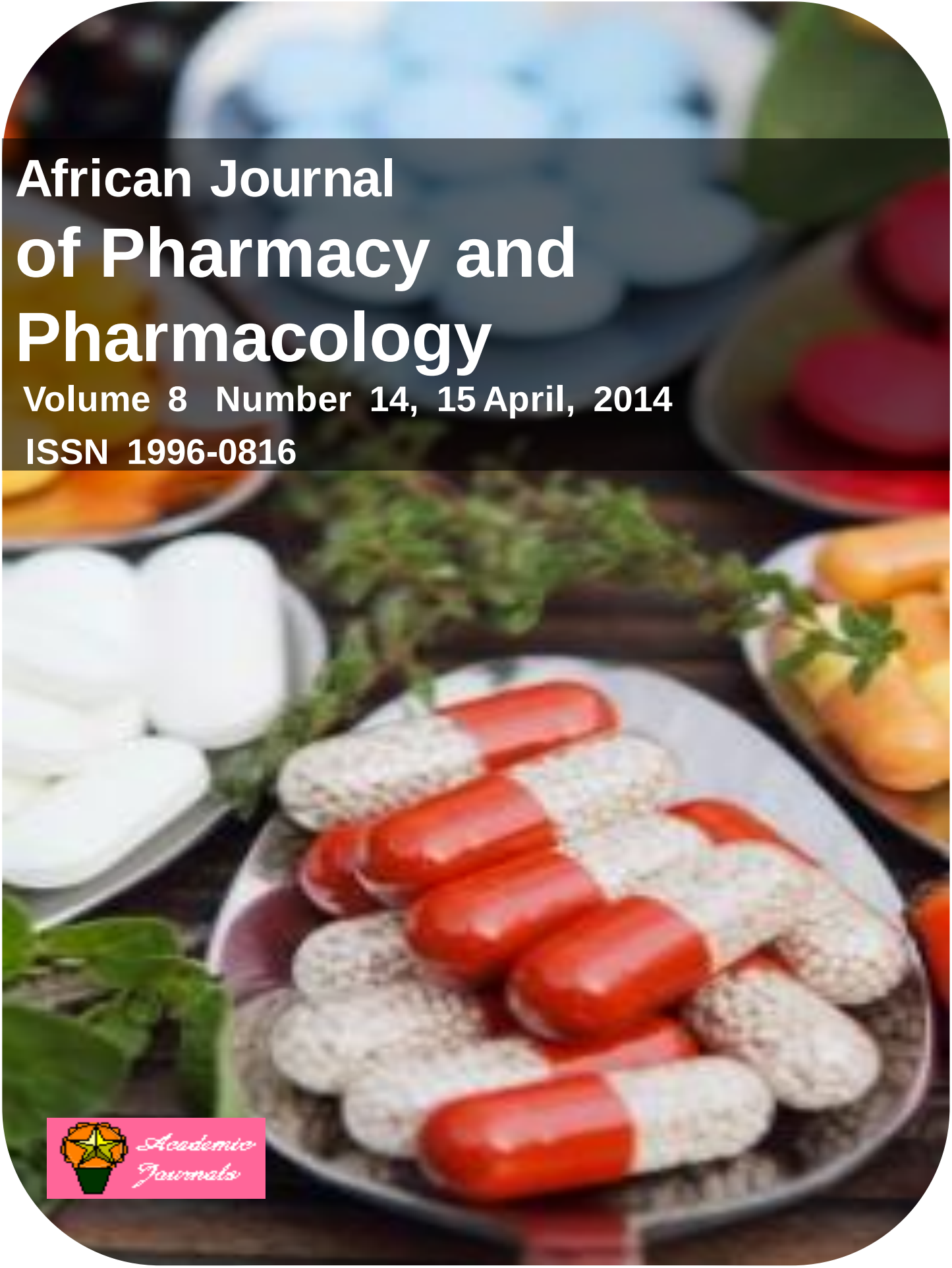

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Full Length Research Paper

Evaluation of the effects of long-term of pharmacotherapeutic follow-up intervention on clinical and humanistic outcomes in diabetes mellitus patients

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This study aimed to evaluate the long-term effects of pharmacotherapeutic follow-up on clinical parameters and the quality of life of a group of elderly patients. A longitudinal pilot study was carried out to examine 14 elderly patients with diabetes mellitus 12 months after they completed the pharmacotherapeutic follow-up in a popular Pharmacy in Aracaju-SE, Brazil. Glycosylated hemoglobin, blood pressure, blood glucose capilary level, body index mass and waist circumference and quality of life were measured. Glycosylated hemoglobin level was < 7% in 42.86% of the patients. In addition, baseline and post-reevaluation mean blood pressure values were statistically different ($p < 0.05$). The patients noticed improvement in all domains of quality of life, compared to baseline and reevaluation. Pharmacotherapeutic follow-up trained elderly patients to be capable of controlling their diabetes and this is important for maintaining their clinical parameters and quality of life.

Key words: Elderly, pharmacotherapeutic follow-up, diabetes mellitus, diabetes self-management education.

INTRODUCTION

Diabetes mellitus is a group of metabolic diseases characterized by hyperglycemia resulting from deficiencies in insulin secretion and/or its action; it is associated with complications, dysfunction, and the failure of various organs (American Diabetes Association (ADA), 2002). In 2000, 171 million people had diabetes worldwide; the number will reach 366 million in 2030, with the disease acquiring epidemic characteristics in several countries, particularly in developing countries that encounter barriers that make diagnosis and treatment difficult. In this scenario, Brazil will have approximately 11.3 million people with diabetes (Wild et al., 2004). The factors that

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contribute to the increasing incidence, prevalence and mortality rate of this disease include a sedentary lifestyle, improper eating habits, socio-behavioral changes and the accelerated rate of aging of the general population (BDS, 2005). Although diabetes can occur at any age, its prevalence significantly increases among the elderly population (Marcondes et al., 2005). Approximately 10% of people over 70 years of age have diabetes and thus, diabetes represents the fourth most common chronic condition for this group, greatly hindering the functional capacity, independence and quality of life for the elderly (Alves et al., 2007; Wermeille et al., 2004).

Therefore, the need to improve the clinical management of diabetes and the quality of life of patients is an opportunity for pharmacists to be more involved in the treatment of this disease. In this context, in 1990, the "Pharmacotherapeutic Follow-up" (PF) was defined by Hepler and Strand (1990) and aimed at ensuring a convenient, safe and cost-effective use of pharmacotherapy that offers pharmaceutical guidance to patients, pharmacotherapeutic monitoring and health education (Hepler and Strand, 1990; Lyra Jr. et al., 2008).

Health education is defined as a set of values that promotes training and participation in the process of care (Auld et al., 1998). According to Lyra Jr. et al. (2007), the relationship between a pharmacist and a patient should be guided by ethical principles, mutual respect, confidentiality and above all, co-responsibility. Based on this, the pharmacist should try to establish a dialogue to understand the past and present morbid history of the patients and their needs; the pharmacist must use health education as an essential and effective tool for this in order to ensure good therapeutic results (Lyra Jr. et al., 2007).

In recent years, PF programs that use educational intervention for patients and health professionals have obtained positive results in the control of diabetes (Planas et al., 2009; Doucette et al., 2009; Kiel and Mccord, 2005; Flores, 2005). For example, Al Mazroui et al. (2009) argued that educational intervention is as important as the clinical intervention because it provides the patients with better knowledge about drug therapy, diet and physical activity, allowing more effective self-monitoring of blood glucose to control diabetes (Al Mazroui et al., 2009; Speight and Bradley, 2001). McWhorter et al. (2002) confirmed that medicines reached the target goal established for glycosylated hemoglobin (HbA1c) level < 7% in patients who are oriented by a pharmacist about their illness.

On the other hand, a review by Cooper et al. (2001) about educational intervention promoted by many health professionals for chronic diseases such as diabetes shows that despite the improved metabolic levels reached in the first 6 months of monitoring, the levels revert 6 months after the education process. Therefore, the authors recommend continuous monitoring and reassessment of the program to measure the effects of educational intervention over time (Cooper et al., 2001; Funnell, 2009). Another review by Machado et al. (2007) points out the lack of studies evaluating the effect of long-term clinical and humanistic outcomes achieved by educational intervention promoted by pharmacists in the care of diabetes patients (Machado et al., 2007). Thus, the aim of this study was to evaluate the long-term effects of PF on the clinical and humanistic aspects of a group of elderly patients with diabetes mellitus.

METHODOLOGY

Study design and location

We conducted a longitudinal and prospective study with an intervention divided into 3 stages. In this study, we describe the results obtained in the 3rd evaluation, which was a continuation from a previous study that describes the 1st and the 2nd ones (Balisa-Rocha et al., 2012). The study was conducted in a Popular Pharmacy in Brazil, a community pharmacy, located at Estância Street, Aracaju-SE, Brazil. The Popular Pharmacy was launched by the Federal Government to augment the acquisition of essential medicines by the by low-income population of Brazil. At present, there are 423 Popular Pharmacies in Brazil and the PF is planned as one of the main guidelines for the care setting (BRASIL, 2005). This study was approved by the Ethics Committee in Research from the University Hospital at Universidade Federal de Sergipe, under protocol No. 0137.0.107.000-07.

Patients

All patients (n = 34) who attended the Popular Pharmacy of the PF from January to November, 2009 were invited for the 3rd evaluation (November, 2010). These patients were elderly patients between 60 and 75 years of age and included men and women diagnosed with diabetes. The study population was recruited over a period of approximately 2 months through telephone contact. All patients signed an informed consent document in accordance with Resolution CNS No. 196/96.

Data collection

One year after the end of the program (3rd evaluation), individual consultations were scheduled within 1 month, each lasting 40 to 60 min. The period for reassessing the clinical and humanistic results was 1 year, which doubled the amount of time suggested by Cooper et al. (2001) during which the parameters evaluated after the educational intervention declined. During the consultations, sociodemographic and pharmacotherapeutic data (that is, the number of medicines, specifically for hypoglycemia) and clinical data equivalent to data obtained during the PF one year later (that is, HbA1c level, blood glucose level, blood pressure (BP), body index mass (BMI) and waist circumference (WC)) were obtained (ADA, 2013a; BDS, 2013; Lipschitz, 1994; WHO, 1998).

In addition, to assess the humanistic outcomes, quality of life was evaluated in all 3 stages of the study by using the same generic tool, the Portuguese version of the Medical Outcomes Studies 36-Item Short Form (SF36®), used in the PF in 2009 (Ciconelli et al., 1999). This instrument has 8 domains that measure physical capacity, pain, general health, vitality, social, physical and emotional

Table 1. The 3 stages of development of the longitudinal and prospective study with an intervention, 2009 - 2010.

Evaluation	Period	Phase of study
1st	January, 2009	Beginning of the pharmacotherapeutic follow-up
2nd	November, 2009	Final of the pharmacotherapeutic follow-up
3rd	November, 2010	Evaluation performed 12 months after the pharmacotherapeutic follow-up

Table 2. Sociodemographic and pharmacotherapeutical profiles of the elderly patients (n = 14) treated at the Popular Pharmacy in Aracaju-SE, Brazil during the 3rd assessment in November, 2010.

Parameter	Value
Age (years)*	64.28 (2.63)*
Gender	**N (%)**
Women	7 (50)
Men	7 (50)
Marital status	
Married	10 (71.43)
Single	2 (14.28)
Divorced	2 (14.28)
Education	
Elementary school	9 (64.28)
Middle school	4 (28.57)
High school	1 (7.14)
Quantity of drugs	6.57 (2.28)*
Hypoglycemic drugs	1.93 (0.8)*

emotional aspects and mental health. Each domain was transformed into a scale from 0 to 100 where lower scores represent a better quality of life.

Pharmaceutical and educational intervention

During the PF (January to November, 2009), educational interventions were performed through oral and written instructions, including folders and slides (Balisa-Rocha et al., 2012). The intervention was based on the previous experience and reality of the patients, and involved dialogue and co-responsibility in the process of health care and decision making (ADA, 2013b; Roter et al., 2001; Freire, 1983). The patients were oriented about diabetes and its complications, proper dosage, medication side effects and storage, changes in lifestyle-particularly with regard to diet and physical exercise and the importance of managing the signs and symptoms of diabetes through self-monitoring (Al Mazroui et al., 2009; ADA, 1996). The educational intervention was reinforced by the pharmacist during each visit. In addition, changes in drug therapy were discussed with patients and their physicians when necessary, and suggestions were made according the American Diabetes Association (ADA) (1996). For the 3rd evaluation (November, 2010), the educational intervention was reinforced through oral and written instructions.

Statistical analysis

Data were collected and entered twice in a BioEstat® version 5.0 database. The frequency, means and standard deviations were obtained. Changes in clinical and humanistic outcomes pre- and post-intervention and during reevaluation were analyzed using bivariate Friedman's test for dependent samples. A p value less than 0.05 was considered statistically significant, with a confidence interval of 95%.

RESULTS

Fourteen of the 34 patients (41.17%) recruited for the 3rd evaluation in November, 2010, 22 months after the start of the program, attended the consultations. Regarding the use of medications, the polypharmacy phenomenon was identified in 12 (85.71%) patients. Table 2 shows the sociodemographic features and the pharmacotherapeutic profile of the participants. The mean values obtained for HbA1c, systolic blood pressure (SBP) and diastolic blood pressure (DBP) significantly improved in relation to the 1st and 2nd evaluations, that is before and after the pharmaceutical intervention ($p < 0.05$), as shown in Table 3. In addition, in the 2nd evaluation, 10 (71.42%) patients presented HbA1c level < 7% and 13 (92.85%) patients presented HbA1c level < 8%. In the 3rd evaluation, 6 (42.86%) of the patients achieved HbA1c level < 7% and 12 (85.71%) of the patients achieved HbA1c level < 8%. Blood pressure values also reduced over the sessions of the program and remained stable during the 3rd evaluation ($p < 0.05$). In addition, in the 2nd evaluation, capillary blood glucose values achieved the targets recommended by the literature (American Diabetes Association, 2005). It is noteworthy that during the 3rd evaluation, blood glucose values were significant ($p < 0.05$) compared to those at the 1st evaluation (not statistically significant). The SF-36 scores changed significantly ($p < 0.05$) between the 1st and 2nd evaluations after the pharmaceutical intervention in the fields "pain" and "vitality," as shown in Table 4. Although there were no statistically significant differences in the other parameters, their averages increased from the 1st to 3rd evaluations.

Table 3. Clinical parameters in the 1st, 2nd, and 3rd evaluations of elderly patients (n = 14) at the Popular Pharmacy in Aracaju-SE, Brazil in November 2010.

Statistical indicator	1st evaluation	2nd evaluation	3rd evaluation	*P** 1st × 2nd evaluation	*P** 1st × 3rd evaluation	*P** 2nd × 3rd evaluation
SBP (mmHg)	150.35	134	131.5	<0.05*	<0.05*	nss
DBP (mmHg)	86.57	78.21	78.85	<0.05*	nss	nss
Capillary glycemia	200.64	167.92	140.43	nss	<0.05*	nss
BMI	28.61	29.13	29.49	nss	nss	nss
HbA1C	8.59	6.94	6.92	<0.05*	nss	nss
WC (women)	104.43	102.86	104.57	nss	nss	nss
WC (men)	97.62	96.5	96.81	nss	nss	nss

*Statistical significance: $p < 0.05$. nss: not statistically significant.

Table 4. SF-36 scores related to quality-of-life parameters in the 1st, 2nd, and 3rd evaluations of elderly patients (n = 14) in Aracaju-SE, Brazil in November, 2010.

Statistical indicator	1st evaluation	2nd evaluation	3rd evaluation	P* 1st × 2nd evaluation	P* 1st × 3rd evaluation	P* 2nd × 3rd evaluation
Functional capacity	69.6	70.6	81	nss	nss	nss
Physical aspects	58.3	61.6	73.3	nss	nss	nss
Pain	48.9	67.7	61.3	<0.05*	nss	nss
General health	66	76.0	75.8	nss	nss	nss
Vitality	68	80.6	77.6	<0.05*	nss	nss
Social aspects	75.8	85.8	90	nss	nss	nss
Emotional aspects	59.9	86.6	75.5	nss	nss	nss
Mental health	69.8	81.8	79.7	nss	nss	nss

*Statistical significance: $p < 0.05$. nss: not statistically significant.

DISCUSSION

The sociodemographic characteristics and use of polypharmacy presented by the elderly patients are concordant with the data reported in the literature (Wermeille et al., 2005; Al Mazroui et al., 2009; Rosa et al., 2003). Polypharmacy, which is the use of 5 or more drugs simultaneously, increases the risk of adverse events that result in hospitalization, such as hypoglycemia (Picone et al., 2008; Jyrkka et al., 2009). In Europe, 20% of the elderly patients are attended to in clinics and other 20% are admitted in geriatric hospitals owing to adverse reactions caused by drugs (Laroche et al., 2006). Thus, the pharmacist must ensure that the patient is aware of the risks of polypharmacy, handle the medicine schedule and report to physicians about possible drug interactions and therapeutic duplicity.

The results obtained in this study suggest that pharmaceutical intervention generates clinically relevant improvement because several patients with hypertension and diabetes concomitantly were able to achieve and maintain clinical goals recommended by the literature (130/80 mmHg) (Balisa- Rocha et al., 2012; Wild et al., 2004). Patients exhibited significant reductions in mean SBP (18 mmHg) and DBP (12 mmHg) after the educational intervention; similar results were obtained by Lyra Jr. et al. (2008). According to the UK Prospective Diabetes Study (UKPDS), a difference of 10/5 mmHg reduces the risks of stroke, microvascular complications and diabetes-related mortality by 44, 37 and 32%, respectively (UKPDS, 1998).

In the 3rd assessment, even after 1 year of pharmaceutical educational intervention, HbA1c level had changed but not significantly. Similarly, Al Mazroui et al. (2009) showed that 45.4% of patients from the group who received pharmaceutical intervention achieved the target HbA1c levels (< 7%) (Al Mazroui et al., 2009).

Nevertheless, a decrement of at least 0.5% in HbA1c

levels leads to estimated 18.5 and 10.5% reductions in microvascular complications and diabetes-related mortality, respectively (UKPDS, 1998). Although the results suggest these parameters were improved and maintained, it is necessary to periodically strengthen the educational process to account for the possibility of other interferences related to the natural aspects of the disease or external factors such as diet and sedentary lifestyle; the goal of this is to avoid any reduction in the effectiveness of the long-term intervention (ADA, 2013b). In the 3rd evaluation, some patients had HbA1c levels higher than the recommended levels (≤ 7%). However, patients who present complications in advanced stages or other clinical conditions that reduce the quality of life may have slightly higher HbA1c levels as a treatment goal. In Brazil, HbA1c level ≤ 8% is considered acceptable for the elderly and other patients in whom the risks of more intensive glycemic control are greater than the potential benefits of tight control (BDS, 2013).

In this study, educational intervention aimed to persuade patients about the need for self-care and health co-responsibility as well as the active role of referring to physicians and pharmacists when they have problems. The self-management of glycemic control should keep patients cognizant about the possibility of the loss of control of clinical parameters; they should be able to identify symptoms and prevent complications and squeals (ADA, 2013b). Therefore, the need to strengthen the educational process in order to stimulate the self-care of patients and conscious improvement of these parameters is necessary.

The body mass index (BMI) and waist circumference (WC) parameters did not differ significantly before and after educational intervention as well as in the 3rd assessment. This can be explained by the fact that the elderly are less involved in physical activities and the reduction of weight gain than younger patients. Thus, the need to include other health professionals such as doctors, nutritionists, and physical trainers in the support team for the continuous care of elderly people is necessary (ADA, 2013a; Guimarães and Ciolac, 2004; Ahrens et al., 2003).

Regarding the assessment of quality of life, our results are similar to those obtained by Elnour et al. (2008) and Al Mazroui et al. (2009). The presence of diabetes is a factor that can influence the quality of life because patients with this chronic condition use more medicines than healthy ones do, have higher blood pressure, higher rates of cardiovascular complications, worse self-perception of real quality of life, lower scores on physical scales and functional capacity of the SF-36 and higher mortality (Martinez-Casteano et al., 2004). Grincenkov et al. (2011) assessed the quality of life of hemodialysis patients and found that elderly patients with diabetes have the worst results and need to understand the limitations and perspectives of the treatment process (Grincenkov et al., 2011). Thus, it is necessary to consider the living conditions and health of the elderly with diabetes; considering these may allow the creation of proposals for specific educational intervention, promoting wellness in this age group.

ADVANTAGES AND LIMITATIONS

This study has some advantages and limitations. The advantages are the positive impact of pharmaceutical intervention on most clinical and humanistic parameters even after 12 months without communication with a pharmacist. In addition, the educational intervention combined the optimization of the use of medicines, self-monitoring of the disease, diet information and physical activity, and included the patient in the care process such that they could attend to their own needs; therefore, the intervention was effective in the treatment of diabetes.

The reduced sample size and the absences of patients (58.82%) were considerably high in this study; this may have influenced the statistical analysis. The limited time window (1 month) for performing the reassessment could be a reason for the small number of recruited patients, because many were available to participate in this study on a later date.

The percentage of patients with HbA1c level < 7% in the 2nd evaluation (the end of the Pharmaceutical Care Program) was 71.42%; in the 3rd assessment, this rate dropped to 42.86%. This fact may indicate that the PF period (10 months) was too short to ensure the sustainability of the educational, clinical and humanistic benefits demonstrated in this study.

The pharmaceutical educational intervention can also be a factor to be re-evaluated by researchers. According to the National Standards for Self-Management Education DM (Funnell et al., 2008), diabetes education is effective for improving clinical outcomes and the quality of life of patients. However, the need for continuous support to sustain the progress made by the participants of the educational program was discussed. It is important to emphasize that for diabetes to be a chronic condition, the disease requires continuous monitoring by physicians and other health professionals with new appointments every 3 months (Funnell, 2009; BDS, 2013). This frequency may also be necessary for the care of patients participating in the PF.

In conclusion, PF trains elderly patients to be capable of controlling their diabetes and this is important for maintaining their clinical parameters and quality of life long term. In this study, we observed that the PF contributed to maintaining the levels of BP after 12 months. Moreover, in the 3rd evaluation, HbA1c level and quality of life were similar to those at the 2nd evaluation, suggesting that the program contributed to developing the self-management of diabetes in some patients.

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ABBREVIATIONS

PF, Pharmacotherapeutic follow-up; **HbA1c,** glycosylated hemoglobin; **BP,** blood pressure; **BMI,** body index mass; **WC,** waist circumference; **SF36®,** medical outcomes studies 36-item short form; **SBP,** systolic blood pressure; **DBP,** diastolic blood pressure.

Conflict of interest

Authors reported none.

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Full length research paper

Pakistani physicians' knowledge and attitude towards reporting adverse drug reactions

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The present study was designed to investigate the knowledge and attitude of Pakistani physicians towards adverse drug reaction (ADR) reporting. In this study, five hundred and fifty survey forms were distributed among the physicians belonging to different private and public sector clinics and hospitals of Karachi through email or direct correspondence. Two hundred and twenty five physicians provided consent to show their responses for research purposes. Hence, the response rate for filling the questionnaire was 40.9%. Most of the doctors that participated in the study were consultants. The previously validated questionnaire was adopted that sought the demographics of the physicians, their knowledge and attitudes towards ADR reporting. Descriptive statistics were employed to report the response of respondents to questionnaire items. The association of the position and organization on the responses of participants towards ADR reporting was determined by using a chi-square. Majority of the participants (88%) were aware about the ADRs; 31.5% were aware of pharmacovigilance; 7.5% had an access to ADR reporting system; and only 9.7% were informed about the availability of ADR reporting system. Physicians (64%) were considered to be the most qualified health professionals to report ADRs. The knowledge of ADRs among physicians working in different hospitals of Karachi was quite sufficient, but their attitude toward ADR reporting was lacking. Physicians strongly suggested the need of training through frequent continuous medical education sessions to improve reporting.

Key words: ADR reporting, knowledge, attitude, physicians.

INTRODUCTION

Adverse drug reaction (ADR) can be defined as "an appreciably harmful or unpleasant reaction, resulting from an intervention related to the use of a medicinal product, which predicts hazard from future administration and warrants prevention or specific treatment, or alteration of the dosage regimen, or withdrawal of the product" (Edwards and Aronson, 2000). Trend concerning ADR as an area of major health concern was developed after thalidomide disaster in 1960s (D'arcy and Griffin, 1994). ADRs are the major health tribulations considered globally since every drug provides evidence to have adverse effects, even if utilized appropriately. Drug related morbidity and mortality are the major causes of patient hospitalization affecting the status of public health (Lazarou et al., 1998). It also imposes a considerable fiscal burden on the health care systems of society

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(Ayani et al., 1999; Wu and Pantaleo, 2003) Healthcare professionals can play a vital role in detecting and reporting of ADR if they are encouraged to execute it appropriately (DACA, 2008). It is anticipated that the ratio of ADRs that are reported is only 6 to 10% (Smith et al., 1996; Edwards and Aronson, 2000). Different factors including medical professionals' knowledge and attitudes to reporting are associated with ADRs under-reporting which consequently impart negative impact on the public health (Lopez-Gonzalez et al., 2009). Initially, the 20th world's assembly adopted a resolution to begin a project on the feasibility of global system of monitoring adverse reaction of drugs. For the first time, an international data base was established at WHO head quarter in Geneva in 1971 which later shifted to Uppsala, Sweden in 1978 (WHO, 2000, 2001). Since then, Uppsala Monitoring Centre (UMC) located in Sweden is carrying out this imperative job for managing of the WHO-PIDM which is a collaborating centre for maintaining international ADR database, Vigibase. The major focus of UMCs is to support high-quality decision-making concerning the benefits and risks associated with medicines. WHO Programme has 105 countries as an official member and 35 countries as associate member. Pakistan was also one of an associate member of WHO program (Waller, 2006; WHO, 2001, 2000; Wiffen, 2002). Numerous countries of the world have well-developed drug safety survellance program owing to the recognition of the importance of this program (Yadav, 2008). ADR reporting system focuses on the ways to decrease ADR risks associated with FDA approved medications (Wiffen, 2002). Spontaneous and voluntary reporting is the most effective methods of acquiring ADR information (Waller, 2006). The medical professionals can play a very significant role in reporting suspected ADRs that they encountered in their clinical practice. But still, there is substantial divergence in the patterns of ADR reporting phenomena in some countries (Kharkar and Bowalekar, 2012).

It is realized that creating awareness on the relevance and importance of ADR monitoring is an assurance for establishing and sustaining sound ADRs reporting program (Ernst and Grizzle, 2001). Physicians are the key components of healthcare system encountering ADRs in their daily practice. Several studies have been conducted with an aim of recognizing physician's attitude and perception about ADR reporting worldwide (Gupta and Udupa, 2011; Okezio, 2008; Aziz et al., 2007). ADR reporting system has yet not received the deserving importance in Pakistan owing to the lack of its basic knowledge among the health professionals. Ghulam et al. (2013) conducted a study in Lahore, Pakistan to investigate the factors contributing to ADR under reporting among different healthcare professionals (Ghulam et al., 2013). Therefore, the present study was designed to explore their knowledge and attitudes towards ADR reporting in different hospitals of Karachi, the largest city of Pakistan and also to find out the ways of improving spontaneous reporting.

MATERIALS AND METHODS

Study design and study period

The present study was cross-sectional study and was conducted from September, 2012 till February, 2013.

Study population

The study population comprised of physicians working in different public and private sector hospitals and clinics of Karachi selected by non probability convenience sampling technique. Physicians were surveyed with a 31 items questionnaire to assess attitudes and perception of medical practitioners towards ADR reporting.

Study tool

A prevalidated questionnaire was adapted from previous studies to assess attitudes of medical practitioners to ADR reporting (Bateman et al., 1992; Belton, 1997). In addition to the demographic information of the physicians, the questionnaire consisted of two parts. The first part consisted of thirteen questions that explored the perception of physicians towards ADR, the most qualified healthcare professional to whom ADR should be reported, the frequency of ADR encountered in daily practice and the purpose of reporting ADR from physicians point of view. Part two comprises eighteen questions; exploring the attitude and the factors that hinder physicians to report ADR, the most appropriate method of improving ADR reporting, training on ADR reporting and the most reliable source of information about ADRs.

Ethical approval

Prior permission was taken from the various heads of departments in the hospitals and clinics before initiating the study. The questionnaires were distributed to the physicians after explaining th them the purpose of the study. Their verbal consent was taken and the questionnaires were left with them for a period of 1 week. After the given time period the filled questionnaires were collected back.

Data analysis

The retrieved questionnaires were entered into Statistical Package for Social Sciences (SPSS 20.0, Chicago, IL) for analysis. The demographic data of the participants was estimated in frequencies and percentages. Descriptive statistics were employed to report the response of respondents to questionnaire items. The association of the position and organization with the responses of participants towards ADR reporting was determined using a chi-square at 0.05 significant level.

RESULTS

In the present study, five hundred and fifty survey forms were distributed among the physicians belonging to different private and public sector clinics and hospitals of Karachi through email or direct correspondence. Two hundred and twenty five physicians provided consent to

Table 1. Characteristics of the physicians participated in the study.

Characteristic	Number (%)
Gender	
Male	86 (38.2)
Female	139 (61.7)
Age (Years)	
25-30	81 (36)
31-35	51 (22.6)
36-40	27 (12)
41-50	33 (14.6)
51 and above	33 (14.6)
Organization	
Private	150 (66.6)
Public sector	75 (33.3)
Position	
Consultant	116 (51.5)
Chief medical officer	18 (8)
Medical officer	29 (12.8)
Resident medical officer	38 (16.8)
Head of department	24 (10.6)

show their responses for research purposes. Hence, the response rate for filling the questionnaire was 40.9%. Majority of the respondents were female 61.7%, while 38.2% were male. Most of the respondents (66.6%) who participated were rendering their services privately and 33.3% were employed in public sector hospitals. Most of the doctors (51.5%) who participated in the study were consultants (Table 1).

Perception of physicians regarding ADRs is recorded in Table 2. Majority of the participants (88%) were aware about the ADRs. Almost all physicians (90.6%) considered that reporting ADRs to ministry of health is necessary. Physicians (85.7%) also agreed that all ADRs should be reported for newly marketed as well as for established drugs. In view of respondents (74.6%), ADR reporting system should be improved in Pakistan. On the other hand, only 31.5% of the participants were aware of the term pharmacovigilance and 9.7% were informed about the availability of ADR reporting system. 29.6% of the participants encounter 0 to 5 ADRs/week, while 65.6% did not encounter a single ADR in their daily practice.

Most of the participants (83.5%) considered that they should report a recognized ADR. About 80% agreed that ADR reporting is a professional obligation; 70.4% opined that managing patient is more important than reporting ADR. 70.6% agreed that they can confidently discuss an ADR with other colleagues. Only 15.5 and 16% knew where to report and how to report, respectively. Only 7.5% have an access to ADR reporting system; 48% thought that ADR reporting generates an extra work. About 20% of the respondents considered that reporting of a single ADR makes no significant contribution to the ADR reporting system. A small number of participants (7.5%) had ever been trained on how to report an ADR (Table 3).

The prime purpose of ADR reporting from physicians' point of view is to improve patient safety (69.6%), and to identify safe drugs (20%) (Figure 1). Respondents (68.8%) agreed that they will be encouraged to report ADR if the reaction is serious. Mostly physicians (80%) considered that ADR reporting should be compulsory. Continuous Medical Education (CME) was considered as the most appropriate method for the improvement of ADR reporting by physicians (52%), while increased collaboration with other health care professionals (11.2%) and having an ADR specialist in every department (10.4%) were considered next to CME (Figure 2). The reliable sources of information about ADR reporting as considered by physicians included seminars (33.6%), internet (24%), journals (18.4%) and drug advertisement (10.4%).

The influence of position and organization on physicians' response was analyzed statistically by Chi square. Results showed that the position of participant had a significant impact on their responses, that is, it was difficult for them to decide whether an ADR has occurred or not ($\chi 2 = 11.075$, $p = 0.004$); reporting of a single ADR makes no significant contribution to the ADR reporting system ($\chi 2 = 12.174$, $p = 0.002$) and ADR reporting system should improve in Pakistan ($\chi 2 = 14.291$, $p = 0.001$).

The influence of participants' organization also had a significant impact on their responses. Knowledge about Drug Regulatory Authority of Pakistan (DRAP) form of ADR reporting ($\chi 2 = 20.594$, $p < 0.0001$) their perception that ADR reporting generates an extra work ($\chi 2 = 17.905$, $p < 0.0001$) and time to actively look for ADR at work ($\chi 2 = 21.765$, $p < 0.0001$) were the most significant reasons of ADR under reporting.

DISCUSSION

Adverse reactions are predictable risk of drug remedy. Some ADRs are negligible which may be resolved without any significant squeal, while some ADRs can be fatal or may be the cause of enduring disability. Pharmacovigilance is the science and activities relating to the detection, assessment, understanding and prevention of adverse effects or any other drug-related problem. It encompasses recognizing, reporting, and responding to risk-benefit issues associated with marketed drugs(WHO, 2002). The information generated in this post- marketing

Table 2. Physicians' perception about ADRs.

Statement	Yes	No	Don't know
Awareness about ADRs	198 (88)	18 (8)	9 (4)
Knowledge about pharmacovigilance	71 (31.5)	92 (40.8)	62 (27.5)
Knowledge about any drug that has been banned due to ADR	115 (51.1)	72 (32)	38 (16.8)
All ADRs should be reported	193 (85.7)	6 (2.6)	26 (11.5)
Serious ADRs should be reported	193 (85.7)	11 (4.8)	21 (9.3)
Availability of ADR reporting system	22 (9.7)	67 (29.7)	136 (60.4)
Reporting ADRs to ministry of health is necessary	204 (90.6)	2 (0.8)	19 (8.4)
Knowledge about DRAP form of ADR reporting	63 (28)	74 (32.8)	88 (39.1)
Should ADR reporting system improve in Pakistan	168 (74.6)	17 (7.5)	40 (17.7)

Table 3. Physicians' attitude towards reporting ADRs.

Statement	Yes	No	Don't know
Know where to report ADR	35 (15.5)	108 (48)	82 (36.4)
Know how to report ADR	36 (16)	108 (48)	81 (36)
Have an access to ADR reporting system	17 (7.5)	170 (75.5)	38 (16.8)
Have time to fill ADR form	101 (44.8)	105 (46.6)	19 (8.4)
Managing patient is more important than reporting ADR	159 (70.6)	40 (17.7)	27 (12)
ADR reporting generates an extra work	108 (48)	89 (39.5)	28 (12.4)
Have time to actively look for ADR at work	76 (33.7)	125 (55.5)	24 (10.6)
Is it difficult to decide whether an ADR has occurred or not	90 (40)	92 (40.8)	43 (19.1)
Can confidently discuss an ADR with other colleagues	159 (70.6)	56 (24.8)	10 (4.4)
Should report a recognized ADR	188 (83.5)	11 (4.8)	26 (11.5)
ADR reporting may have negative impact on the company that marketed drug	24 (10.6)	162 (72)	39 (17.3)
ADR reporting is professional obligation	180 (80)	18 (8)	27 (12)
Reporting of a single ADR makes no significant contribution to the ADR reporting system	45 (20)	146 (64.8)	34 (15.1)
Have ever been trained on how to report an ADR	17 (7.5)	162 (72)	46 (20.4)

surveillance can be used to revise products' labels and to reconsider the approval decision of such drug. Even the information provided can be the indication of probable harms related with the utilization of certain drugs. Therefore, the transmission of this information is also a critical aspect of pharmacovigilance, needed for safe prescribing of drugs (Brewer and Colditz, 1999). Every healthcare professional can play his/her role in upgrading patients' safety particularly medical practitioners, who are the primary component of ADR reporting system depending on their knowledge, attitudes and perceptions about ADR (Vallano et al., 2005; Vessal et al., 2009; Rawlins, 1994). Several studies have been conducted regarding the knowledge and attitude of physicians in different countries of the world which showed the inadequate knowledge of physicians about ADR reporting (Bateman et al., 1992; Belton, 1997; Enwere and Fawole, 2008; Milstein, 1986; Rogers, 1998). Therefore, the present study was conducted with the objective of investi-gating the knowledge and attitudes of physicians to ADR reporting in different hospitals of Karachi. It was observed in our study that only some participants were aware of ADR reporting and its availability in Pakistan.

Response rate was very low in the present study. This outcome could be a reflection of the importance attached to the problem of ADRs by this category of medical personnel and this is not very encouraging. Similar behavior of physician was also observed in other studies (Fadare et al., 2011). The considerable numbers of physicians in the present study never reported an ADR; majority of the participants did not encounter a single ADR in their daily practice, that is comparable with other studies (Cosentino et al., 1997; Figueiras et al., 1999; Vallano et al., 2005). A study performed in Barcelona/ Spain showed that lack of time to report an ADR, unavailability of ADR reporting system in hospitals and lack of information about the spontaneous reporting system were the main reasons of under reporting ADRs (Evans et al., 2006). Similar trends were also observed in our studies which were the prime reasons of under reporting of ADRs in the view point of the physicians.

Our study revealed that only 15.5 and 16% knew where

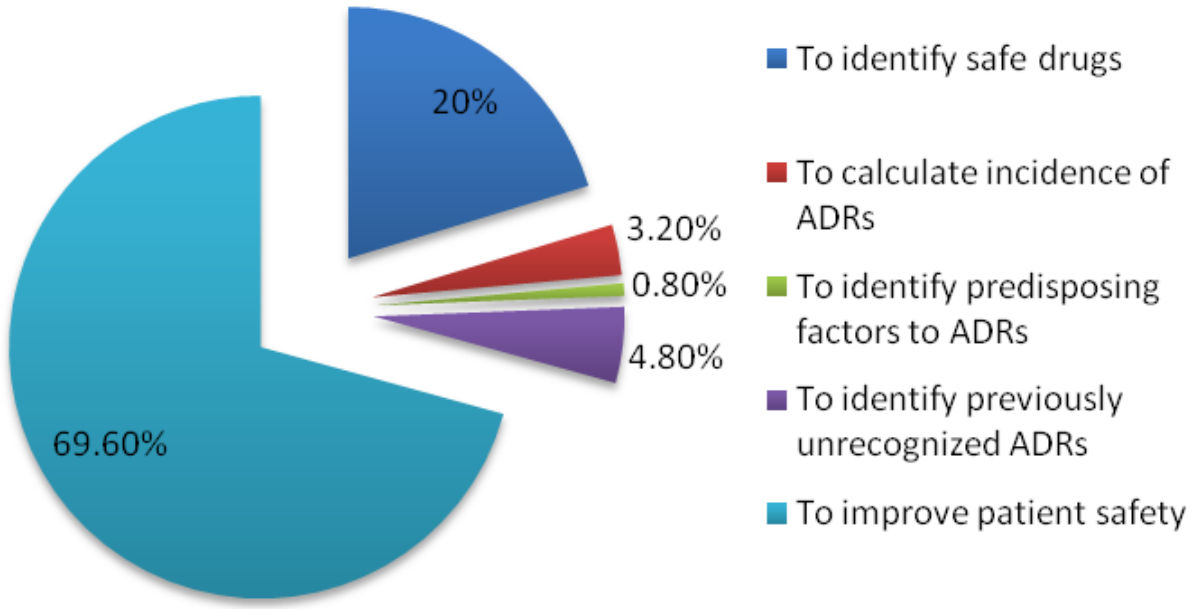

Figure 1. Purpose of reporting ADR in physicians' point of view.

to report and how to report, respectively. Merely 7.5% have an access to ADR reporting system; 48% thought that ADR reporting generates an extra work. A small number of participants 7.5% had ever been trained on how to report an ADR. Previous studies reveals that under-reporting of ADRs is a worldwide phenomenon (Williams and Feely, 1999; Hazell and Shakir, 2006; Lopez-Gonzalez et al., 2009; Nichols et al., 2009). The major factors contributing to under-reporting ADR includes lack of knowledge of the forms for reporting, ignorance of the rules and procedure for reporting, and not being sure of the type of reactions to be reported. The results are similar to the studies carried out in China, Nigeria, and Malaysia (Li et al., 2004; Aziz et al., 2007; Okezie, 2008).

A study conducted in Lagos, Nigeria on physicians' perceptions to ADR reporting documented that 89.9% of them considered physicians as the most qualified health professionals to report ADR (Oshikoya and Awobusuyi, 2009). Analogous trend was observed in our study that 64% of participants considered physicians to be the most appropriate person to report an ADR, while 31.2% considered pharmacist as more suitable person to report an ADR. Physicians' attitude showed that 83.5% considered that they should report a recognized ADR. This study showed an overwhelming result that 80% agreed that ADR reporting is a professional obligation; these results are nearly similar to study conducted in India (Gupta and Udupa, 2011).

Different educational platforms like pharmacovigilance training and workshops conducted for healthcare professionals are essential for improving physicians' knowledge, attitudes and perceptions about ADRs (Salehifar et al., 2007). The present study revealed that CME was considered the most appropriate method for the improvement of ADR reporting. Other considerable ways to improve such reporting system in view of physicians included increased collaboration with other health care professionals and having an ADR specialist in every department.

Limitation of the present study included that the physicians who participated in the present study were only from Karachi. Therefore, the present data did not provide us the comprehensive picture of ADR reporting attitude among physicians of the entire country. It is strongly recommended to conduct a nationwide survey to gather baseline physicians' knowledge and attitude about ADRs.

ADR reporting system has not been flourished in most of the under developing countries like Pakistan. In order to implement such system in its full form, an initial step has to be taken, that is, gather information about physicians' knowledge and attitude of the community. Such information will help the government of Pakistan to successfully implement the ADR reporting system; thereby achieving reduced hospitalization and morbidity/ mortality due to ADRs. In such a way, healthcare system of Pakistan will get a new revival.

Conclusion

The current study provided the information that the knowledge of ADRs among physicians working in different hospitals of Karachi is quite sufficient, but their perception toward ADR reporting was lacking and reflected

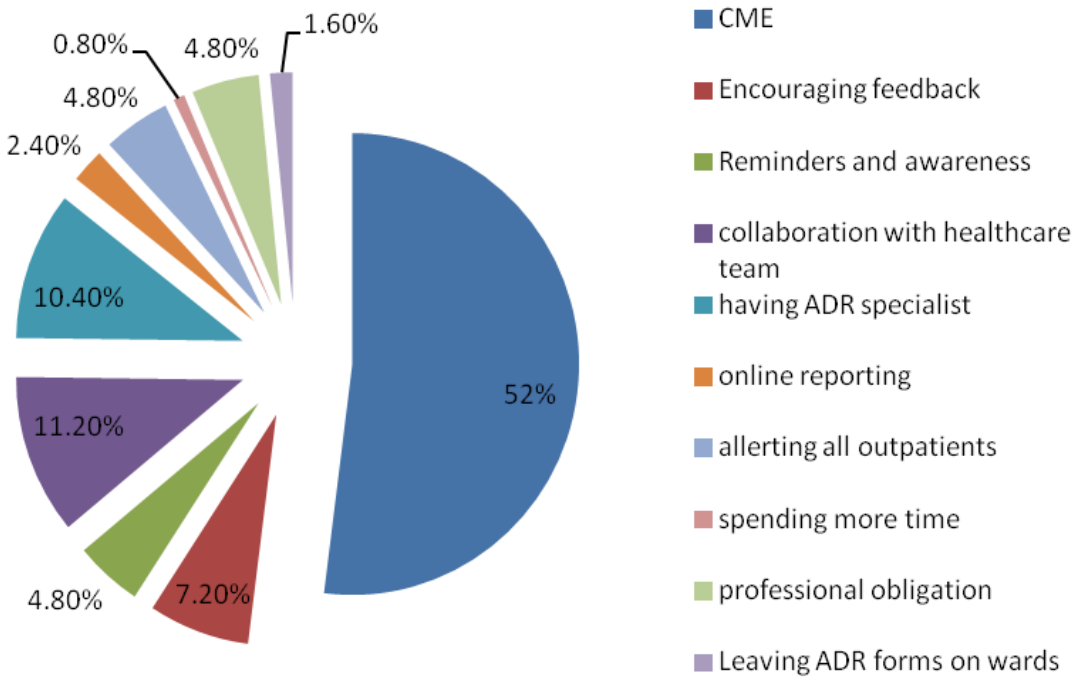

Figure 2. Appropriate method of improving ADR reporting in physicians' point of view.

when it comes to the actively reporting of ADRs. Healthcare professionals should be meticulously involved in pharmacovigilance activities in their daily practice which will set a concrete foundation in healthcare system of Pakistan. Physicians who participated in the study also suggested for the need for training through frequent CME lectures and integration of ADR reporting into the clinical activities of the physicians that would improve reporting.

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Conflict of interest

Authors declare no conflict of interest.

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Full Length Research Paper

In vitro cytotoxic and genotoxic evaluation to ascertain toxicological potential of ketoprofen

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Analgesic and anti-inflammatory properties of ketoprofen are well documented but little is known about its cytotoxic activity and the potential to damage the DNA. The present study was designed to evaluate the cytotoxic and genotoxic potential of ketoprofen. MTT dye (3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) was used to assess cytotoxicity in which confluent monolayer of vero cells were incubated in the presence of increasing concentrations of ketoprofen. Genotoxicity was evaluated by single cell gel electrophoresis (SCGE) assay or comet assay. Lymphocytes were separated from the mice blood and treated with different concentrations of ketoprofen. Lymphocytes were incorporated in agarose gel on cavity slides and visualized for strand break to asses DNA damage. Ketoprofen concentrations 8, 6, 4.5, 3.3, 2.5, 1.8, 1.4, 1 and 0.5 mM were used for both cytotoxic and comet assay. The results of cytotoxic assay showed significant ($p < 0.001$) cytotoxicity at 8 and 6 mM concentrations. The cytotoxic concentration for 50% of cells (CC_{50}) value was calculated at 5.2 mM concentration. In case of the comet assay, ketoprofen presented DNA damaging potency, creating significant ($p < 0.001$) DNA damage at 8 mM concentration, a moderate damage at 6 mM concentration and a mild damage at 4.5 mM concentration which was evident from the comet tail lengths and changes in head diameter. DNA damage index was calculated for each concentration of ketoprofen and compared with the control. The data advocates that ketoprofen possesses cytotoxic and genotoxic potential at higher concentrations and its dosage should be carefully monitored to avoid its toxicity.

Key words: Ketoprofen, cytotoxic, genotoxic, MTT assay (3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide), comet assay, DNA damage.

INTRODUCTION

Ketoprofen possess well documented anti-inflammatory, analgesic (Akural et al., 2009), antipyretic and antirheumatic properties (Celebi et al., 2009; Liu et al., 2007; Shinkai et al., 2008). Most of the effects of are ketoprofen due to the

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inhibition of prostaglandins which are produced by various cell types and regulate various body activities (Hata and Breyer, 2004). Ketoprofen can be given in the form of injectables, taken orally or applied topically to the skin. Photosensitization and contact eczemas are the side effects reported with the use of ketoprofen gel as cutaneous application (Baudot et al., 1998). There are no side effects with the solution containing ketoprofen used as intraoral solution (Liccardi et al., 2003). Ketoprofen developed in the form for nano-emulsion for topical application on the skin is considered very effective (Sakeena et al., 2010).

The cytotoxic assay using MTT dye is considered to be the most sensitive method to assess the nonsteroidal anti-inflammatory drugs (NSAIDs) induced toxicity. MTT assay is mostly used in lab to determine cytotoxicity of various drugs at different concentrations. MTT assay has a number of advantages than clonogenic assay as large number of assays can be performed in one batch (Plumb, 1999). MTT assay is extensively used to study cell viability and cytotoxic effects of the test substance on cell lines under *in vitro* conditions (van Meerloo et al., 2011). Toxicity studies are mostly performed on cell culture models which are an effective ways to evaluate test compounds (Hong et al., 2007). The phosphoramidate derivatives of NSAIDs (fenoprofen, ketoprofen, ibuprofen, indomethacin, diclofenac) were evaluated for their cytostatic and antiviral activity by using malignant tumor cell lines. It was found that phosphoramidate derivatives possessed viral inhibitory activities (Wittine et al., 2009).

Genotoxicity describes that cells integrity is affected due to damaging action on a cell's genetic material which disrupt cell survival. The comet assay is a micro-electrophoretic technique for the direct visualization of damage to DNA of individual cells (Ostling and Johanson, 1984). Comet assay is unique method for assessing the DNA damage caused by certain drugs, environmental factors and other substances (Blaisak et al., 1999). In comet assay the comets are formed and their tail lengths are measured. These tail lengths give an estimation of extent of DNA damage (Fairbairn et al., 1995). Previously, different NSAIDs were tested for their genotoxicity and comet assay was performed for estimation of DNA damage. Cell of HEC/UGT cell lines were treated with different NSAIDs and the cytotoxicity and genotoxicity caused by these NSAIDs was assessed. In these studies, no cytotoxicity and genotoxicity were observed at ketoprofen (1 mM concentration). It was suggested that ketoprofen at 1 mM concentration is safe for use (Koga et al., 2011).

The objective of present study was to determine the cytotoxic and genotoxic activity of ketoprofen at its various concentrations by adopting various *in vitro* bioassays such as MTT assay and comet assay. Ketoprofen different concentrations were tested both for its cytotoxic and genotoxic potential. The CC_{50} value and DNA damage index was also calculated.

MATERIALS AND METHODS

Preparation of drug concentrations

Ketoprofen was obtained from Sigma Aldrich. Stock solution of drug was prepared by dissolving the drug in sterile PBS. Different concentrations 8, 6, 4.5, 3.3, 2.5, 1.8, 1.40, 1 and 0.5 mM were used for both MTT and comet assay. For MTT assay vero cell lines were used and in comet assay lymphocytes from the mice were exposed to different concentrations of ketoprofen.

Cell lines

Vero cell lines were obtained from quality operation laboratory (QOL), University of Veterinary and Animal Sciences, Lahore in freeze dried form and were revived for propagation of cells using Dulbecco's modified eagle's medium (DMEM) (Sigma Aldrich, Germany) media and trypsin solution. Cells were counted for their viability by hemocytometer method using the modified neubar chamber. Cell viability was calculated by following formula.

$$\% \text{ viable cells} = \frac{\text{Number of viable cells / ml}}{\text{Total number of cells / ml}} \times 100$$

Cytotoxicity assay

We used MTT dye to assess cytotoxicity of ketoprofen. In the assay, vero cells along with cell culture media were kept as negative control, however vero cells, dimethyl sulphoxide (DMSO) (10%) and cell culture media were taken as positive control, respectively. After development of confluent monolayer in 96 well plate, media in the cells was regularly changed and afterward 100 ul of ketoprofen different concentrations were added in triplicate to the 96 well plates and incubated for 48 h at 37°C. After incubation media was removed and after washing, new media was added to the wells and then 100 ul of MTT solution (98% catalog#194592, MP Biomedical USA) was added to each well and plates were then incubated for 4 h. The MTT solution was afterwards removed and DMSO was added to each well and incubated for 2 h at 37°C. Finally the enzyme linked immunosorbent assay (ELISA) reader (Type 355, Model 2005-05, Thermo, China) was used to measure the optical density at 570 nm (Raheel et al., 2013). Cell survival percentages were calculated.

Comet assay

Preparation of reagents

Lysing solution, electrophoresis buffer and neutralizing solutions were prepared for comet assay. Staining of slides was carried out by Ethidium bromide solution (Singh et al., 1988).

Lymphocytes separation

Lymphocytes for comet assay were separated on the principle of density gradient using Histopaque 1077 (Sigma-Aldrich, USA) (Yildiz et al., 2008).

Preparation of slides

For preparation of slides, low melting point agarose and normal melting agarose were prepared. Slides were dipped in methanol and burn over a blue flame to remove the machine oil and dust. Cavity slides were used for comet assay. Normal melting agarose was added to the cavity of the slide. For quick drying, slides were air dried. After preparing the slides, ketoprofen exposed lymphocytes were placed on them and electrophoresis of micro gel slides and evaluation of DNA damage was carried out (Blasiak et al., 1999). The slides were then stained with 80 µl of ethidium bromide (2 µg/ml) for 10 min and examined at 40× magnification of fluorescent microscope (Nikon, Japan) equipped with excitation filter of 515 to 560 nm and barrier filter of 590 nm. The damage to DNA was analyzed by quantifying the tail length, head diameter changes and was expressed as damage index. To measure the tail length we used the image J software. The images of 100 randomly chosen nuclei (50 cells from each of two replicate slides) were analyzed visually. Depending on the tail length, four damaging categories were established. Those nuclei which did not presented any DNA damage or tail length were considered no damaged nuclei and were labeled class 0. For damage level arbitrary categories were considered as class 1, presenting smaller tail length when tail length are shorter or equal to head diameter, class 2 when tails lengths are greater or equal to head diameter, class 3 when tail lengths are double of head diameter. Using this protocol, the comet assay was used to test which concentration of ketoprofen showed genotoxicity (Parolini et al., 2009).

Damage index

The damage index was calculated for each concentration of ketoprofen and it was compared with the control. The damage index was calculated by the following formula (Sallustio et al., 2006).

Damage index = No. of cells in Class.1 + (2 × No. of cells in Class.2) + (3 × No. of cells in Class.3)

Statistical analysis

Data collected was analyzed using the statistical package for social sciences (SPSS) for Windows version 13. One-way analysis of variance (ANOVA) and post hoc tests were applied to see statistical differences between groups. Differences were considered significant at $P < 0.05$.

RESULTS

Cytotoxicity testing

Cell survival percentages were calculated for determination of cytotoxic activity of different concentrations of ketoprofen. Cell viability % of vero cells was calculated using Trypan blue dye exclusion technique. Viability of vero cells was 92.45% which was suitable for cytotoxicity testing of ketoprofen. Nine different concentrations in the range of 0.5 to 8 mM were used for ketoprofen cytotoxicity analysis. For ketoprofen, with concentrations 8, 6, 4.5, 3.3, 2.5, 1.8, 1.4, 1 and 0.5 mM, the cell survival percentages are shown in Figure 1 which present significant reduction ($p < 0.001$) in cell survival percentage; however concentration of 1.4 mM with cell survival percentage of 92.75% resulted in significant reduction ($p < 0.05$) in cytotoxic effect. Ketoprofen concentrations in the range of 0.5 to 4.5 M were non cytotoxic for vero cell line. Ketoprofen showed cytotoxicity at the concentration of 6 and 8 mM because cell survival percentage at these concentrations was less than 50% that is, 30.76 and 14.3%, respectively. Concentrations of 1 and 0.5 mM showed no significant reduction with cell survival (%) of 96.11 and 99.62. The cytotoxic concentration CC_{50} for 50% of cells was seen at 5.2 mM concentration. The complete data of cell survival percentage of ketoprofen different concentrations is shown in Figure 1.

Comet assay

Genotoxicity of ketoprofen of nine different concentrations ranging from 0.5 to 8 mM was evaluated using the comet assay. Lymphocytes from mice were used for *in vitro* evaluation of genotoxicity of ketoprofen. Lymphocytes were characterized and their viability was assessed. Ketoprofen showed gentotoxicity at 4.5, 6 and 8 mM concentrations. Image J software was calibrated by stage micrometer and was used to evaluate the comet tail lengths and changes in head diameter. The comet tail length and head diameter at different concentrations of ketoprofen are shown in Table 1. Ketoprofen showed concentration dependent DNA damage. No DNA damage was observed at 3.3, 2.5, 1.8, 1.4, 1 and 0.5 mM concentrations of Ketoprofen. There was significant difference ($p < 0.001$) in DNA tail length between 4.5, 6 and 8 mM concentration of ketoprofen. DNA tail length was 6.25 µm at 4.5 mM, 11.05 µm at 6 mM and 18.06 µm at 8 mM concentration. DNA damage index increased with increase in ketoprofen concentrations, with highest values of damaging index at 8 mM concentration (Table 2). There was no significant difference in damage index with ketoprofen concentration from 0.5 to 3.3 mM when compared with control. The damage observed with 4.5, 6 and 8 mM showed marked difference with control ($p < 0.01$).

DISCUSSION

Identification of toxicity scale and contamination of certain chemicals which require more investigation are mostly carried out using *in vitro* testing approach which is a more easy and reliable method for testing the chemicals (Raheel et al., 2013). We also used *in vitro* methods for evaluating the cytotoxicity and genotoxicity potential of ketoprofen. We used MTT assay for evaluating cytotoxicity

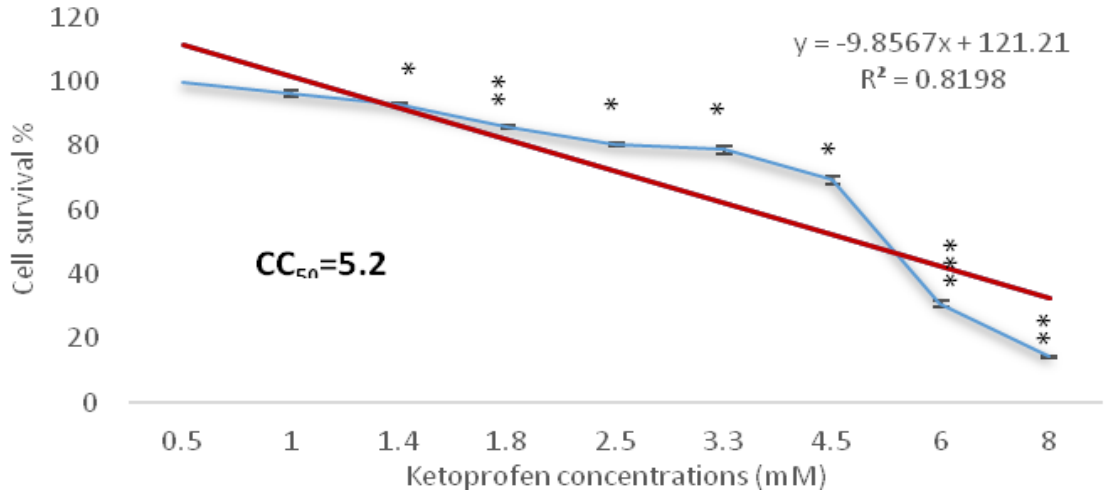

Figure 1. Cytoxicity of ketoprofen at different concentrations. Graphical representation of cytotoxic potential of various concentrations of ketoprofen. Cell survival percentage of Vero Cell line after 48hr exposure to different concentrations of ketorpofen with CC_{50} was observed at 5.2mM concentration.
ANOVA, LSD Post hoc test; * < 0.05, *** < 0.001.

Table 1. Results of genotoxic activity of ketoprofen.

Concentrations (mM)	Tail Length (µm)	Head diameter (µm)
4.5	6.25±1.498	16.87±2.574
6	11.05±2.438	13.89±1.894
8	18.06±2.572	12.72±2.078

Table 2. DNA damage induced by different concentrations of ketoprofen evidenced from comet assay.

Conc. (mM)	Class 0	Class 1	Class 2	Class 3	Damage index
Control	98	2	0	0	2
0.5	97	3	0	0	3
1	97	3	0	0	3
1.4	94	6	0	0	6
1.8	91	9	0	0	9
2.5	87	12	1	0	14
3.3	85	11	2	2	21
4.5	21	73	3	3	88*
6	21	30	44	5	133*
8	27	27	24	22	141*

n = 100 nuclei in two experiments. Nuclei with damage DNA were registered from 0 (undamaged nuclei) to 3 (damaged nuclei). * = significant difference ($p<0.01$) as compared to control analyzed by SPSS Windows Version 13 Tukey's test.

of ketoprofen and comet assay for evaluating genotoxicity of ketoprofen. The result showed cytotoxicity and genotoxicity in concentration dependent manner at higher concentrations. Different attempts were previously made using various methods for testing the cytotoxicity and genotoxicity of ketoprofen. Photogenotoxicity potential of ketoprofen was assessed using the alkaline comet assay (Parolini et al., 2009). Comet assay and MTT assay were previously used for evaluating the cytotoxicity and genotoxicity of ketoprofen (Rafael et al., 2012). Sallustio

et al. (2006) used murine hepatocytes for evaluating cytotoxicity and genotoxicity of ketoprofen along with other NSAIDs. The results showed no cytotoxicity and genotoxicity at 0.5 mM concentrations of ketoprofen. They declared that 0.5 mM concentration of ketoprofen is safe and no cytotoxicity and DNA damage was observed at this concentration. Our results suggested that 0.5 mM showed no cytotoxicity when vero cell lines were used. Lymphocyte DNA was also not affected by this concentration.

Allen et al. (1991) used 1 mM concentration of ketoprofen using MTT assay for assessing the ketoprofen cytotoxicity. The results of their study suggested ketoprofen has partial toxicity. Their results did not claim that cytotoxicity is observed at 1 mM concentration, only partial toxicity was observed. Our study suggested that no cytotoxicity or genotoxicity is observable at 1 mM concentration which is in contrast to the result of Allen et al. (1991).

Previously, very few concentrations of ketoprofen were tested for the study of cytotoxic and genotoxic effects of the drug. In our study, we evaluated the cytotoxic potential of ketoprofen and according to our results ketoprofen shows cytotoxic potential at a dose of 5.2 mM against the vero cell lines. The CC_{50} is 5.2 mM. CC_{50} value of ketoprofen was not previously reported. Our results suggest that ketoprofen cause cytotoxicity at higher concentrations. Various concentrations tested for genotoxicity showed that ketoprofen causes genotoxicity at higher concentration and at 8 mM concentration considerable comet tails can be seen. To the best of our knowledge no previous studies have shown gentoxic concentrations of ketoprofen. Our results suggested that ketoprofen at higher concentrations causes genotoxicity which is evident from comet tails formation and changes in the head diameter of murine lymphocyte DNA.

Conclusion

Ketoprofen, a non-steroidal anti-inflammatory drug, causes cytotoxicity and genotoxicity at higher concentrations. Its dose should be carefully monitored in order to avoid its accumulation in the body which may cause cytotoxicity and genotoxicity.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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Full Length Research Paper

Chemical composition and antimicrobial activity of essential oil from Brazilian plants *Acanthospermum australe*, *Calea fruticosa* and *Mikania glauca*

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The essential oils of leaves of *Acanthospermum australe*, *Calea fruticosa* and *Mikania glauca* (Asteraceae) from southeastern Brazil were obtained by hydrodistillation and analyzed by gas chromatography-mass spectrometry (GC-MS). Oxygenated sesquiterpenes were predominant in *C. fruticosa* (47.8%) whereas sesquiterpenes hydrocarbons constituents predominated in *A. australe* (85.1%) and *M. glauca* (63.3%) oils. Caryophyllene oxide, α-cadinol and selin-11-en-4-α-ol were the most abundant components in *C. fruticosa*. Germacrene D, (*E*)-caryophyllene and bicyclogermacrene were the major components observed in the essential oil obtained from the leaves of *A. australe* and *M. glauca*. The antimicrobial capacity of the oils was tested. The results showed that the oils have antimicrobial activity against Gram-negative bacteria and *Candida glabrata,* with minimal inhibitory concentration (MIC) values between 50 and 1000 µg/ml.

Key words: Asteraceae, essential oil, gas chromatography-mass spectrometry (GC-MS), antimicrobial activity, Brazilian flora.

INTRODUCTION

Essential oils are complex natural mixtures of volatile secondary metabolites, which sometimes can be isolated from different parts of plants. Most of them are used as flavours in the food and beverage industry, as well as in perfumery, and they are also recognized as having several therapeutic applications. They demonstrate pharmacological effects, such as anti-inflammatory, antioxidant, cytotoxic, and they are biocides against a broad range of organisms, such as bacteria, fungi, viruses, protozoa, as well as insects and plants

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(Vagionas et al., 2007). The main constituents of essential oils, for example monoterpenes and sesquiterpenes and phenylpropanoids including carbohydrates, alcohols, ethers, aldehydes and ketones, are responsible for the fragrant and biological properties of aromatic and medicinal plants (Astani et al., 2010).

Asteraceae is the largest family of angiosperms with 1600 to 1700 genera widely distributed and 24,000 species (Funk et al., 2009). *Acanthospermum*, *Calea* and *Mikania* comprise around 6, 110 and 430 species, respectively (Bremer, 1994).

Acanthospermum australe (Loefl.) Kuntze is an annual shrub widely distributed in South America. In Brazil, where it is popularly known as “carrapichinho” or “carrapicho-de-carneiro” it grows vigorously in agricultural fields, pasture and fallow soil. Its aerial parts are used in folk medicine as a tonic, diaphoretic, eupeptic, vermifuge, antidiarrheal, antimalarial, antigonorrheal, febrifuge and antianemic (Lorenzi and Matos, 2002). Previous phytochemical investigations of *A. australe* have led to the isolation of germacranolides, melampolides, diterpene lactones and 6-methoxyflavonoids (Bohlmann et al., 1979, 1981a; Matsunaga et al., 1996). *Calea fruticosa* (Gardner) Urbatsch, Zlotsky and Pruski is a synonym of *Calea morii* H. Rob (Urbatsch et al., 1986). The plant is not used in folk medicine, although other species of the same genus are used for stomach disease (Martinez et al., 1987; Steinbeck et al., 1997; Kato et al., 1994). Chemical studies carried out on *Calea* species have revealed the occurrence of a variety of compounds including sesquiterpene lactones (Ober et al., 1985), *p*-hydroxyacetophenone derivatives (Bohlmann et al., 1981b), thymol derivatives (Metwally and King, 1985), benzofurans (Bohlmann et al., 1982), chromenes (Steinbeck et al., 1997) and others.

Mikania glauca Mart. ex Baker is native and endemic in Brazil and its geographic distribution includes the South-eastern, particularly the State of Minas Gerais (Ritter et al., 2012). The plant is not used in folk medicine, although other species of the same genus known as “guaco” are used to treat fever, rheumatism, flu, asthmatic bronchitis, cough and hoarseness (Oliveira et al., 1984; Vilegas et al., 1997; Soares e Silva et al., 2012). Studies of the chemical composition of species of genus *Mikania* demonstrated the presence of sesquiterpene lactones and diterpenes, mainly of the *ent*-kaurene type (Herz, 1998).

In the present study, the chemical composition of the essential oils of three Asteraceae species from Brazil was investigated. The study included hydrodistillation of the leaves and gas chromatographic/mass spectrometric analysis of the essential oils from leaves of *A. australe*, *C. fruticosa* and *M. glauca*, and also an evaluation of the essential oils against a panel of microorganisms strains. There are no reports in the literature concerning the chemical composition of essential oil from *C. fruticosa*. To our knowledge, no study has shown the antimicrobial activity of the essential oils of *A. australe*, *C. fruticosa* and *M. glauca*.

MATERIALS AND METHODS

Plant

Samples of leaves from three Brazilian Asteraceae species were collected in the city of Ouro Preto, State of Minas Gerais (March to May, 2012). Voucher specimens have been deposited at the Herbarium José Badini, Universidade Federal de Ouro Preto-UFOP, voucher No. OUPR 25895 for *A. australe*, OUPR 26290 for *C. fruticosa* and OUPR 26457 for *M. glauca*.

Extraction of the essential oils

Fresh leaves were steam distilled using a modified clevenger apparatus for 4 h and the essential oils obtained were stored in sealed amber ampules at 4°C until chromatographic analysis could be performed. Oil yields were determined (w/w) based on the fresh plant material.

Gas chromatography-mass spectrometry (GC-MS) analysis

Analyses were performed on a Shimadzu QP-2010 gas chromatograph interfaced to a mass spectrometer (GC-MS). The following conditions were used: ZB-5MS column Phenomenex Zebron (30 m × 0.25 mm × 0.25 µm); helium (99.999%) carrier gas at a constant flow of 1.1 ml/min; 1 µl injection volume; injector split ratio of 1:40; injector temperature 240°C; electron impact mode at 70 eV; ion-source temperature 280°C. The oven temperature was programmed from 100°C (isothermal for 5 min), with an increase of 10°C/min to 250°C (isothermal for 5 min), and 10°C/min to 280°C (isothermal for 15 min).

Identification of constituents of essential oils

Individual identification of the constituents was accomplished by comparison of their GC retention indices determined with reference to a homologous series of normal C_9-C_{25} alkanes and comparison of the fragmentation patterns in the mass spectra with those from the software database (Wiley 7 lib and Nist 08 lib). The Kovats index was calculated for each constituent as previously described (Van den Dool, 1963) and the data were compared to the literature (Adams, 2009). The oil compositions are presented in Table 1.

Antimicrobial activity

The antimicrobial properties of the essential oils were examined using the broth microdilution method (96-well microtiter plates) as previously described by Salvador et al. (2002), to give a concentration between 12 and 5000 µg/ml. The minimal inhibitory concentration (MIC) was calculated as the lowest concentration showing complete inhibition of microbial growth. In these tests, chloramphenicol and ketoconazole were used as experimental positive controls for bacteria and fungi strains, respectively, while the solution of dimethyl sulphoxide (DMSO)-sterile distilled water (5:95, v/v) served as the negative control. Each sensitivity test was performed in duplicate for each microorganism evaluated and repeated 3 times. The strains of microorganisms utilized are shown in Table 2.

Table 1. Relative abundance of the constituents in the essential oils from the leaves of three species of Asteraceae from Brazil.

RI (lit.)[a]	RI [b]	Constituent	Relative peak area (%)		
			AA	CF	MG
932	939	α-Pinene	-	0.15	0.11
975	975	Sabinene	0.02	-	0.09
979	977	β-Pinene	-	-	0.38
990	988	Myrcene	0.12	-	2.55
1002	1007	α-Phellandrene	0.04	-	-
1024	1023	*p*-Cymene	-	0.12	-
1029	1028	Limonene	0.51	1.18	3.93
1037	1033	(*Z*)-β-Ocimene	-	-	0.15
1039	1033	Lavender lactone	-	0.50	-
1050	1044	(*E*)-β-Ocimene	0.18	-	1.01
1052	1048	*cis*-Arbusculone	-	0.16	-
1070	1067	*trans*-Arbusculone	-	0.23	-
1072	1069	*cis*-Linalool oxide	-	0.20	-
1086	1085	*trans*-Linalool oxide	-	0.15	-
1088	1085	Terpinolene	0.30	-	0.09
1096	1100	Linalool	0.05	1.28	0.06
1177	1180	Terpinen-4-ol	0.06	0.18	0.71
1182	1187	*p*-Cymen-8-ol	-	0.42	-
1188	1194	α-Terpineol	-	-	0.29
1216	1217	*trans*-Carveol	-	0.17	-
1243	1242	Carvone	-	0.20	-
1295	1297	Perilla alcohol	-	-	0.07
1338	1333	δ-Elemene	0.14	0.12	0.08
1351	1345	α-Cubebene	-	-	0.29
1371	1366	Cyclosativene	-	0.53	-
1376	1373	α-Copaene	0.12	1.36	0.61
1388	1381	β-Bourbonene	-	0.24	0.11
1388	1386	β-Cubebene	0.39	0.66	1.47
1390	1387	β-Elemene	0.16	0.79	0.88
1419	1417	(*E*)-Caryophyllene	9.92	0.25	8.58
1432	1427	β-Copaene	0.15	0.57	0.16
1439	1432	α-Guaiene	-	-	0.47
1450	1446	*cis*-Muurola-3,5-diene	-	-	0.52
1454	1452	α-Humulene	3.25	0.15	1.41
1460	1457	allo-Aromadendrene	0.19	0.27	0.07
1472	1472	Dauca-5,8-diene	-	1.49	-
1476	1470	*trans*-Cadina-3(6),4-diene	-	-	0.82
1479	1473	γ-Muurolene	0.53	-	0.23
1481	1481	Germacrene D	58.65	-	25.90
1484	1483	α-Amorphene	0.01	-	-
1490	1486	β-Selinene	0.16	1.47	-
1493	1489	*trans*-Muurola-4(14),5-diene	-	-	2.16
1494	1492	epi-Cubebol	-	1.14	-
1495	1489	γ-Amorphene	0.04	-	-
1500	1494	Bicyclogermacrene	7.80	-	11.77
1500	1496	α-Muurolene	0.90	1.14	0.92
1509	1499	α-Bulnesene	-	-	0.34
1509	1504	Germacrene A	0.34	-	0.23

Table 1. Contd.

1513	1510	γ-Cadinene	0.24	0.67	-
1515	1512	Cubebol	0.17	1.02	3.19
1522	1519	*trans*-Calamenene	-	0.45	0.79
1523	1516	δ-Cadinene	1.61	-	4.27
1529	1520	Zonarene	-	-	0.92
1534	1529	*trans*-Cadina-1,4-diene	0.04	-	0.13
1538	1533	α-Cadinene	0.18	-	-
1548	1545	Hedycaryol	-	-	0.91
1561	1555	Germacrene B	0.32	-	0.17
1563	1560	(*E*)-Nerolidol	3.40	1.54	-
1578	1574	Spathulenol	**-**	2.38	3.53
1583	1578	Caryophyllene oxide	0.27	15.01	1.41
1587	1584	Gleenol	**-**	0.31	-
1590	1587	Globulol	0.29	0.32	1.15
1592	1590	Viridiflorol	0.25	-	-
1595	1593	Cubeban-11-ol	0.03	-	-
1600	1593	Guaiol	-	-	0.21
1600	1603	Rosiflorol	0.09	-	-
1602	1599	Ledol	-	-	0.17
1608	1606	Humulene epoxide II	0.05	3.36	-
1619	1612	1,10-di-epi-Cubenol	0.04	-	-
1619	1618	Junenol	0.44	1.14	-
1628	1624	1-epi-Cubenol	0.26	3.71	1.48
1640	1638	Epi-α-Cadinol	0.61	-	-
1642	1641	Epi-α-Muurolol	1.18	-	2.27
1646	1643	α-Muurolol	0.43	1.38	0.56
1650	1651	β-Eudesmol	-	**-**	2.83
1653	1654	Pogostol	-	**-**	1.62
1654	1652	α-Cadinol	2.45	7.88	**-**
1659	1655	Selin-11-en-4-α-ol	-	7.32	-
1661	1660	*cis*-Calamenen-10-ol	-	0.88	0.08
1677	1669	Mustakona	-	-	0.32
1686	1681	Germacra-4(15),5,10(14)-trien-1-α-ol	0.13	-	0.54
1689	1689	Shyobunol	-	-	0.17
1736	1739	Eremophilone	-	0.44	-
1741	1731	Mint sulfide	-	-	0.33
2010	2007	13-epi-Manool oxide	-		0.09
		Monoterpenes	-	-	-
		Hydrocarbons	1.17	1.45	8.31
		Oxygenated	0.11	2.60	1.13
		Monoterpenes total	1.28	4.05	9.44
		Sesquiterpenes	-	-	-
		Hydrocarbons	85.14	10.16	63.30
		Oxygenated	10.09	47.83	20.44
		Sesquiterpenes total	95.23	57.99	83.74
		Others	-	0.89	0.42
		Total identified	96.51	62.93	93.60

[a]RI = Retention Indices. See Adams, 2009. [b]RI = Retention Indices on ZB-5MS column (relative to *n*-alkanes). AA = *Acanthospermum australe* (Loefl.) Kuntz; CF = *Calea fruticosa* (Gardner) Urbatsch, Zlotsky & Pruski; MG = *Mikania glauca* Mart. ex Baker. In bold: most representative components (>5%).

Table 2. Antimicrobial activity of the essential oils from the leaves of three species of Asteraceae from Brazil.

Microorganism	MIC[a] (µg/ml)			
	AA	CF	MG	Controls[b]
Proteus vulgaris (Pv)[c]	-	100	1000	50
Escherichia coli (ATCC 10538)[d]	-	-	-	50
Staphylococcus epidermidis (ATCC 12228)[d]	-	-	1000	50
Staphylococcus aureus (ATCC 14458)[d]	-	-	-	25
Candida glabrata (ATCC 30070)[c]	100	-	500	12
Candida tropicalis (CT)[c]	-	-	-	12
Candida albicans (ATCC 10231)[d]	-	-	-	12
Candida dubliniensis (ATCC 778157)[d]	50	-	-	12

[a]MIC: minimum inhibitory concentration in µg/mL; [b]Positive controls: chloramphenicol for bacterial strains and ketoconazole for yeast strains; [c]standard strain; [d]field strain; -: no inhibition of microbial development. AA = *Acanthospermum australe* (Loefl.) Kuntz; CF = *Calea fruticosa* (Gardner) Urbatsch, Zlotsky & Pruski; MG = *Mikania glauca* Mart. ex Baker.

RESULTS AND DISCUSSION

Extraction of the essential oils

Of all the plants analyzed in relation to their essential oil extraction, *M. glauca* presented the highest yield (0.227%). The yield was lower than presented by Guimarães et al. (2012), 0.65% for the same species. The yield of the essential oil of *A. australe* (0.030%) was also lower than that presented in the literature (0.13% of fresh mass) by Morais et al. (1997). *C. fruticosa* presented a yield of 0.041%.

Chemical composition

The compositions of the essential oils obtained by hydrodistillation from the leaves of *A. australe*, *C. fruticosa* and *M. glauca* analyzed by GC-MS are listed in Table 1. The percentage and retention indices of components are given. For the three species studied, 62.9 to 96.5% of all chemical constituents were identified. Sixteen compounds were common to these three species: limonene, terpinen-4-ol, δ-elemene, α-copaene, β-cubebene, β-elemene, (*E*)-caryophyllene, β-copaene, α-humulene, allo-aromadendrene, α-muurolene, cubebol, caryophyllene oxide, globulol, 1-epi-cubenol and α-muurolol.

Forty five compounds were identified in the essential oil of *A. australe*, accounting for 96.5% of the total oil. The oil from fresh leaves of *A. australe* was characterized by a high amount of sesquiterpenes hydrocarbons (85.1%), followed by oxygenated sesquiterpenes (10.1%). The major components were germacrene-D, (*E*)-caryophyllene and bicyclogermacrene which constitute, respectively 58.7, 9.9 and 7.8% of the total oil composition. In another investigation on chemical composition of essential oil from the leaves of *A. australe* collected in the Southern part of the Amazon Forest in Brazil, *E*-caryophyllene (16.0%), β-elemene (14.4%), γ-cadinene (13.0%), germacrene A (10.1%) and δ-cadinene (5.5%) were the major components in this oil, however it did not contain germacrene D (Morais et al., 1997). This difference probably occurred as a result of various factors that can affect the composition of the essential oils, such as genetic factors, growing location, the regional climate and the time of day at which it is collected (Burt, 2004).

The *C. fruticosa* essential oil was characterized by 43 constituents, representing 62.9% of the total oil composition. The essential oil is dominated by the presence of oxygenated sesquiterpenes constituting 47.8%, followed by sesquiterpenes hydrocarbons (10.2%) and the most abundant components were caryophyllene oxide (15.0%), α-cadinol (7.9%) and sellin-11-en-4-α-ol (7.3%). Other chemical constituents in lower quantities in the oil were ketones (0.4%) and lactone (0.5%). Among the species of the genus, only *C. pinnatifida*, *C. clematidea* and *C. serrata* were previously studied for the essential oil content. The major compounds found in the essential oil from aerial parts of *C. pinnatifida* were (*E*)-caryophyllene (15.2%), α-cadinene (8.2%) and α-coprene (4.9%) (Kato et al., 1994). The essential oil of the leaves from *C. clematidea* showed a high content of a natural epoxy terpenoid named clemateol (70.5%), with minor amounts of others compounds (Flach et al., 2002). Ribeiro et al. (2011) characterized the essential oil of *C. serrata* and encountered higher concentrations of precocene II (29.6%) and germacrene D (26.4%). The essential oils of these four species, including *C. fruticosa* are quite different, having in common only the sesquiterpene (*E*)-caryophyllene, indicating a considerable chemodiversity in the essential oils of *Calea* species. There are no data about the chemical volatile composition of specie *C. fruticosa* in the literature, which made difficult the discussion of the results.

The total number of chemical constituents identified in essential oils was 55 for *M. glauca*, representing 93.6%

of the total oil content. In essential oil, sesquiterpenes hydrocarbons were the main class of constituents (63.3%), followed by oxygenated sesquiterpenes (20.4%), and the most abundant components were germacrene D (25.9%), bicyclogermacrene (11.8%) and (*E*)-caryophyllene (8.6%). Previous report on essential oil composition of *M. glauca* is not completely in agreement with the present study. Guimarães et al. (2012) demonstrated that monoterpene hydrocarbons were the most abundant components in the essential oil of fresh leaves of *M. glauca*. The principal constituents were α-pinene, β-pinene, myrcene, (*E*)-caryophyllene and bicyclogermacrene. The predominance of terpene hydrocarbons has been reported for the essential oils of several species of *Mikania*. Germacrene-D and β-caryophyllene sesquiterpenes are commonly encountered as the principal constituents of the essential oils from several species of the genus.

Antimicrobial activity

The oils were tested against two Gram-positive and two Gram-negative bacterial strains and four yeast strains (Table 2). The results show that essential oil of *M. glauca* inhibited both Gram-negative (*Proteus vulgaris*, field strain) and Gram-positive (*Staphylococcus epidermidis* ATCC 12228, standard strain) bacteria and inhibited one yeast *Candida glabrata*. The antibacterial activity could have resulted from the presence of caryophyllene oxide, α-pinene, α-terpineol and linalool compounds that are known to possess antibacterial activity. Although present in low concentrations, these compounds could have imparted a significant effect on the antibacterial activity of the oil (Sivasothy et al., 2011; Magiatis et al., 1999).

The *C. fruticosa* essential oil exhibited antimicrobial activity only against Gram-negative bacteria *P. vulgaris* and no antimicrobial activity against yeast strains, suggesting its selectivity. The essential oil *C. fruticosa* showed a minimum inhibitory concentration (MIC) value greater than the essential oil of *M. glauca* to the same bacteria. This profile could be attributed to the high concentration in the oil of caryophyllene oxide (15.0%) with known antimicrobial activity (Vagionas et al., 2007). The antibacterial activity of the oil is suspected also to be associated with α-pinene and linalool, via a synergistic effect (Sivasothy et al., 2011). The essential oil of another plant of the same genus, *C. clematidea*, showed a moderate antifungal activity against multiple dermatophytes (Flach et al., 2002).

The results showed that the essential oil from *A. australe* was the only one that inhibited two of the four yeasts evaluated with MIC between 50 and 100 µg/ml. The most susceptible yeast was *C. glabrata* whose growth was inhibited by the essential oils of *A. australe* and *M. glauca* at concentrations of 100 and 500 µg/ml, respectively. The antimicrobial activities of the essential oils of *A. australe*, *C. fruticosa* and *M. glauca* confirm that the Asteraceae species are source of biologically active compounds. Further investigations are necessary to confirm the potential of these essential oils as bioactive agents useful for *in vivo* applications.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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