhakal B, Ghos SK. Estimation and validation of stability indicating UV spectrophotometric methos for the determination of guaifenesin in presence of its degradant products. Int. J Pharm. Pharm. Sci. 2013; 5(Suppl 1): 262-268.

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Simple UV Spectrophotometric Assay of Atorvastatin API Formulation and their Comparative Study

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Abstract - A rapid, simple, accurate, and economical least time consuming rosuvastatin spectrophotometric method has been developed for the assay of atorvastatin and then compare assay of brand available in Karachi,Pakistan. The assay is based on the ultraviolet UV absorbance maxima at about 244nm wavelength of atorvastatin using methanol as solvent. A sample of drug was dissolved in methanol to produce a solution containing atorvastatin. Similarly, a sample of ground tablets of different brand were extracted with methanol and diluted with the same methanol. The absorbance of sample preparation was measured at 244 nm against the solvent blank and the assay was determined by comparing with the absorbance of available brand. The method can be applied for the routine QC quantitation of atorvastatin in tablet formulation and active.

Keywords: *atorvastatin, assay, uv pectrophotometry.*

GJMR-B Classification: *NLMC Code: QV 55, WB 330*

Strictly as per the compliance and regulations of:

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I. Introduction

Atorvastatin figure 1 is an HMG-CoA reductase inhibitors (3-hydroxy,3-methylglutaryl-CoA), called statins. It was a breakthrough for the prevention of hypercholesterolemia and related diseases.1-3 Cholesterol has an important role in the daily functioning of the body. But, it can also have a negative effect to the development of atherosclerosis. These plaques can block the arteries, disturb blood flow, or may rupturing and causing a clot that increases blockage. The results of these blockages are very serious and can cause angina, claudication, stroke and heart attack.4 Hyperlipidemia and hypertension are correlated to each other and have additional effect on CHD coronary heart disease and associated mortality rate, since CV cardiovascular disease is closely related to some factors such as high cholesterol levels, hypertension or diabetes. In literature there are many evidences which suggest additive beneficial effects of statin combined with losartan in the treatment of hypercholesterolemia, hypertensive patients.5

There are several methods reported by HPLC with the statin 6-10 but there is study found that show the comparison of available brands in market.Because the therapy is very expensive an person who has any cardiovascular disorder take medicine life time when he started.Therefor it is important that they use medicine should not be expensive and give hundred % result.

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There for in the mind of this I have checked the % assay of different brands available in the market .

The aim of this study is to investigate the assay of commercially available six brands of atorvastatin in Karachi, Pakistan.

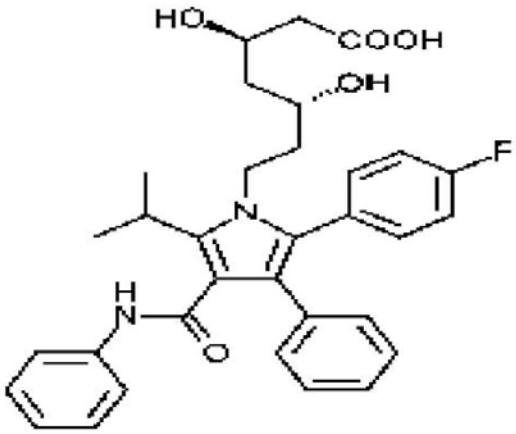

Figure 1 : Structure of Atorvastatin

II. Experimental

UV visible 1601 Shimadzu double beam spectrophotometer was used to measurement of spectra. The solvent which are used for the assay was spectroscopic-grade methanol.

a) Wavelength Selection

About 100 ppm of atorvastatin was accurately prepared in spectroscopic-grade methanol . This solutions were scanned in the 200-400 nm UV region. The wavelength maxima (λmax) was observed at 244 nm and this wavelength was adopted for absorbance measurement.

b) Standard Stock solution

Accurately weighed 10 mg of atorvastatin standard was transferred to a volumetric flask and add add suffi-cient methanol to produce 100 ml. This was sonicated 5 min to dissolve it.

c) Sample Preparation

The six different brands were purchased from different Public medical store located in Karachi, Pakistan. All tablets of each brand have same batch

number and were labeled to conatin atorvastatin 10mg per tablet. All the six brands have 5 year shelf life.

The serial number as an identification of purchased brands are given in Tabel 1.20 tablets of six different brand of atorvastatin from the marketed sample were weighed and crushed uniformly with the help of a mortar and pestle. By calculating the average weighed sample powder equivalent to 10 mg of atorvastatin was transferred into a volumetric flask containing 10mL methanol solvent MeOH. The solutions were sonicated for about 5 min and than make up volume upto 100 ml with water.

d) Procedure

After preparation of standard and tablet solutions, strength of solution 100 ppm in 100 ml absorbance of the sample preparation and standard preparation in 1cm cell at the wavelength of maximum absorbance at about 244 nm, using a spectrophotometer, using the blank solution. Calculate the quantity in mg, of atorvastatin per tablet.

III. Results and Discussions

Pharmaceutical assay was carried out by using spectrophotometer on all brands of atorvastatin tablets during the study. Table-1 shows name brand and % assay of different brands. Table-2 ,3 are showing the descriptive within and between groups and shows result are highly significant with p value 0.000.

Test of hypothesis i-e ANOVA and multiple comparison of different brands of atorvastatin are given in table 3 shows highly significant difference of all brands with each other. The proposed method for the assay of commercially available atorvastatin tablet formulation is very simple, accurate ,least time consuming and rapid. It can be easily used for routine quality control QC for monitoring the assay in the API, in-process samples and tablet formulation. ANOVA shows between and within group F value 309348.804 with degree of freedom df value5 and 24 and p value 0.00 which shows significant results.

References Références Referencias

1. Tobert, J. A. Nat. Rev. Drug Discov. 2003, 2, 517-526.
2. Jones, P. H.; Davidson, M. H.; Stein, E. A.; Bays, H. E.;McKenney, J. M.; Miller, E. Am. J. Cardiol. 2003, 92, 152-160.
3. Caslake, M. J.; Stewart, G.; Day, S. P.; Daly, E. Atherosclerosis 2003, 171, 245-253.
4. McKenney, J. M. Am. J. Health-Syst. Pharm. 2005, 62,1033-1047.
5. Koh, K. K.; Quon, M. J.; Han, S. H. J. Am. Heart Assoc.2004, 110, 3687-3692.
6. Sultana N, Arayne MS and and Safila Naveed (2010) Simultaneous Determination of Captopril and Statins in API, Pharmaceutical Formulations and in Human Serum by RP-HPLC J. Chin. Chem. Soc., 57, 378-383.
7. Sultana N, Arayne MS, Shah SN and Safila Naveed (2010) Simultaneous determination of Prazosin, Atorvastatin, Rosuvastatin and Simvastatin in API, Dosage Formulations and Human serum by RP-HPLC. Journal of the Chinese Chemical Society, 57(6), 1286-1292. I
8. Sultana N, Arayne MS and Safila Naveed (2011) Validated Method for the Simultaneous Determination of Lisinopril, Pravastatin, Atorvastatin and Rosuvastatin in API, Formulations and Human Serum by RP-HPLC Chinese Journal of Chemistry 29, 1216-1220.DOI: 10.1002/cjoc.201190226.
9. Sultana N, Arayne MS and Safila Naveed (2011) simultaneous Determination of Enalapril and Statins In Pharmaceutical Formulations by RP- HPLC, Chilean chemical society 56(3),734-737.
10. Arayne MS, Sultana N, Arman Tabassum Saeeda Nadir Ali and Safila Naveed (2012). Simultaneous LC Determination of Rosuvastatin, Lisinopril, Captopril, and Enalapril in API, Pharmaceutical Dosage Formulations, and Human Serum Medicinal Chemistry Research 21:4542-4548 DOI 10.1007/s00044-012-9997-

Table 1 : % assay of different brands of atorvastatin

Brand Name	Serial no	Average wt of tablet mg	Wt for 100 ppm	Absorbance at 244 nm	% assay
Prostatin	ATR- 1	16.43	16.43	0.157	104.66
Statin	ATR- 2	16.6	16.6	0.137	91.33
Fopsec	ATR- 3	15.9	15.9	0.099	66.00
Winstor	ATR- 4	15.6	15.6	0.118	78.66
Survive	ATR- 5	18.8	18.8	0.059	39.33
Lipiget	ATR- 6	18.8	18.8	0.093	62.00

Table 2 : Descriptive statistics of different brands of atorvastatin

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	13328.351	5	2665.670	309348.804	.000
Within Groups	.207	24	.009		
Total	13328.557	29			

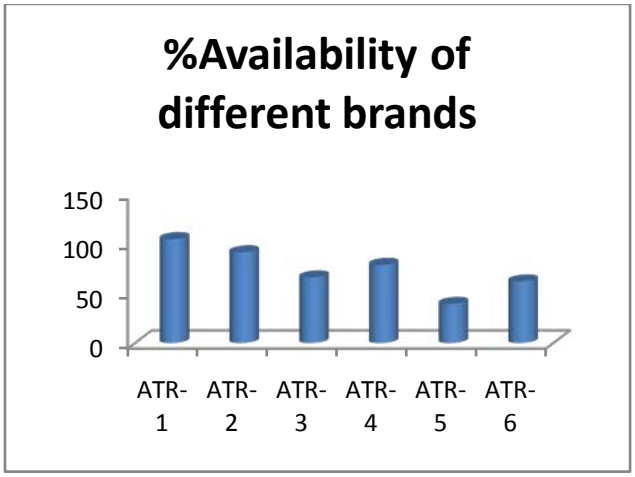

Figure 1

Table 5 : Multiple Comparisons of different brands of acetaminophen

(I) Brand Name	(J) Brand Name	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
ATR1	ATR2	13.30467*	.05871	.000	13.1835	13.4258
	ATR3	38.56733*	.05871	.000	38.4462	38.6885
	ATR4	25.96600*	.05871	.000	25.8448	26.0872
	ATR5	65.29267*	.05871	.000	65.1715	65.4138
	ATR6	42.53133*	.05871	.000	42.4102	42.6525
ATR2	ATR1	-13.30467*	.05871	.000	-13.4258	-13.1835
	ATR3	25.26267*	.05871	.000	25.1415	25.3838
	ATR4	12.66133*	.05871	.000	12.5402	12.7825
	ATR5	51.98800*	.05871	.000	51.8668	52.1092
	ATR6	29.22667*	.05871	.000	29.1055	29.3478
ATR3	ATR1	-38.56733*	.05871	.000	-38.6885	-38.4462
	ATR2	-25.26267*	.05871	.000	-25.3838	-25.1415
	ATR4	-12.60133*	.05871	.000	-12.7225	-12.4802
	ATR5	26.72533*	.05871	.000	26.6042	26.8465
	ATR6	3.96400*	.05871	.000	3.8428	4.0852
ATR4	ATR1	-25.96600*	.05871	.000	-26.0872	-25.8448
	ATR2	-12.66133*	.05871	.000	-12.7825	-12.5402
	ATR3	12.60133*	.05871	.000	12.4802	12.7225
	ATR5	39.32667*	.05871	.000	39.2055	39.4478
	ATR6	16.56533*	.05871	.000	16.4442	16.6865
ATR5	ATR1	-65.29267*	.05871	.000	-65.4138	-65.1715
	ATR2	-51.98800*	.05871	.000	-52.1092	-51.8668
	ATR3	-26.72533*	.05871	.000	-26.8465	-26.6042
	ATR4	-39.32667*	.05871	.000	-39.4478	-39.2055
	ATR6	-22.76133*	.05871	.000	-22.8825	-22.6402

ATR6	ATR1	-42.53133*	.05871	.000	-42.6525	-42.4102
	ATR2	-29.22667*	.05871	.000	-29.3478	-29.1055
	ATR3	-3.96400*	.05871	.000	-4.0852	-3.8428
	ATR4	-16.56533*	.05871	.000	-16.6865	-16.4442
	ATR5	22.76133*	.05871	.000	22.6402	22.8825

**. The mean difference is significant at the 0.05 level.*

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Assessment of Adequate use of Asthma in halational Medication Administration in Children in Gondar University Teaching Hospital, Northwest Ethiopia

By Belayneh Kefale Gelaw & Yitayih Kefale Gelaw
Ambo University, Ethiopia

Abstract - *Introduction:* Asthma and other chronic airway diseases can be effectively treated by inhaler therapy. Inhaler therapy depends on appropriate use of the inhaler. For asthma, inhalation therapy is the foundation of treatment. Yet all too often, patients do not get the full value of their inhaled medications because they use their inhaler incorrectly. The objective of this study was to evaluate the knowledge among asthmatic children and their parents regarding asthma inhaler therapy and appropriateness of its use.

Method: Cross-sectional study was conducted on assessment of adequate use of asthma inhalational medication in children.

Results: Sixty one asthmatic children were involved in the study with a mean age of 4.67 + 3.69 years; 35 (57.4%) were males and 26 (42.6%) were females. Of 61 asthmatic children 44 (72.1%) were preschool children, 32 (52.5%) asthmatic children were living in a number of family 1-5, 28 (45.9%) were living in a family number of 5-10 and only 1 (1.6%) lived in a family number of >10. Nineteen (31.1%) asthmatic children had a family history of asthma.

GJMR-B Classification: *NLMC Code: WF 553, WB 342*

Strictly as per the compliance and regulations of:

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Belayneh Kefale Gelaw [α] & Yitayih Kefale Gelaw [σ]

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Conclusion and Recommendations: There were significant mistakes related to inhaler use that are easy to avoid. These mistakes were due to the device used, lack of giving appropriate instructions, some parents give too much responsibility to the child for monitoring and treating their asthma. The inhalation device used in our setting should be modernized, i.e. spacer with/ without mask. Provide simple verbal and written instructions and information on treatment for children and their parents and check their understanding

I. Introduction

a) Background

Asthma is the condition of subjects with wide spread narrowing of the bronchial airways, which changes in severity over short period of time (either spontaneously or under treatment). Asthma causes wheezing, and often cough and breathlessness too, due to airway obstruction with smooth muscle contraction and airway inflammation. In children it is most commonly intermittent, but may be persistent. Asthma affects an estimated 300 million people worldwide, causing an estimated 250,000 deaths annually (all ages) (1).

Around 15 million disability adjusted life years (DALYs) are lost annually (1).

The optimal treatment of asthma depends on a number of factors, including child's age, severity and frequency of asthma attacks. For most children, asthma treatment can control symptoms, allowing the child to participate fully in activities and sports. Successful treatment of asthma involves three components:

1. Controlling and avoiding asthma triggers
2. Regularly monitoring asthma symptoms and lung function
3. Understanding how to use medications to treat asthma

Asthma and other chronic airway disease can be effectively treated by inhaler therapy (2). Inhaler devices come in a variety of types, such as metered dose inhalers (MDI) or dry powder inhalers (DPI). Irrespective of the type of inhaler device used, the outcome of inhaler therapy largely depends on appropriate use of inhaler. Appropriate use primarily involves the correct inhalation technique. A poor inhalation technique reduces drug deposition in the lungs (3). Moreover, the more mistakes made in the inhalation technique the lower the beneficial effect on lung function (4). From adults it is known that 89% of the patients make at least one mistake in the inhalation technique (5).

Appropriate inhaler use also involves actual use compared with advised regimen of the prescriber. Several studies shown that, even with adequate inhaler use (between 50 and 80% of prescribed doses), compliance with inhalation corticosteroids (ICS) is far from perfect (6). When inhaled therapy is used the administered therapeutic dose is small as compared with other routs of administration and consequently the incidence of systemic side effects is very low. This is particularly important in the case of ICS treatment compared with oral administration; delivery of the drug directly to the airways by inhalation has a more rapid

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onset of action which is advantageous when bronchodilators are used to treat acute attack of bronchoconstriction. Also, inhalation of a beta-2 agonist offers marked protection against exercise induced asthma which is common in children. In contrast oral administration of high doses of the same drug has no or a marginally protective effect against this condition (7). For this reasons inhalation therapy constitutes the cornerstone of asthma management in children of all ages.

More than 100 different inhaler/ drug combinations are now available for the treatment of asthma. Although such a variety increases the likelihood of finding an appropriate inhaler for each individual patient, it also increases the complexity of inhaler choice for clinicians and it may also reduce the physician's or nurse's experience in each individual inhaler. Therefore, it may be better for the individual clinician to focus on a limited number of inhalers to get better experience with the devices used.

The following three inhalation systems constitute the cornerstone of inhalation therapy in children with asthma.

1. Conventional Pressurized metered dose inhaler (pMDI).
2. PMDI with a spacer attached
3. Dry powder inhaler (DPI)

These three inhaler systems differ with respect to construction, aerosol cloud generation, optimal inhalation technique and ease of use. Still, with appropriate tuition and training, virtually all pediatric patients including children less than one year old can be taught effective inhaler use with one of these three systems. The precondition for this is accurate knowledge about nature and magnitude of the problems that children of various age groups experience when using these devices correctly (7).

b) Statement of Problem

Asthma is a chronic disease and increasing in all parts of the globe, especially in children and older people. Three hundred million people have asthma worldwide (1).

In Europe vaccination programs, better nutrition, and antibiotic treatment have reduced mortality from acute respiratory infection while the asthma incidence increased at the same time (8).

According to the International Study of Asthma and Allergy in Children (ISAAC) the prevalence of data is limited in developing countries. The study conducted prevalence data in seven African countries and found out that Ethiopia 9.1%, Kenya 15.8%, Nigeria 13%, South Africa 20.3%, Algeria 8.7%, Morocco 10.4% and Tunisia 11.9%) (9).

For asthma, inhalation therapy is the foundation of treatment. Yet all too often, patients do not get the full value of their inhaled medications because they use their inhaler incorrectly. Faulty inhaler technique is the major problem in public health (4).

Most asthmatic children and their caregivers do not give stress the importance of exhaling gently; for a few second before inhaling (deeply and slowly for MDI, deeply and rapidly for most DPI) (10).

Forgetting to exhale before inhaling is a common and significant mistake regardless of the type of the device. For MDI users, poor timing described earlier is another common and serious mistake (11).

c) Significance of the Study

Asthma is one of the most common diseases in children in the world at large and in Ethiopia. But, little is known about the current situation regarding the appropriate use of inhalers by children in Ethiopia. Children are more prone to use inhalation devices incorrectly if they are not monitored closely to use correctly. Pressurized MDI with and without a spacer were more prone to errors compared with DPIs, children prescribed a new device were more prone to usage errors. Many asthmatic children use their inhaler devices too poorly to result in reliable drug delivery, even after inhalation instruction. Comprehensive inhalation instruction and repeated check-up are needed to assure reliable inhalation technique.

This study will assess the inhalation technique in children and it helps to give clue for the inhalation technique. And for the other researchers to study the problems of inhalation technique in children. In addition, it will initiate Gondar University Hospital as well as the country as a whole to give priority attention of adequate use of asthma inhalation medications.

II. Objectives

a) General Objective

- To assess the knowledge of asthmatic children and their parents regarding asthma inhaler therapy and appropriateness of its use in Gondar University Teaching Hospital

b) Specific Objectives

- To identify the most common problems experienced by various age groups of children in using their inhalers correctly
- To evaluate the knowledge of asthmatic children and their parents/guardians towards their inhalation technique
- To evaluate the correct use of inhalational medications in children

III. Methods and Materials

a) Study Area and Period

The study was conducted in Gondar University Teaching Hospital in pediatrics ward and pediatrics chronic illness outpatient department, Amhara region, Northwest Ethiopia which is 738 km from Addis Ababa.

The study was conducted from October 15 to May 20, 2013.

b) Study Design

A cross- sectional study was conducted on adequate use of asthma inhalation medication in children in Gondar University Teaching Hospital.

c) Population

i. *Source of Population*

All asthmatic children attending Gondar University Teaching Hospital.

ii. *Study Population*

All asthmatic children aged 0- 14 years who had been prescribed inhalation medication attending Gondar University Teaching Hospital.

❖ *Inclusion Criteria*
- ➢ Patients who started asthma inhalational medication
- ➢ Patients whose age is 0-14 years

❖ *Exclusion Criteria*
- ➢ Patients who did not start inhalational medication
- ➢ Patients whose age is > 14 years
- ➢ Patients who are involuntary to participate

d) Study Variables

➢ *Independent variables*
- ✓ sex
- ✓ age
- ✓ educational level
- ✓ religion

➢ *Dependent variables*
- ✓ Practice
- ✓ Knowledge

e) Sampling Technique and Sample Size

Convenience sampling technique was used to select 61 asthmatic children from out of the total 70 asthmatic children who were expected at the pediatric department of university of Gondar teaching hospital.

f) Data Collection Procedure

Data was collected by interview guide composed of closed and open ended questions and a questionnaire was prepared to gather the necessary information from respondents. Data was collected by a data collector (health professional).

g) Data Analysis

All data collected by questionnaire was checked for the completeness and fulfillment daily. The data was processed by SPSS and analysis was done using descriptive and analytical method (binary logistic regression), the result was presented by tables and the necessary conclusion was made.

h) Ethical Considerations

Before starting to collect data for the study we obtained formal letter from Gondar University Research Office, permission letter from school of pharmacy, department of clinical pharmacy and willingness from pediatrics department. Representation sample was taken and kept free from any bias. Confidentiality, neutrality, accountability and academic honesty was maintained throughout the study.

i) Dissemination Plan

The final report of this study will be given to the concerned bodies (School of Pharmacy, pediatrics department, CEO of Gondar University Teaching Hospital, regional health bureau, etc) through seminar presentation, provision of hard copy, and other means.

j) Operational Definitions

Metered dose inhaler - is a device that delivers a specific amount of medication to the lungs, in the form of a short burst aerosolized medicine that is inhaled by a patient.

Dry Powder Inhalers - are inhalers that deliver medication in a dry powder form.

Spacer - is an add–on device used to increase the ease of administering aerosolized medication from a MDI. In this study called 'traditional plastic bottle.'

Mask - is a device used to deliver medications which is attached to a spacer that goes over the child's mouth and nose.

Intermittent - is the mildest form of asthma which occurs sporadically. Example: symptoms ≤ 2 days per week.

Pediatrics department- is part of the University of Gondar teaching hospital which includes the pediatric outpatient department and pediatric wards.

Persistent - another type of asthma which classified in to three and occurs repeatedly.

a) Mild persistent- symptoms occur > 2 days per week.
b) Moderate persistent- symptoms occur daily.
c) Sever persistent- symptoms occur throughout the day.

Triggers - are factors those set-off/worsen asthma symptoms.

Breathing rate - a speed at which the children breathe in through the spacer.

a) *Fast*- breathing within < 2 sec.
b) *Normal*- breathing between 2-5 sec.
c) *Slow*- breathing > 5 sec.

IV. Results

Of 61 asthmatic children who took inhalational medications, 35 (57.4%) males and 26 (42.6%) females were included in the study with the mean age of 4.67 ± 3.69 years. Among 61 asthmatic children 44 (72.1%) were preschool children. Thirty two (52.5%) asthmatic children live in a family member of 1-5, 28 (45.9%) live in a family member of 5-10, and only 1 (1.6%) lives in a family member of > 10. Nineteen (31.1%) asthmatic children had a family history of asthma (Table 1).

Table 1: Socio Demographic Characteristics of Asthmatic Children, Pediatrics Department, Gondar University Teaching Hospital, October-May, 2013.

		Frequency (%)
Age	< 1 year	12 (19.7%)
	1-5 years	31 (50.8%)
	5-10 years	10 (16.4%)
	10-14 years	8 (13.1%)
Sex	Male	35 (57.4%)
	Female	26 (42.6%)
Religion	Orthodox	48 (78.7%)
	Muslim	11 (18%)
	Protestant	2 (3.3%)
Occupation	Student	17 (27.9%)
	Preschool	44 (72.1%)
No of family in a house	1-5	32 (52.5%)
	5-10	28 (45.9%)
	>10	1 (1.6%)
Family history of Asthma	Yes	19 (31.1%
	No	42 (68.9%)

The most frequently noticed errors during inhalational medications in asthmatic children were: not rinsing the mouth after inhaling 53 (86.9%), not cleaning the plastic bottle at all 44 (72.1%), the rate of breathing through the inhaler was slow 40 (65.6%), not to shake before use 23 (37.7%) (Table 2).

Table 2 : Appropriateness of Inhalational Medications Use of Asthmatic Children, Pediatrics Department, Gondar University Teaching Hospital, October-May, 2013.

Shake before use		Frequency (%)
	Yes	38 (62.3%)
	No	23(37.7%)
Mask placed	Mouth	18 (29.5%)
	Nose	2 (3.3%)
	Both	41 (67.2%)
Mask fit well with face	Yes	41 (67.2%)
	No	20 (32.8%)
Dose at a time	2-5 puff	32 (52.5%)
	6-9 puff	29 (47.5%)
Rate of breathing during inhalation	Fast	2 (3.3%)
	Normal	19 (31.1%)
	Slow	40 (65.6%)
Pattern of breathing during multiple dosing	Bring all doses in spacer and start breathe	3 (4.9%)
	Bring one dose in the spacer and start breathe, then bring the other	58 (95.1%)
Rinse the mouth after inhaling	Yes	8 (13.1%)
	No	53 (86.9%)
Cleaning the plastic bottle	Not clean	44 (72.1%)
	By soft	10 (16.4%)
	Changing	7 (11.5%)

Of 61 asthmatic children 57 (93.4%) got instructions from GPs, 56 (91.8%) assessed the remaining dose by spraying into the air, 46 (75.4%) inhalation technique was checked during follow up appointment, 45 (73.8%) of asthmatic children inhaler use was decided by their parents. Most (51 (83.6%)) asthmatic children and their parents did not read leaflet (Table 3).

Table 3 : Assessing the Knowledge and Practice of Asthmatic Children on Inhalational Medications Use, Pediatrics Department, Gondar University Teaching Hospital, October-May, 2013.

Gives instructions		Frequency (%)
	GP	57 (93.4%)
	Nurse	4 (6.6%)
Inhalation technique checked during follow up appointment	Yes	46 (75.4%)
	No	15 (24.6%)
Read leaflet	Yes	10 (16.4%)
	No	51 (83.6%)
Assessing remaining dose	Spraying into the air	56 (91.8%)
	Don't know	5 (8.2%)
When inhaler use	Daily	19 (31.1%)
	As needed	42 (68.9%)
decides inhaler use	Parent	45 (73.8%)
	Child	16 (26.2%)

Of 61 asthmatic children 12 (19.7%) were less than 1 year. Among these 4 (33.3%) were shaking before use and 8 (66.7%) were not shaking before use. Of 61 asthmatic children 8 (13.1%) were age 10-14 years, among these 7 (87.5%) were shaking before use and 1(12.5%) were not shaking before use. Other independent variables did not have statistically significant association with dependent variables. The likelihood of shaking inhalational medications before use was 14 times more in 10-14 years of age than that of <1 year age (Table 4).

Table 4 : Association Between Age and Shaking Before Use in Asthmatic Children, Pediatrics Department, Gondar University Teaching Hospital, October-May, 2013.

Age	Frequency (%)	Odds ratio	p-value	Shake before use	
				Yes	No
< 1 year	12 (19.7%)	14	0.032	4 (33.3%)	8 (66.7%)
1-5 years	31 (50.8%)	3.85	0.234	20 (64.5%)	11 (35.5%)
5-10 years	10 (16.4%)	3	0.388	7 (70%)	3 (30%)
10-14 years*	8 (13.1%)	0.14	0.69	7 (87.5%)	1 (12.5%)

P-value= 0.032; *- reference

V. Discussion

The aim of the study was to summarize the most common problems experienced by various age groups of asthmatic children in using their inhalers correctly and to assess the knowledge of asthmatic children and their parents towards their inhalation technique.

Inhaler therapy is very important if we use appropriately. A variety of mistakes concerning therapy adherence, the inhalation technique and mistakes in the handling of devices were made by Gondar University Teaching Hospital asthmatic children. When we compared with the other countries like Dutch children, the mistakes were different. The most significant mistakes in this hospital during the use of inhalation were: not rinsing the mouth after inhaling (86.9%), not cleaning the plastic bottle (72.1%), slow rate of breathing through the inhaler (65.6%), not shaking before use (37.7%) but in Dutch children "when I need two doses, I can activate the inhaler (MDI) twice before starting to inhale through the spacer (43%) and not shaking before use (20%) were the most frequently noticed mistakes (12).

The most frequently noticed inhalational problems by various age groups of asthmatic children: slow rate of breathe through the inhaler was the most frequent problem in our study (65.6%). When we

compare with a study in Thailand asthmatic children also shows that slow breathing after activation of MDI was the major problem (40.5%), which is the same problem but higher in our study (13).

The device used to take the medication was plastic bottle which is traditional (not used by other countries). This makes the children not to improve within short period of time. Because the dose was not taken fully as a result of the plastic bottle not fit well with the face in addition to their incorrect technique. So, the modern device which is used by other countries must replace this plastic bottle so as to get the correct prescribed full dose and improved within short period of time. Children with the same severity of disease and take the same dose might not get relief from symptoms at the same time, due to the difference of device used.

Cleaning the plastic bottle was very important but in this study, most (44 (72.1%)) were not cleaning it, even they did not know the plastic bottle is to be cleaned. Actually there were children who know how to clean: some 10 (16.4%) children clean the plastic bottles by soft which was incorrect and the other 7 (11.5%) have changed the plastic bottle totally so as to get clean plastic bottle. But a study in Dutch children, the method of cleaning the inhaler was soaping inhaler, dry in air which is the correct method of cleaning (49%), but 10% were not cleaning at all (12).

Shaking before use is mandatory to take the medication but there were significant numbers of asthmatic children 23 (37.7%) who did not shake before use due to forgetting and not aware the importance of shaking. This mistake also was frequently made by other countries like Dutch children (20%) (4).

Knowing the correct route of taking the inhalational medication is very crucial for asthmatic children to get the required therapeutic effect of the drug but there were children 2 (3.3%) who took the medication by nose which is not effective. Taking the inhalational medication by mouth is the appropriate route. If the children whose age is < 5 years, they can use both nose and mouth.

Decision concerning with the inhaler use, the involvement of parents with clear and simple instructions were important when and how to use because most 43 (70%) asthmatic children were less than 5 years of age.

In this study 56 (91.8%) of participants were assessing the remaining doses by spraying into the air but the other 5 (8.2%) did not know how to assess the remaining dose. Spraying into the air is incorrect method of assessing remaining dose, but spraying into the dark background is the correct method of assessing remaining dose. A study in Dutch children the method of assessing the remaining doses were spraying against dark background (9%), if inhaler floats in water, it is empty (4%), counting the remaining doses using agenda (7%) and looking on counter (7%) which were correct method of assessing and the others spraying in the air (28%), feeling inhaler weight while shaking (20%), listening to inhaler while shaking (9%), not assessing at all (14%) and if inhaler sink in water, it is empty (2%) were incorrect methods (6). There is significant association between age and shaking before use (p= 0.032). This shows that the age group of 10-14 years had good practice of shaking before use as compared to <1 years of age. This might be because of the age groups of 10-14 years were matured enough to accept the instructions told by health professionals and parents were forgetting the instructions told by health professionals because they were responsible for many activities in the house.

VI. Limitations of the Study

- *This Study had Potential Limitations*
- ✓ Absence of asthmatic children during their follow up appointment which was not included during our data collection period. So, we did not include all patients.
- ✓ Some parents were not voluntary to respond for our questionnaire
- ✓ As the study was cross-sectional interview based, there might be recall bias in the study subjects side.

VII. Conclusion

There were significant mistakes/errors related to inhaler use that are easy to avoid. These mistakes were due to different factors like the device used, lack of giving appropriate instructions and parents give too much responsibility to the child for monitoring and treating their asthma. These lead to inadequate use of inhalational medications. Selecting the right inhalational device in University of Gondar Teaching Hospital, pediatric department is crucial because their technique was incorrect, they would not get the benefit from the drug. This must be checked by the doctors, nurses or pharmacists.

VIII. Recommendations

- The inhalation device used in Gondar University Teaching Hospital should be modernized, i.e. spacer with/ without mask.
- The device also should be based on age in order to fit with the face.
- Parents should be involved during the use of inhaler devices with clear and simple instructions by health professionals.
- Teach patients and their parents when and how to use asthma medications and observe the patients inhaler technique regularly.
- Provide simple verbal and written instructions and information on treatment for children and their parents and check their understandings.
- If there is misunderstandings and bad experience, clarify for them.

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Ethical Approval: obtained

References Références Referencias

1. WHO. Global Burden of Diseases, 2004 update. WHO 2008.
2. Masoli F, *et al.* Global Burden of Asthma, 2004. (http://www.ginasthma.org).
3. O'connel EJ. optimizing inhaled corticosteroid therapy in children with chronic asthma. *Pediatr pulmonol* 2005, 39: 74-83.
4. Pedersen S, Frost L, Arnfred T. Errors in inhalation technique and efficiency in inhaler use in asthmatic children. *Allergy* 1986, 41: 118-24.
5. Van Beerendonk I, Mesters I, Mudde AN, Tan TD: Assessment of the inhalation technique in outpatients with asthma or chronic obstructive disease using a metered dose inhaler or dry powder device. *J Asthma* 1998, 35: 273-9.
6. Gibson NA, Ferguson AE, Aitchison TC, Paton JY. Compliance with inhaled asthma medication in preschool children. *Thorax* 1995, 50: 1274-9.
7. Fuglsang G, Hertz B, Holm B. No protection by oral terbutaline against exercise – induced asthma in children; a dose response study. *Eur Respir* 1993, 6:27-30.
8. Farooqi IS, Hopkin JM.: Early Childhood Infection and Atopic Disorder. *Thorax,* 1998, 53: 927-932.
9. Asher MI, montefort S, Bjorksten B, Laick, Strachan DP, *et al.* worldwide time trends in the prevalence of asthma and allergic rhinconjunctivitis and eczema in childhood: ISAAC phases one and three repeat multicountry cross sectional survey *Lacent, 2006,* 368: 733-743.
10. Centers for Diseases Control and prevention. National Center for Health Statistics: National health interview survey raw data. Analysis performed by American Lung Association Research and Program Services, 2008.
11. American Lung Association chronic Obstructive Pulmonary Respiratory Diseases- helping the missing millions, Feb 24, 2010.
12. Hagmolen of Ion Have W, Van de Berg WN, Bindles PJ, Van Alderen WM, Vander Palen J, *J Asthma;* 2008, 45(1): 67-71.
13. Asian pacific Journal of Allergy and Immunology/ launched by the allergy and immunology society of Thailand volume: 27.

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New Process Based on the Coupling of an Electrochemical Sensor and Bioanalytical Column for Determining Antioxidant Capacity

By Ngono Thérèse Rosie Lauriane, Rachida Najih & Abdelilah Chtaini
Université Sultan Moulay Slimane, Maroc

Abstract - A new approach, for antioxidant capacity determination was proposed. It is based on the using of the xanthine-xanthine oxidase system coupled with H2O2 electrochemical sensor. The paper presents the preparation and characterization of the H2O2 amperometric sensor and its utilization for antioxidant evaluation of some real samples (Garlic, tea and coffee). The obtained results were found in good correlation with reality.

Keywords: *voltammetry; antioxidant capacity; xanthine; xantine oxidase.*

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Abstract- **A new approach, for antioxidant capacity determination was proposed. It is based on the using of the xanthine-xanthine oxidase system coupled with H_2O_2 electrochemical sensor. The paper presents the preparation and characterization of the H_2O_2 amperometric sensor and its utilization for antioxidant evaluation of some real samples (Garlic, tea and coffee). The obtained results were found in good correlation with reality.**

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I. Introduction

Antioxidant can be defined as substances that inhibit the oxidation of the other molecules. Reactive oxygen species (ROS), naturally generated during the metabolism, can damage biological structures such as proteins, lipids or DNA. Normally, cells defend themselves against ROS damage with enzymes, but sometimes the natural defenses are overwhelmed by an excessive generation of ROS and a situation of oxidative stress occurs. In this case, cellular and extracellular macromolecules (proteins, lipids, and nucleic acids) can suffer oxidative damage, causing tissue injury [1, 2].

Antioxidant compounds in food are found to have a health protecting factor. Primary sources of naturally occurring antioxidants are whole grains, fruits and vegetables.

Garlic (*Allium sativum*) is an herb. It is best known as a flavoring for food [15]. But over the years, garlic has been used as a medicine to prevent or treat a wide range of diseases and conditions. The fresh clove or supplements made from the clove are used for medicine [16]. Garlic is used for many conditions related to the heart and blood system. These conditions include high blood pressure, high cholesterol, and coronary heart disease, heart attack, and "hardening of the arteries" (atherosclerosis) [17]. Some of these uses are supported by science. Garlic actually may be effective in slowing the development of atherosclerosis and seems to be able to modestly reduce blood pressure.

Some people use garlic to prevent colon cancer, rectal cancer, stomach cancer, breast cancer, prostate cancer, and lung cancer. It is also used to treat prostate cancer and bladder cancer. Garlic has been tried for treating an enlarged prostate (benign prostatic hyperplasia; BPH), diabetes, osteoarthritis, hayfever (allergic rhinitis), traveler's diarrhea, high blood pressure late in pregnancy (pre-eclampsia), cold and flu. It is also used for building the immune system, preventing tick bites, and preventing and treating bacterial and fungal infections. Other uses include treatment of fever, coughs, headache, stomach ache, sinus congestion, gout, rheumatism, hemorrhoids, asthma, bronchitis, shortness of breath, low blood pressure, low blood sugar, high blood sugar, and snakebites. It is also used for fighting stress and fatigue, and maintaining healthy liver function. Some people apply garlic oil to their skin to treat fungal infections, warts, and corns. There is some evidence supporting the topical use of garlic for fungal infections like ringworm, jock itch, and athlete's foot; but the effectiveness of garlic against warts and corns is still uncertain. There is a lot of variation among garlic products sold for medicinal purposes. The amount of allicin, the active ingredient and the source of garlic's distinctive odor, depends on the method of preparation. Allicin is unstable, and changes into a different chemical rather quickly. Some manufacturers take advantage of this by aging garlic to make it odorless. Unfortunately, this also reduces the amount of allicin and compromises the effectiveness of the product. Some odorless garlic preparations and products may contain very little, if any, allicin [18, 19].

Several amperometric biosensors have already been proposed for antioxidant capacity evaluation [3-14]. Most of them are based on the amperometric detection of H_2O_2, resulting from the catalyzed dismutation of superoxide radicals ($O_2^{\cdot-}$) in presence of superoxide dismutase.

In this work, electrochemical deposition of copper on paste carbon electrode is carried out to develop stable recognition layers for the voltammetric detection of antioxidant capacity of domestic garlic, tea and coffee samples.

The antioxidant capacity was evaluated, by coupling an amperometric sensor for H_2O_2 detection, obtained by modification of paste carbon graphite electrode with copper, with xanthine oxidase (XOD) immobilized at silice – xanthine (XA) enzymatic system,

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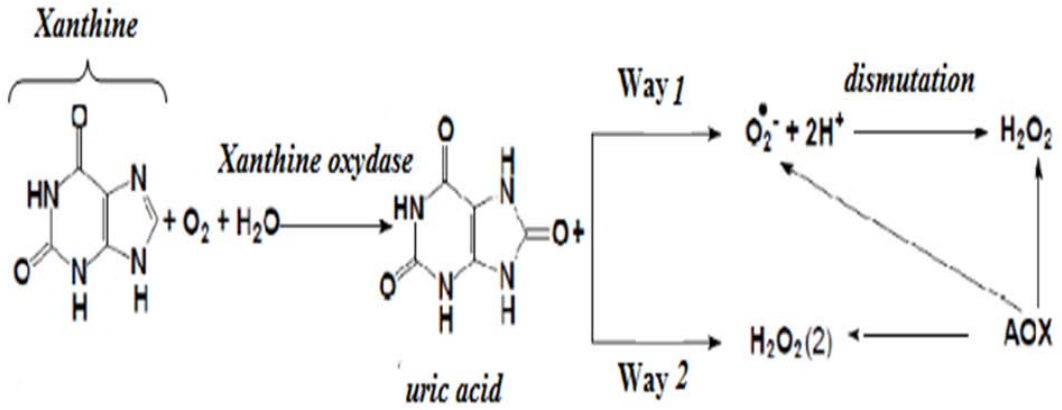
as generator of O_2-. radicals. The advantages of this strategy consist to:

- It works at low applied potential, allowing a significant decrease of the risk of electrochemical interferences;
- The antioxidant capacity evaluation, requiring the monitoring of H_2O_2 concentration in presence of antioxidant sample as well as in its absence, will enhance global estimation of free radicals (O_2-.) or no radical reactive species, (H_2O_2) (Reaction 1).



Reaction 1

II. Experimental Section

a) Apparatus

Electrochemical experiments were performed using a voltalab potentiostat (model PGSTAT 100, Eco Chemie B. V., Utrecht, The Netherlands) driven by the general purpose electrochemical systems data processing software (voltalab master 4 software).

All the electrochemical experiments were performed in a standard one-compartment three-electrode cell. The reference electrode was SCE and the counter electrode was platinum. All electrode potentials were referred to this reference electrode. The working electrode was copper modified carbon paste electrode (Cu-CPE).

b) Reagents and Solutions

All chemicals were of the highest quality. Graphite powder (spectroscopic grade RWB, Ringsdorff-Werke GmbH, Bonn-Bad Godesberg, Germany) was obtained from Aldrich and was used without further purification. CuSO4 was obtained from Merck chemicals. Deionised water was used to prepare all solution.

c) Preparation of the Electrochemical Sensor

The carbon paste unmodified was prepared by adding paraffin oil to carbon powder and thoroughly hand –mixing in a mortar and pestle. The resulting paste was packed into the electrode and the surface was smoothed. The electrochemical sensor was developed by depositing the copper at fixed potential (0.1 V for 1 hour) onto the carbon paste electrode surface.

d) Procedure

The device constructed for the measurement of the antioxidant capacity is given in Figure 1. The free radical was generated in column following the reaction 1, a calibration curve; giving current density of H_2O_2 reduction versus [H_2O_2] is recorded. In the second test, the investigated antioxidant associated to xanthine solution, were pouring in column and electrochemical response behaviour was recorded, the [H_2O_2] no consumed was evaluated from calibration curve already established.

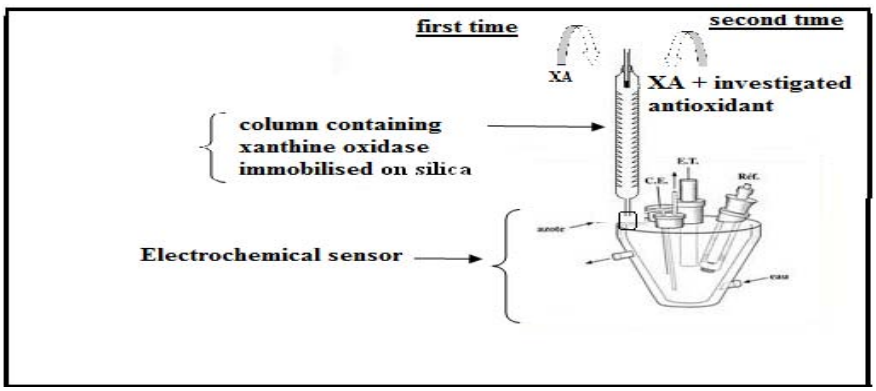


Figure 1 : Scheme of the Device Constructed for the Measurement Antioxidant Capacity.

III. Results and Discussion

a) Characterization of Prepared Electrode

The cyclic voltammograms (CVs) of the copper modified carbon paste electrode (Cu-CPE) and carbon paste electrode (CPE) were recorded in the supporting electrolyte (phosphate buffer solution) (Fig. 2).

We can see that the shape of the cyclic voltammogram was modified in the presence of copper at CPE surface, suggesting that the carbon paste electrode was effectively modified by copper. The surface structure of copper modified carbon paste surface was observed using scanning electron microscopy (Fig. 3). The film layer of copper was formed on the surface of carbon paste electrode; it was not disintegrated or detached from the surface when immersed in the buffer solution.

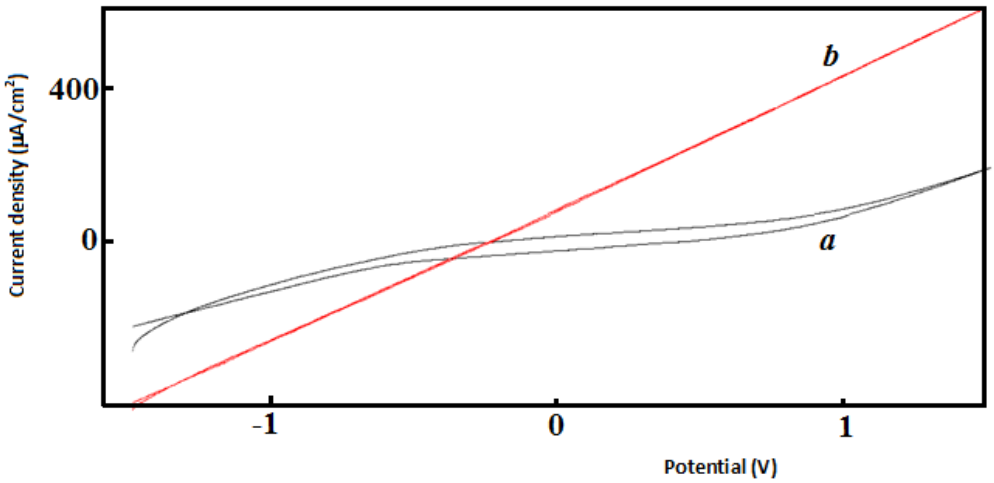


Figure 2 : Cyclic voltammograms recorded in buffer solution at 100 mV/s, at a- carbon paste electrode, b- copper modified carbon paste electrode.

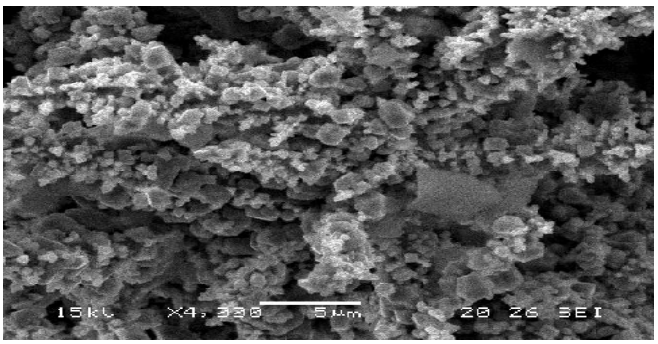


Figure 3 : Scanning Electron Micrograph of Cu-CPE.

b) Calibration Graph

The detection of H_2O_2, generated in the silica column, was examined by square wave voltammetry, in the electrochemical sensor. The electrode response was tested for different amounts of H_2O_2, in the range from 1µL/100mL (buffer tampon solution) to 100 µ L/100mL (buffer tampon solution). Figure 4 shows some typical square wave voltammetry curves recorded at Cu-CPE electrode. A calibration graph was then constructed from the observed peak currents. The square wave voltammetric response was almost linear dependent on the concentration of H_2O_2 (Fig. 5). The linear regression analysis gave:

$$i_p = -0.042[H2O2] +- 20.59$$

with a correlation coefficient of 0.9498

c) Antioxidant Capacity Determination

i. Garlic juice

After drawing the calibration curve relative to a reduction of H_2O_2, we introduced into the silica column a solution containing xanthine and the antioxidant considered.

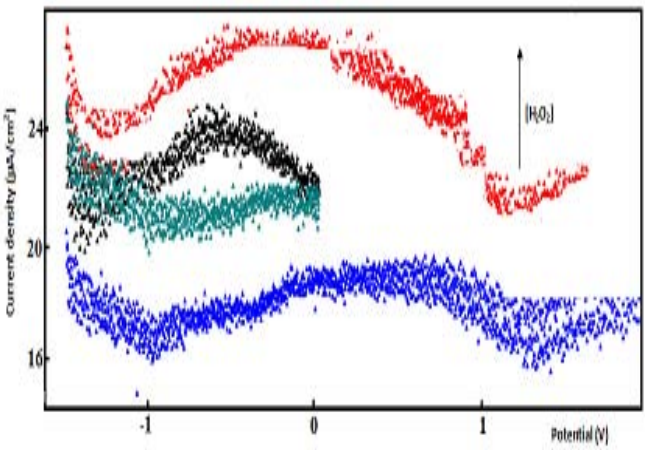


Figure 4 : Square Wave Voltammograms of H_2O_2, in Buffer Tampon Solution Ph ≈ 7.4 At Cu-CPE, Scan Rate 50 Mv/S.

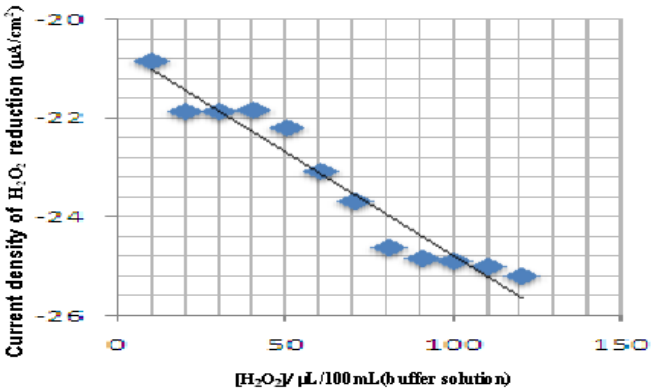


Figure 5 : Calibration Graph

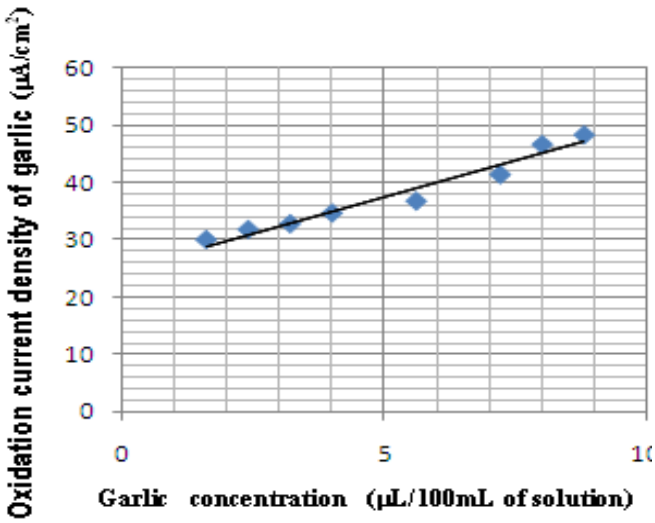


Figure 6 : Garlic Calibration Graph

Figure 6 shows the garlic juice calibration plots. As can be seen, the garlic oxidation current density increases with concentration. The addition of garlic juice to xanthine solution caused a decrease in H_2O_2 reduction current signal (Fig. 7). We can conclude that garlic inhibited the reductive effect of H_2O_2.

ii. *Antioxidant Capacity of Tea*

The developed system was also applied to the determination of the antioxidant capacity of tea. Figure 8 shows the evolution of the oxidation current density of tea with concentration, the peak current have a linear relationship with concentration. For constant concentration of H_2O_2, the dependence of square wave voltammetric peak current on the addition of tea in silica column was studied (Fig. 9). The reduction peak of H_2O_2 decreases in presence of tea.

iii. *Antioxidant Capacity of Coffee*

The working procedure consisted in adding xanthine and coffee to a column containing xanthine oxidase immobilized onto silica. The presence of coffee will induce a decrease of H_2O_2 concentration, resulting in a decrease of the H_2O_2 electrochemical sensor (currentdensity). Figure 10 shows evolution of the H_2O_2 reduction current density with concentration of coffee.

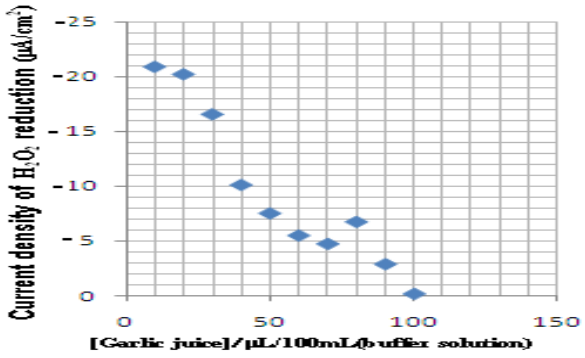


Figure 7 : Evolution of the H_2O_2 Reduction Current Density with Concentration of Garlic.

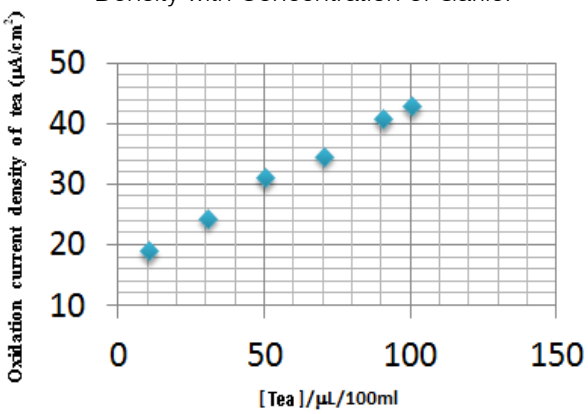


Figure 8 : Calibration Curve for Tea at Cu-CPE in Buffer Solution.

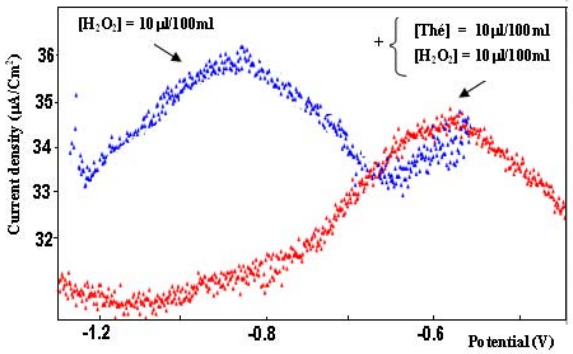


Figure 9 : Square Wave Voltammograms Recorded at Cu-CPE in Buffer Solution.

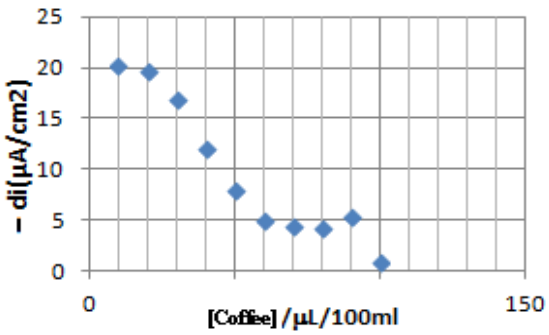


Figure 10 : Inhibition of the H_2O_2 Reduction by the Coffee

The corresponding antioxidant capacity values, was calculated using the relation:

$$AOC = \frac{I_{H2O2} - I_{H2O2\text{-antioxidant sample}}}{I_{H2O2}} \times 100$$

Where I_{H2O2}, is the current density due to H_2O_2 reduction and $I_{H2O2\text{-antioxidant sample}}$ represent the current density due to antioxidant sample addition. The results are summarized in Table 1.

Table. 1

Sample	AOC%
Garlic	25.6
Tea	13.15
Coffee	6.6

IV. Conclusion

A bioanalytical system for the evaluation of the antioxidant capacity has been developed. The main advantage of the new approach is based on coupling the production of radicals, generated by the xanthine-xanthine oxidase enzymatic system, with the electrochemicale sensor, for H_2O_2 detection. The immobilization of xanthine oxidase (XOD) on the silica increased the sensitivity of the system in comparison with those where the XOD remained in solution. The results obtained show that the proposed system is fast, sensitive and better suited than conventional methods.

References Références Referencias

1. B. Halliwell, J. Gutteridge, "Free Radicals in Biology and Medicine", Oxford University Press, New York USA, **2007**.
2. B. Haliwell, O. I. *Aruoma, FEBS Letters*, **1991**, 281 (1-2), 9-19.
3. L. Mello and L. Kubota, *Talanta*, **2007**, 72, 335-348.
4. L. Campanella, A. Bonnani, E. Finotti and M. Tomassetti, Biosens. Bioelectron., 2004, 19, 641-651.
5. L. Campanella, G. Favero, L. Persi and M. Tomassetti, *Anal. Lett.,* **1999**, 32, 2559-2581.
6. A. Bonnani, L. Campanella, T. Gatta, E. Gregoria and M. Tomassetti, *Food Chem*., **2007**, 102, 751-758.
7. L. Campanella, A. Bonnani, G. Favero, and M. Tomassetti, *Bioanal. Chem.,* **2003**, 375, 1011-1016.
8. J. Di, S. Bi and M. Zhang, *Biosens. Bioelectron.,* **2004**, 19, 1497-1486.
9. Y. Tiam, T. Okajima, F. Kitamura and T. Ohsaka, *J. Korean Electrochem. Soc.,* **2002**, 5, 212-215.
10. E. Emregul, *Anal. Bioanal. Chem.,* **2005,** 383, 947-954.
11. S. Mesaros, Z. Vankova, A. Mesarosova,P. Tomcik and S. Grufeld, Bioelectrochem. Bioenergy., **1998**, 46, 33-37.
12. S. Ignatov, D. Shishniashvili, B. Ge, F. Scheller and F. Lisdat, *Biosens. Bioelectron.,* **2002**, 17, 191-199.
13. F. Lisdat, B. Ge, R. Rezeka and E. Kozniewska, Fresentius. *J. Anal.Chem.,* **1999**, 365, 494-498.
14. M. Beissenhirtz, F. Scheller and F. Lisdat, *Electroanal.*, **2003**, 15, 1425-1435.
15. M.I. Cervantes, P. M. Balderas, J. J. Banos, M. O. Ibarra, B. F. Rojas, O. N. Campos, M. E. Rojo, M. R. Tachiquin, A. O. Plata, *Food chemistry*, **2013**, 140, 343-352.
16. P. Aguilera, M. E. Cardenas, A. O. Plata, D. L. Aparicio, D. Barrera, M. E. Rojo*, Phytomedecine*, **2010**, 17, 241-247.
17. A. S. Mousa, S. A. Mousa, Nutrition *Resaerch*, **2007**, 27, 119-123.
18. Y. Nodo, C. Asado, C. Sasaki, S. Hashimoto, Y. Nakamuro, *Biochemical Engineering Journal*, **2013**, 73, 1-4.
19. Sami G. Alsabri, Hanan M. El-Basir, Nouri B. Rmeli, Salah B. Mohamed, Aemen A. Allafi, Abdulmottaleb A. Zetrini, Asma A. Salem, Sofian S. Mohamed, Abdul Gbaj and Mokhtar M. El-Baseir, Journal of Chemical and Pharmaceutical Research, 2013, 5(1):32-36.

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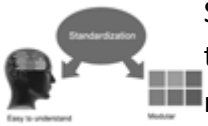

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The Board can also play vital role by exploring and giving valuable suggestions regarding the Standards of "Open Association of Research Society, U.S.A (OARS)" so that proper amendment can take place for the benefit of entire research community. We shall provide details of particular standard only on receipt of request from the Board.

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Note :

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- In future, if the board feels the necessity to change any board member, the same can be done with the consent of the chairperson along with anyone board member without our approval.
- In case, the chairperson needs to be replaced then consent of 2/3rd board members are required and they are also required to jointly pass the resolution copy of which should be sent to us. In such case, it will be compulsory to obtain our approval before replacement.
- In case of "Difference of Opinion [if any]" among the Board members, our decision will be final and binding to everyone.

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1. General,

2. Ethical Guidelines,

3. Submission of Manuscripts,

4. Manuscript's Category,

5. Structure and Format of Manuscript,

6. After Acceptance.

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Numerical Methods: Numerical methods used should be clear and, where appropriate, supported by references.

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27. Refresh your mind after intervals: Try to give rest to your mind by listening to soft music or by sleeping in intervals. This will also improve your memory.

28. Make colleagues: Always try to make colleagues. No matter how sharper or intelligent you are, if you make colleagues you can have several ideas, which will be helpful for your research.

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Key points to remember:

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- Please note the criterion for grading the final paper by peer-reviewers.

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Writing a research paper is not an easy job no matter how trouble-free the actual research or concept. Practice, excellent preparation, and controlled record keeping are the only means to make straightforward the progression.

General style:

Specific editorial column necessities for compliance of a manuscript will always take over from directions in these general guidelines.

To make a paper clear

· Adhere to recommended page limits

Mistakes to evade

- Insertion a title at the foot of a page with the subsequent text on the next page
- Separating a table/chart or figure - impound each figure/table to a single page
- Submitting a manuscript with pages out of sequence

In every sections of your document

· Use standard writing style including articles ("a", "the," etc.)

· Keep on paying attention on the research topic of the paper

· Use paragraphs to split each significant point (excluding for the abstract)

· Align the primary line of each section

· Present your points in sound order

· Use present tense to report well accepted

· Use past tense to describe specific results

· Shun familiar wording, don't address the reviewer directly, and don't use slang, slang language, or superlatives

· Shun use of extra pictures - include only those figures essential to presenting results

Title Page:

Choose a revealing title. It should be short. It should not have non-standard acronyms or abbreviations. It should not exceed two printed lines. It should include the name(s) and address (es) of all authors.

Abstract:

The summary should be two hundred words or less. It should briefly and clearly explain the key findings reported in the manuscript--must have precise statistics. It should not have abnormal acronyms or abbreviations. It should be logical in itself. Shun citing references at this point.

An abstract is a brief distinct paragraph summary of finished work or work in development. In a minute or less a reviewer can be taught the foundation behind the study, common approach to the problem, relevant results, and significant conclusions or new questions.

Write your summary when your paper is completed because how can you write the summary of anything which is not yet written? Wealth of terminology is very essential in abstract. Yet, use comprehensive sentences and do not let go readability for briefness. You can maintain it succinct by phrasing sentences so that they provide more than lone rationale. The author can at this moment go straight to shortening the outcome. Sum up the study, with the subsequent elements in any summary. Try to maintain the initial two items to no more than one ruling each.

- Reason of the study - theory, overall issue, purpose
- Fundamental goal
- To the point depiction of the research
- Consequences, including definite statistics - if the consequences are quantitative in nature, account quantitative data; results of any numerical analysis should be reported
- Significant conclusions or questions that track from the research(es)

Approach:

- Single section, and succinct
- As a outline of job done, it is always written in past tense
- A conceptual should situate on its own, and not submit to any other part of the paper such as a form or table
- Center on shortening results - bound background information to a verdict or two, if completely necessary
- What you account in an conceptual must be regular with what you reported in the manuscript
- Exact spelling, clearness of sentences and phrases, and appropriate reporting of quantities (proper units, important statistics) are just as significant in an abstract as they are anywhere else

Introduction:

The **Introduction** should "introduce" the manuscript. The reviewer should be presented with sufficient background information to be capable to comprehend and calculate the purpose of your study without having to submit to other works. The basis for the study should be offered. Give most important references but shun difficult to make a comprehensive appraisal of the topic. In the introduction, describe the problem visibly. If the problem is not acknowledged in a logical, reasonable way, the reviewer will have no attention in your result. Speak in common terms about techniques used to explain the problem, if needed, but do not present any particulars about the protocols here. Following approach can create a valuable beginning:

- Explain the value (significance) of the study
- Shield the model - why did you employ this particular system or method? What is its compensation? You strength remark on its appropriateness from a abstract point of vision as well as point out sensible reasons for using it.
- Present a justification. Status your particular theory (es) or aim(s), and describe the logic that led you to choose them.
- Very for a short time explain the tentative propose and how it skilled the declared objectives.

Approach:

- Use past tense except for when referring to recognized facts. After all, the manuscript will be submitted after the entire job is done.
- Sort out your thoughts; manufacture one key point with every section. If you make the four points listed above, you will need a least of four paragraphs.

- Present surroundings information only as desirable in order hold up a situation. The reviewer does not desire to read the whole thing you know about a topic.
- Shape the theory/purpose specifically - do not take a broad view.
- As always, give awareness to spelling, simplicity and correctness of sentences and phrases.

Procedures (Methods and Materials):

This part is supposed to be the easiest to carve if you have good skills. A sound written Procedures segment allows a capable scientist to replacement your results. Present precise information about your supplies. The suppliers and clarity of reagents can be helpful bits of information. Present methods in sequential order but linked methodologies can be grouped as a segment. Be concise when relating the protocols. Attempt for the least amount of information that would permit another capable scientist to spare your outcome but be cautious that vital information is integrated. The use of subheadings is suggested and ought to be synchronized with the results section. When a technique is used that has been well described in another object, mention the specific item describing a way but draw the basic principle while stating the situation. The purpose is to text all particular resources and broad procedures, so that another person may use some or all of the methods in one more study or referee the scientific value of your work. It is not to be a step by step report of the whole thing you did, nor is a methods section a set of orders.

Materials:

- Explain materials individually only if the study is so complex that it saves liberty this way.
- Embrace particular materials, and any tools or provisions that are not frequently found in laboratories.
- Do not take in frequently found.
- If use of a definite type of tools.
- Materials may be reported in a part section or else they may be recognized along with your measures.

Methods:

- Report the method (not particulars of each process that engaged the same methodology)
- Describe the method entirely
- To be succinct, present methods under headings dedicated to specific dealings or groups of measures
- Simplify - details how procedures were completed not how they were exclusively performed on a particular day.
- If well known procedures were used, account the procedure by name, possibly with reference, and that's all.

Approach:

- It is embarrassed or not possible to use vigorous voice when documenting methods with no using first person, which would focus the reviewer's interest on the researcher rather than the job. As a result when script up the methods most authors use third person passive voice.
- Use standard style in this and in every other part of the paper - avoid familiar lists, and use full sentences.

What to keep away from

- Resources and methods are not a set of information.
- Skip all descriptive information and surroundings - save it for the argument.
- Leave out information that is immaterial to a third party.

Results:

The principle of a results segment is to present and demonstrate your conclusion. Create this part a entirely objective details of the outcome, and save all understanding for the discussion.

The page length of this segment is set by the sum and types of data to be reported. Carry on to be to the point, by means of statistics and tables, if suitable, to present consequences most efficiently.You must obviously differentiate material that would usually be incorporated in a study editorial from any unprocessed data or additional appendix matter that would not be available. In fact, such matter should not be submitted at all except requested by the instructor.

Content

- Sum up your conclusion in text and demonstrate them, if suitable, with figures and tables.
- In manuscript, explain each of your consequences, point the reader to remarks that are most appropriate.
- Present a background, such as by describing the question that was addressed by creation an exacting study.
- Explain results of control experiments and comprise remarks that are not accessible in a prescribed figure or table, if appropriate.
- Examine your data, then prepare the analyzed (transformed) data in the form of a figure (graph), table, or in manuscript form.

What to stay away from

- Do not discuss or infer your outcome, report surroundings information, or try to explain anything.
- Not at all, take in raw data or intermediate calculations in a research manuscript.
- Do not present the similar data more than once.
- Manuscript should complement any figures or tables, not duplicate the identical information.
- Never confuse figures with tables - there is a difference.

Approach

- As forever, use past tense when you submit to your results, and put the whole thing in a reasonable order.
- Put figures and tables, appropriately numbered, in order at the end of the report
- If you desire, you may place your figures and tables properly within the text of your results part.

Figures and tables

- If you put figures and tables at the end of the details, make certain that they are visibly distinguished from any attach appendix materials, such as raw facts
- Despite of position, each figure must be numbered one after the other and complete with subtitle
- In spite of position, each table must be titled, numbered one after the other and complete with heading
- All figure and table must be adequately complete that it could situate on its own, divide from text

Discussion:

The Discussion is expected the trickiest segment to write and describe. A lot of papers submitted for journal are discarded based on problems with the Discussion. There is no head of state for how long a argument should be. Position your understanding of the outcome visibly to lead the reviewer through your conclusions, and then finish the paper with a summing up of the implication of the study. The purpose here is to offer an understanding of your results and hold up for all of your conclusions, using facts from your research and generally accepted information, if suitable. The implication of result should be visibly described. Infer your data in the conversation in suitable depth. This means that when you clarify an observable fact you must explain mechanisms that may account for the observation. If your results vary from your prospect, make clear why that may have happened. If your results agree, then explain the theory that the proof supported. It is never suitable to just state that the data approved with prospect, and let it drop at that.

- Make a decision if each premise is supported, discarded, or if you cannot make a conclusion with assurance. Do not just dismiss a study or part of a study as "uncertain."
- Research papers are not acknowledged if the work is imperfect. Draw what conclusions you can based upon the results that you have, and take care of the study as a finished work
- You may propose future guidelines, such as how the experiment might be personalized to accomplish a new idea.
- Give details all of your remarks as much as possible, focus on mechanisms.
- Make a decision if the tentative design sufficiently addressed the theory, and whether or not it was correctly restricted.
- Try to present substitute explanations if sensible alternatives be present.
- One research will not counter an overall question, so maintain the large picture in mind, where do you go next? The best studies unlock new avenues of study. What questions remain?
- Recommendations for detailed papers will offer supplementary suggestions.

Approach:

- When you refer to information, differentiate data generated by your own studies from available information
- Submit to work done by specific persons (including you) in past tense.
- Submit to generally acknowledged facts and main beliefs in present tense.

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Topics	Grades		
	A-B	**C-D**	**E-F**
Abstract	Clear and concise with appropriate content, Correct format. 200 words or below	Unclear summary and no specific data, Incorrect form Above 200 words	No specific data with ambiguous information Above 250 words
Introduction	Containing all background details with clear goal and appropriate details, flow specification, no grammar and spelling mistake, well organized sentence and paragraph, reference cited	Unclear and confusing data, appropriate format, grammar and spelling errors with unorganized matter	Out of place depth and content, hazy format
Methods and Procedures	Clear and to the point with well arranged paragraph, precision and accuracy of facts and figures, well organized subheads	Difficult to comprehend with embarrassed text, too much explanation but completed	Incorrect and unorganized structure with hazy meaning
Result	Well organized, Clear and specific, Correct units with precision, correct data, well structuring of paragraph, no grammar and spelling mistake	Complete and embarrassed text, difficult to comprehend	Irregular format with wrong facts and figures
Discussion	Well organized, meaningful specification, sound conclusion, logical and concise explanation, highly structured paragraph reference cited	Wordy, unclear conclusion, spurious	Conclusion is not cited, unorganized, difficult to comprehend
References	Complete and correct format, well organized	Beside the point, Incomplete	Wrong format and structuring

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