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Full Length Research Paper

The role of nitric oxide in alendronate-mediated acceleration of gastric emptying and gastrointestinal transit in rats

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The effect of the alendronate (ALD; 30 mg/kg, pH 7.0, p.o.) and sodium nitroprusside (SNP; 10 mg/kg, p.o.; a nitric oxide (NO) donor) on gastric dye retention (GDR) and proximal, medial and distal small intestine dye retentions (IDR) was investigated in rats. The drugs were administered once daily for 4 days. On the last day of treatment, 4 h after ALD administration, GDR and IDR were measured. ALD treatment decreased GDR at postprandial intervals of 20 (28.5%) and 30 min (38.3%), while it increased medial IDR (117.2%), as compared to the saline group. ALD had no effect on dye retention in proximal and distal portions of the small intestine. In 30 min, ALD increased medial (50.5%) and distal IDR (149.7%), as compared to the saline group. Pretreatment with SNP prevented ALD from decreasing gastric retention and intestinal transit. The results of this study indicated that ALD accelerates gastric emptying of liquids in rats and support the hypothesis that the inhibition of nitric oxide is of primary importance.

Key words: Gastric emptying, gaseous mediators, nitric oxide, alendronate.

INTRODUCTION

Osteoporosis is a disease that is particularly common in elderly patients and is characterized by increased bone brittleness and fracture risk caused by low bone mineral density (BMD) and degeneration of the bone microarchitecture (Silva, 2003; Brandão et al., 2008). Various drugs are available for the treatment and prevention of osteoporosis. The beneficial effects of bisphosphonates such as alendronate (ALD) on calcium metabolism are well established, and today, they are the major class of drugs used to treat bone diseases associated with excessive resorption. However, these drugs cause serious adverse effects including bleeding, inflammation, and abdominal pain and can through an unknown mechanism, cause additional side effects in the

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upper gastrointestinal tract, including gastroesophageal inflammation and ulceration (Marshall et al., 2000; Graham, 2002).

Nitric oxide (NO) is a major inhibitory neurotransmitter in the gastrointestinal tract, and appears to mediate enteric smooth muscle relaxation (Takahashi, 2003). In healthy subjects, an increase in NO availability has been reported to slow gastric emptying. Exogenous nitric oxide inhibits gastric emptying and antral motor activity (Plourde et al., 1994; Konturek et al., 1995), and the nitric oxide synthase inhibitor, L-N^G-nitroarginine methyl ester (L-NAME), attenuates the delay in gastric emptying (Kuo et al., 2009). Recently, we demonstrated that ALD-induced gastric ulcerogenic responses were mediated by a decrease in NO derived from both endothelial nitric oxide synthase (eNOS) and inducible nitric oxide synthase (iNOS) (Silva et al., 2014).

However, there are few reports concerning the effects of ALD on gastrointestinal motility *in vivo*, and there have been no investigations into its effect on nitrergic mechanisms. As a result, we investigated the effect of ALD on the gastric emptying of liquid meals in rats and evaluated the involvement of NO with this phenomenon.

MATERIALS AND METHODS

Animals

Female Wistar rats (100 to 150 g) were obtained from the Department of Physiology and Pharmacology, Federal University of Ceará and housed in cages with a controlled temperature (25 ± 2°C) and a 12-h light/12-h dark cycle. The animals were deprived of food for 18 to 24 h before the experiment, but had free access to water. All animal treatments and surgical procedures were performed in accordance with the Guide for Care and Use of Laboratory Animals (National Institutes of Health, Bethesda, MD, USA) and were approved by the local Ethics Committee (Protocol No. 0067/10).

Drugs

Alendronate (ALD) and sodium nitroprusside (SNP) were purchased from Sigma Aldrich (St. Louis, MO, USA). ALD was dissolved in saline and adjusted to pH 7.0 using NaOH or HCl (Kanatsu et al., 2004). All other drugs were dissolved in saline (0.9% NaCl).

Experimental protocol

The animals were divided randomly in four groups (n = 6 to 7 animals): Group I, Saline; Group II, SNP (10 mg/kg, p.o.); Group III, ALD (30 mg/kg, pH 7.0, p.o.); and Group IV, SNP (10 mg/kg, p.o.) + ALD (30 mg/kg). The drugs were administered once daily for 4 days, and doses were chosen based on the results from previous work conducted by our research group (Silva et al., 2014). On the last day of treatment, 4 h after saline or ALD administration (Groups I to IV), gastric emptying and intestinal transit were measured as described subsequently.

Evaluation of gastric emptying and intestinal transit

Gastric emptying and intestinal transit were performed according to a method described previously (Reynell and Spray, 1956), with modifications. Initially, the animals received phenol red (1.5 ml; 0.75 mg/ml in 5% glucose). After 10, 20, or 30 min, animals were euthanized, a laparotomy was performed, and the gastro-esophageal, gastroduodenal, and ileocaecal junctions were immediately isolated by ligatures, removed and divided into consecutive segments: stomach, proximal (40%), middle (30%), and distal (30%) small intestine. Then, each segment was placed in a graduated cylinder that contained 0.1 N NaOH (100 ml) solution and total volume was measured. After 20 min, the supernatant (10 ml) was collected and centrifuged at 2800 rpm for 10 min. Subsequently, 20% trichloroacetic acid (TCA; 0.5 ml) was added to the homogenate (5 ml) for protein precipitation and centrifuged at 2800 for 20 min. Finally, samples of supernatant (150 µl) were added to 200 µl of NaOH (0.5 N in distillated water) and the absorbance was measured on a plate reader at 560 nm. The results are expressed as fractional dye retention (%), according to the following equation:

$$\text{Gastric dye retention} = \frac{\text{Phenol red recovered in stomach}}{\text{Phenol red recovered from stomach and intestine}} \times 100$$

Intestinal transit was calculated for gut bowel segments by dividing the amount of phenol red recovered from a given segment by the amount of phenol red recovered from all three segments, and it is expressed as fractional dye retention (%) (Peixoto-Junior et al., 2009).

Role of NO in ALD-enhanced gastric emptying of liquid

To study the role of NO in the effect of ALD on gastric emptying, rats were pretreated with saline or SNP (10 mg/kg, p.o.; a nitric oxide donor). After 1 h, animals received ALD (30 mg/kg, p.o.). The drugs were administered once daily for 4 days. On the last day of treatment, 4 h after saline or ALD, the rats received phenol red (1.5 ml; 0.75 mg/ml in 5% glucose). After 20 min, animals were sacrificed and gastric retention was measured as previously described.

Statistical analysis

All values are expressed as means ± standard error of mean (S.E.M). The analysis of variance (ANOVA) and Student–Newman–Keuls test were used to determine the statistical significance of differences between groups. Differences were considered as significant at $p < 0.05$.

RESULTS

Evaluation of gastric emptying at postprandial intervals

Figure 1 shows that ALD significantly ($p < 0.05$) decreased gastric retention at postprandial intervals of 20

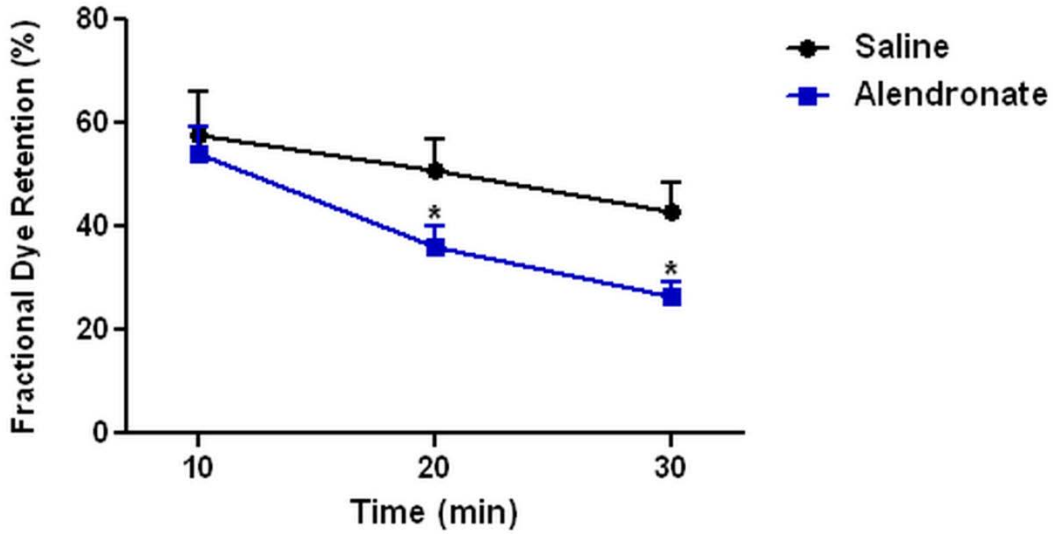

Figure 1. Alendronate treatment accelerates gastric emptying in rats. Rats received saline (control group) or alendronate (30 mg/kg, p.o.) once daily for 4 days. On the last day of treatment, 4 h after administration, the animals received phenol red (1.5 ml; 0.75 mg/ml) and were sacrificed 10, 20 or 30 min later. Values are expressed as mean ± SEM (n= 6 to 7 rats per group). *p < 0.05 versus saline group.

min (36.24 ± 3.76 versus 50.69 ± 6.24 %; 28.5% of reduction) and 30 min (26.47 ± 2.73 versus 48.88 ± 5.57%; 38.3% of reduction), as compared to the saline group.

Effect of alendronate on gastric emptying and intestinal transit

Figure 2 shows the gastric dye retention (GDR) and proximal, medial, and distal small intestine dye retentions (IDR) in saline- or ALD-treated animals 10, 20, and 30 min after gavage. In Figure 2A, the fractional dye retention 10 min after receiving the test meal in ALD-treated animals, did not change in stomach and any portion of the small intestine. However, ALD treatment significantly ($p < 0.05$) decreased GDR (36.24 ± 3.76 versus 50.69 ± 6.24%; 28.5% of reduction) while it significantly ($p < 0.05$) increased medial IDR (18.37 ± 4.16 versus 39.91 ± 3.65% fractional dye retention; 117.2% of increase) 20 min after receiving the test meal (Figure 2B). ALD had no effect on dye retention in proximal and distal portions of the small intestine in this time.

Figure 2C shows the fractional dye retention in saline- or ALD-treated animals 30 min after receiving the test meal. Compared to the saline group, ALD treatment significantly ($p < 0.05$) decreased GDR (48.88 ± 5.57 versus 26.47 ± 2.73%; 38.3% of reduction) while increased medial (29.21 ± 3.62 versus 43.80 ± 4.52%; 50.5% of increase) and distal IDR (5.62 ± 0.78 versus 14.06 ± 2.83%; 149.7% of increase). ALD had no effect on dye retention in the proximal portion of the small intestine.

Role of NO in the effect of alendronate on gastric emptying and intestinal transit

Figure 3 show that SNP (a nitric oxide donor) pretreatment significantly ($p < 0.05$) prevented decreasing GDR (52.65 ± 2.96 versus 36.24 ± 3.76% fractional dye retention) medial IDR (21.80 ± 3.19 versus 39.91 3.64% fractional dye retention) altered by ALD administration.

As shown in Figure 4, it was observed that the marker progressed through the intestine; the center of mass advanced gradually over 20 min after the meal, that intestinal transit was significantly ($p < 0.05$) improved in the ALD group, as compared to the saline group (median geometric center: 2.09 ± 0.04 versus 1.67 ± 0.15), but was normalized by SNP pretreatment (1.78 ± 0.06). In addition, the treatment only with SNP did not alter this parameter.

DISCUSSION

ALD is an aminobisphosphonate, potent inhibitor of osteoclast-mediated bone resorption, which is marketed for the treatment of osteoporosis and Paget's disease of bone (Liberman et al., 1995). Unfortunately, these drugs are also capable of causing injury to the upper gastrointestinal tract, causing gastric injury and delays ulcer healing in rodents (Elliott et al., 1998). In patients, gastrointestinal adverse events reported during ALD

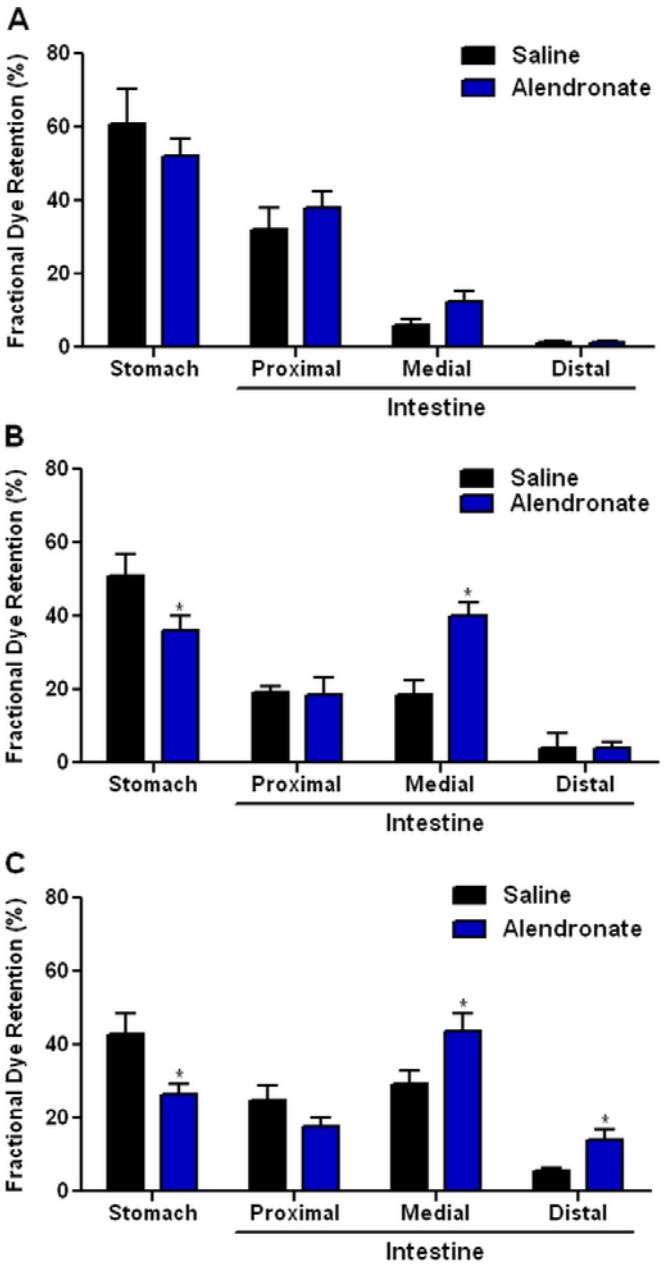

Figure 2. Alendronate treatment accelerates gastric emptying and gastrointestinal transit in rats. Rats received saline (control group) or alendronate (30 mg/kg, p.o.) once daily for 4 days. On the last day of treatment, 4 h after administration, the animals received phenol red (1.5 ml; 0.75 mg/ml) and were sacrificed 20 min later. Values are expressed as mean ± SEM (n= 6–7 rats per group). *p < 0.05 versus saline

therapy also include dyspepsia, dysphagia, and oesophageal ulcers (Thomson et al., 2002). Another side effect seen in patients with the use of alendronate is complications of gastrointestinal transit. However, there are few studies linking alendronate with these effects and the mechanism involved. Thus, in this work, we evaluated the effect of ALD on the gastric emptying (GE) and gastrointestinal transit (GI) of liquid in rats. We also investigated the role of NO in this process.

Analysis of fractional dye retention has been widely used to assess gastric emptying (Medeiros et al., 2012; Gondim et al., 2001; Sharma, 1983). It allowed us to evaluate gastric and intestinal motility in rodents, thus eliminating the need for anesthesia and its associated effects on the cardiovascular and autonomic functions; as a result, our model closely mimicked the clinical scenario. Our results showed that ALD accelerates the GE and GI of liquids in rats. Therefore we can infer that ALD has prokinetic effects on gastric motility.

There are few reports concerning the effects of ALD on gastrointestinal motility *in vivo*. We are one of the first groups to report that ALD can accelerate the GE and GI of liquids in rats. This effect could be due to a decrease in nitric oxide synthesis, since we demonstrated that the nitrergic donor SNP inhibits the effect of ALD on gastrointestinal motility. It, is evident from the literature that NO and nitrergic donors inhibit gastrointestinal motility, both in animals and humans (Sun et al., 1998; Allescher et al., 1992).

Additionally, it was demonstrated that ALD reduced NO generation by modulating NOS expression (Silva et al., 2014). The three enzymatic sources of NO-nNOS, eNOS, and iNOS, have been shown to exist in the gastrointestinal tract. Several studies have demonstrated that NO plays a dual role in the mucosal protection and GI motility depending on the NOS isoform involved; the protective effect of NO is derived from nNOS and eNOS, and the inhibition of these enzymes can accelerate GI motility and induce disturbances in blood flow, secretion, and gastric ulcers. In contrast, iNOS, which generates large amounts of NO under certain pathological conditions, is thought to contribute to mucosal injury and dysfunction (Moncada et al., 1991). Since the administration of alendronate diminishes NO levels in the gastric mucosa (Silva et al., 2014), it is believed that this effect contributes to the prokinetic effects of ALD, and increase in NO levels by SPN abolished the ALD effects in GE and GI.

Conclusion

Conclusively, our results indicate that ALD accelerates gastric emptying of liquids in rats. Although there are many mechanisms by which this effect could occur; our data support the hypothesis that the inhibition of nitric oxide is of primary importance.

Conflict of interests

The authors declare that there is no conflict of interests.

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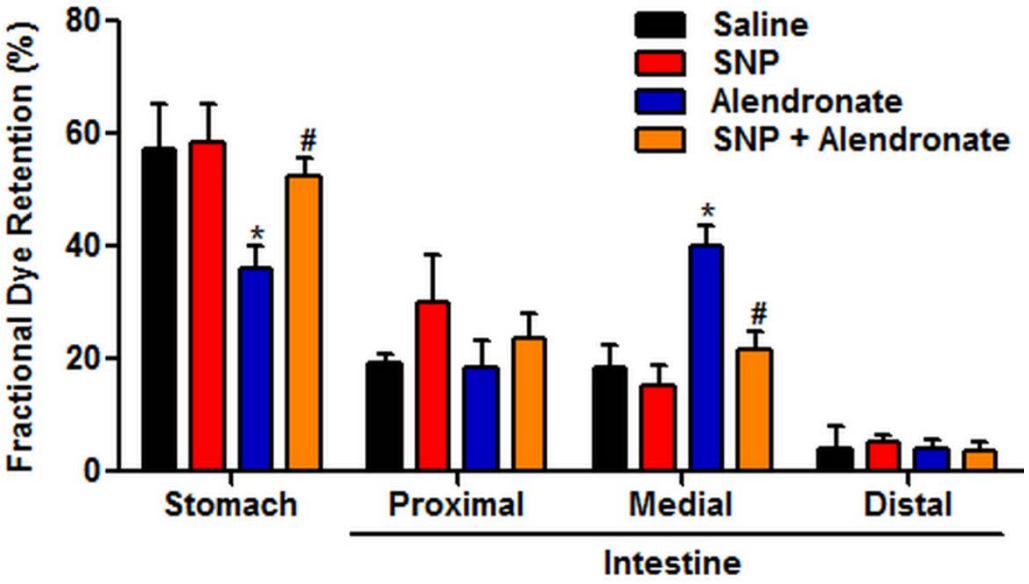

Figure 3. Sodium nitroprusside treatment reverses the effects of alendronate on gastric emptying and gastrointestinal transit. Rats were treated with either saline (control group) or SNP (10 mg/kg, p.o.; a nitric oxide donor) 30 min before alendronate (30 mg/kg, p.o.) administration. All drugs were administered once daily for 4 days. On the last day of treatment, 4 h after saline or alendronate administration, the animals received phenol red (1.5 ml; 0.75 mg/ml) and were sacrificed 20 min later. Values are expressed as mean ± SEM (n= 6–7 rats per group). *p < 0.05 versus saline group; #p < 0.05 versus alendronate group.

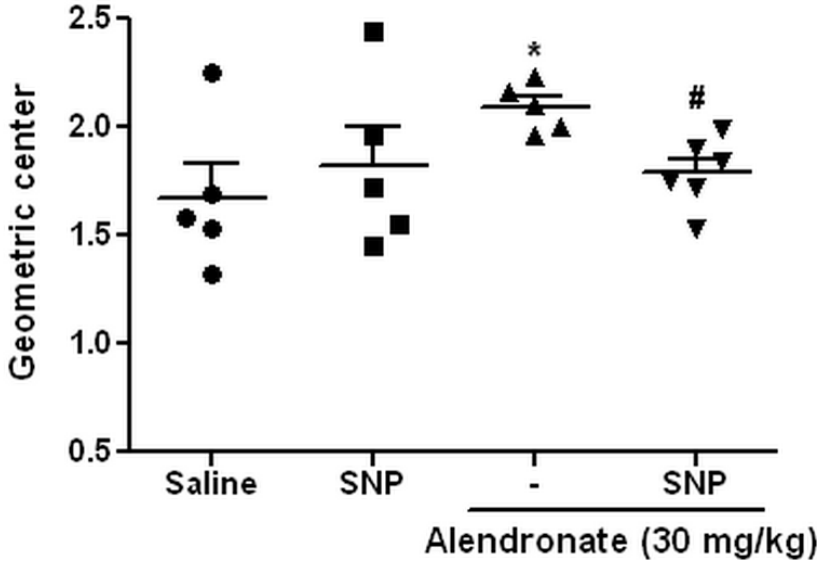

Figure 4. Sodium nitroprusside treatment prevents the detachment of the geometric center of the distribution the test meal induced by alendronate. Rats received saline (control group) or SNP (10 mg/kg, p.o.; a nitric oxide donor) 30 min before alendronate (30 mg/kg, p.o.) administration. All drugs were administered once daily for 4 days. On the last day of treatment, 4 h after administration, the animals received phenol red (1.5 ml; 0.75 mg/ml) and were sacrificed 20 min later. Values are expressed as mean ± SEM (n= 5–6 rats per group). *p < 0.05 versus saline group; #p < 0.05 versus alendronate group.

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Full Length Research Paper

An analysis of the quality of studies that evaluate potentially inappropriate drug therapy

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In the elderly population, increased predisposition to chronic diseases and consequent use of various medications increases the chances of using a potentially inappropriate drug therapy. The purpose of this review was to analyze research that uses tools to study potentially inappropriate drug therapy through the strengthening the reporting of observational studies in epidemiology (STROBE) initiative. A systematic review was undertaken between February and March, 2013. The studies were selected from different combinations of the Medicine's controlled vocabulary thesaurus (MESH) terms - "aged," "elderly," "inappropriate prescribing," and "drug utilization" in English, Spanish and Portuguese, in the Literatura Latino-Americana e do Caribe em Ciências da Saúde, PubMed, Scopus, and Web of Science databases. The papers that satisfied the inclusion criteria for data extraction were examined regarding the following variables: country, sample size, duration, type of study, practice scenario, study limitations and fulfillment of the items proposed by the STROBE initiative. At the end of the selection process, 119 articles met the specific criteria. The US had the highest number of publications in this area. The samples observed were heterogeneous, ranging from patient to database samples, and most studies were cross-sectional. The most frequently used study practice scenarios were hospitals or outpatient clinics. No article completely met the STROBE criteria. It was found that potentially inappropriate drug therapy is studied primarily in developed countries, which reinforces the need for further studies in developing countries. These findings should guide future research in this subject area, providing a more complete approach on aspects related to the use of medications by this specific population.

Key words: Potentially inappropriate drug therapy, inappropriate prescribing, elderly, study quality assessment.

INTRODUCTION

The aging process produces physiological and pathological alterations that increase the predisposition to chronic diseases and consequent use of various medications. This increased consumption of medication raises the odds of the elderly population using five or more drugs (polypharmacy), which increases the

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occurrence of problems related to the use of medication (Ribeiro et al., 2005; Soares et al., 2011). For this reason, pharmacotherapy for the elderly is challenging, especially if potentially inappropriate drug therapy (PIDT) is prescribed, as this increases health risks (Gallagher et al., 2007).

Medication is potentially inappropriate when its risks outweigh its benefits (Beers et al., 1991; Gallagher et al., 2008). Notably, elderly patients consume three times more medications than young adults in industrialized countries. According to Brekke et al. (2008), 10 to 20% of hospital admissions among elderly people are due to PIDT use. This is because elderly persons using PIDT are 1.8 to 1.9 times more likely to be hospitalized (Albert et al., 2010).

Additionally, there is worldwide discussion about whether the standards used in the prescription of pharmacotherapy in older people are inappropriate (Iyer et al., 2008). For example, a study conducted in the south of Ireland with 1.329 patients over 65 years of age, with an average of five drugs per patient, identified 632 prescriptions containing PIDT (Albert et al., 2010). Laroche et al. (2007) showed that the incidence of damage caused by medication was 20.4% among patients with PIDT, compared to 16.4% for patients who use only medications appropriate for the elderly.

Concerns regarding the harmful effects of the use of medication by the elderly led health professionals, such as pharmacists and physicians, to develop and implement various methods and tools to identify PIDT prescription patterns (Ribeiro et al., 2005). Therefore, the adequacy of these techniques should be evaluated by explicit and implicit methods, and the tools validated to reduce PIDT prescription (Iyer et al., 2008; Forsetlund et al., 2011).

Some revisions debate these instruments, but there are few published systematic reviews assessing the quality of studies that use tools to evaluate PIDT in various practice scenarios (Guaraldo et al., 2011; Dimitrow et al., 2011). The purpose of this review was to analyze research that uses tools to assess PIDT through the strengthening the reporting of observational studies in epidemiology (STROBE) initiative.

METHODOLOGY

A review of the scientific literature was performed to identify studies involving inappropriate prescriptions for elderly patients. The Literatura Latino-Americana e do Caribe em Ciências da Saúde (LILACS), PubMed, Scopus and Web of Science databases were reviewed (up to January, 2013). The search strategy included the following keyword terms in various combinations: in English, “aged,” “elderly,” “inappropriate prescribing,” and “drug utilization”; in Spanish, “anciano,” “utilización de medicamentos,” and “prescripción inadecuada”; and in Portuguese, “idoso,” “medicamento inapropriado,” “medicamento inadequado,” and “uso de medicamento”. The research strategies were adapted according to the protocols of each database. The keywords were defined using the National Library of Medicine's controlled vocabulary thesaurus (MeSH). It consists of sets of descriptors, arranged in a hierarchical structure that permits searching at various levels of specificity. In addition to the MeSH terms, other non-standard terms were used to expand the search strategy. The study design followed the guidelines of the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA).

The subsequent screening process was performed in three stages (title, abstract, and full text screening) by two researchers (APALS and DTS); when there was disagreement, a third researcher (DPLJ) analyzed and judged the discrepancy. The measure of agreement between the two reviewers - defined as Cohen’s kappa (κ) was calculated with a confidence interval of 95%. The titles and abstracts were compared using the following predefined inclusion criteria to determine the relevance of the theme: (i) the study involved the use of potentially inappropriate medication for elderly patients, and (ii) the study used a validated tool to make such an assessment.

A researcher (APALS) performed an initial selection, which excluded the titles that did not meet the inclusion criteria. The studies excluded were as follows: (i) reviews and editorials; (ii) studies not written in English, Portuguese, or Spanish; (iii) studies that did not provide the abstract or full text (even with attempts to get them by direct email to the authors); (iv) studies that evaluated only one or two classes of drugs; and (ν) studies evaluating PIDT in only one or two diseases.

The papers that satisfied the inclusion criteria for data extraction were carefully examined regarding the following variables: country, sample size, duration, study type, practice scenario, language of publication, limitations, and fulfillment of the items proposed by the STROBE initiative. The final analysis was performed to assess the methodological rigor of the articles published in this research area; for that purpose, the STROBE tool was used (Malta et al., 2010). The tool’s 22 items were separated into 34 items to perform a more complete and accurate description of observational studies. In this review, each item fulfilled by the article was awarded one point; thus, the score could vary from 0 (0%) to 34 (100%) points.

RESULTS

From the various combinations of keywords, 8,610 articles were found. The first evaluation was performed by one of the evaluators (APALS) who excluded 7,372 articles that did not meet at least one of the inclusion criteria. Of the remaining 1,238 articles, 478 were repeated in the databases. Thus, 760 titles were considered potentially relevant. Of these, 365 were excluded for not meeting the inclusion criteria, leaving 395 items to be evaluated according to the abstracts. In this study, 44 abstracts were not available; therefore, 351 abstracts were read and evaluated. From this evaluation, a further 144 articles were excluded for not meeting the inclusion criteria, leaving 207 articles to be read. At first, 76 articles had no free access, and 50 articles were later retrieved by the bibliographic commutation program of the Brazilian Institute of Science and Technology (IBICT-Comut). Of the articles assessed manually, 62 did not meet the inclusion criteria. At the end of the selection process, 119 articles met the specific inclusion criteria.

Figure 1 shows the progressive selection, number of articles, and reasons for exclusion at each step. The degree of agreement among the researchers was

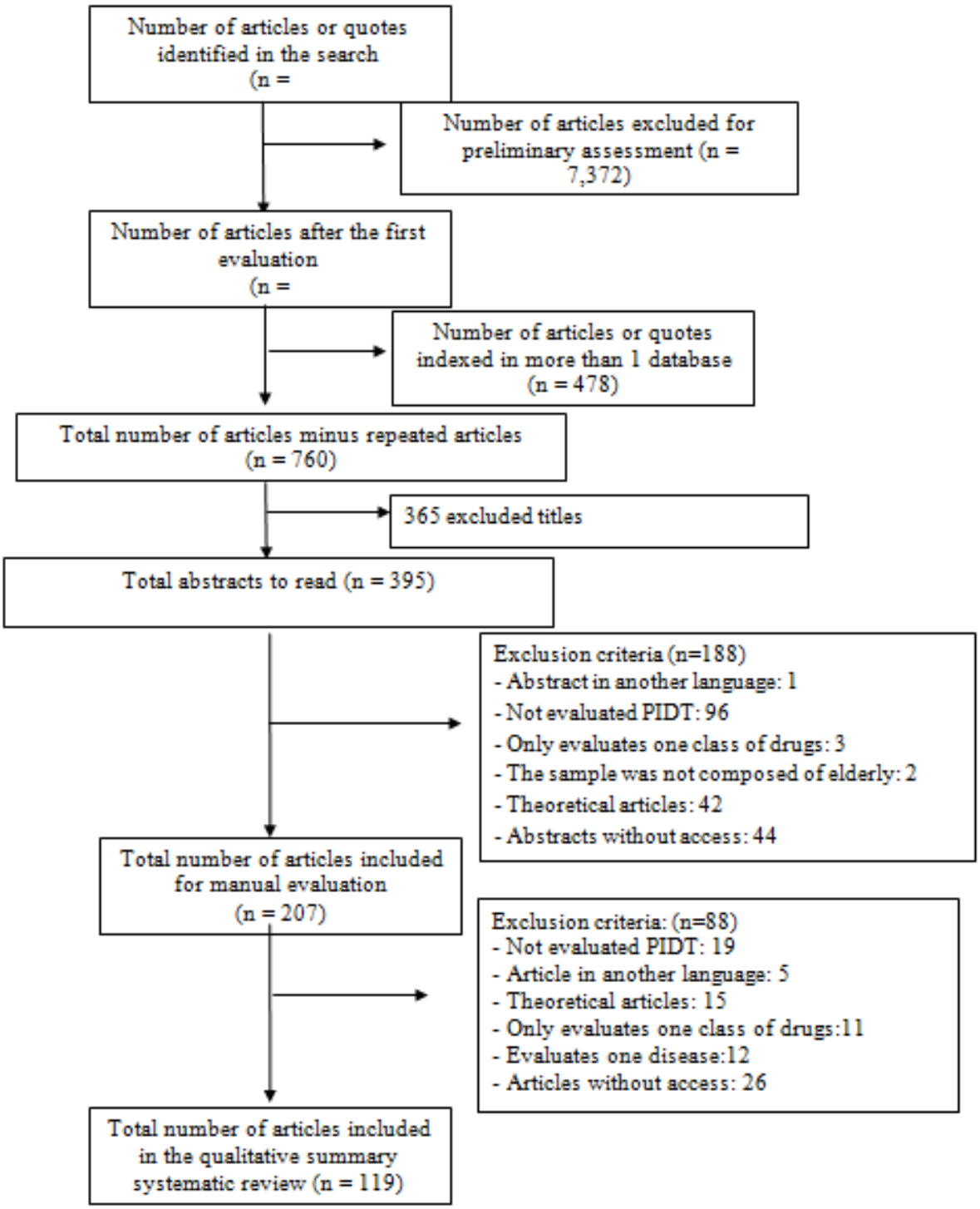

Figure 1. Study selection process.

moderate for the titles (κ = 0.479) and substantial for abstracts (κ_2 = 0.647). At all stages, the intervention of a third evaluator was necessary to resolve disagreements between the two primary researchers (Figure 1).

Of the selected studies, 40.3% were performed in Europe, 32.7% in North America, and 4.2% did not indicate the country where the research was conducted. The samples observed were heterogeneous; some

studies evaluated individual patients, while other studies evaluated prescriptions in databases. Thus, the sample size varied from 30 patients in the study by Stuij et al. (2008) to 33,830,599 prescriptions in the study by Lai et al. (2009). When we grouped the different samples used in "number of reviews," the average number of reviews (patients or prescriptions) across studies was 1,223. Three studies did not specify the sample size. The duration of the studies varied from 1 month to 9 years. Notably, 79% of the studies did not indicate or specify a study duration.

Among the studies, 32.7% were cross-sectional, 19.3% were cohort, and 19.3% did not report which methodological design was used in the study. In addition, 22.6% did not provide a complete description of the methodological design. As for the study scenario, the most frequent were hospitals or outpatient clinics, which accounted for 38.6% of the studies. In 8.4% of the studies, retirement, social security, and health plan databases were used for data collection. Only two studies were undertaken using more than one study scenario (Crotty et al., 2004; Miquel et al., 2010). Additionally, 94.9% of the studies were written in English, and 15.9% of the articles did not mention their limitations in the text. Regarding the fulfillment of the items proposed by STROBE, 49 articles met between 60 and 100% of the 34 items recommended by the initiative (Table 1).

DISCUSSION

Most of the studies included were performed in the US. This may be because the Beers criteria (most used/cited in the literature), STOPP-START criteria, Medication Appropriateness Index (MAI), Assessing Care of Vulnerable Elders (ACOVE), drug use review (DUR), HEDIS criteria, and Zhan criteria were developed there. The prevalence of studies and criteria developed in the US confirms the country as a pioneer in the clinical arena, especially regarding the evaluation of pharmacotherapy (Silva et al., 2010). Additionally, several studies were conducted in Europe, which further indicates the progress of PIDT research in developed countries compared to developing countries. Therefore, it is necessary for developing countries to increase research in this area, focusing on the effectiveness of treatments and above all, the safety of patients.

In the reviewed studies, we found a high variation in sample size, which provided a comprehensive evaluation of the tools used in different sample groups. However, two studies did not clearly describe the size of the sample surveyed (Goulding, 2004; Van der Hooft et al., 2005). In this case, two studies indicated that the lack of information on the sample could reduce the impact of the study (Holmes et al., 2009; Malta et al., 2010). Therefore, the sample in which the hypothesis is being tested should be stated and comprehensively detailed to ensure the robustness of the study.

The largest study samples consisted of retirement and health plan databases to evaluate PIDT. Despite being a viable strategy to assess the situational diagnosis of a sample, it is necessary to question the validity of the results obtained from databases such as these because the use of secondary data can mask possible selection biases. According to Guaraldo et al. (2011), an active data search can decrease the overestimation or underestimation of drug use because it is unknown whether the patient actually used the prescribed pharmacotherapy.

There was a variation of 107 months between studies. Additionally, some of the manuscripts were unclear in differentiating between the time of data collection and the study duration. Thus, in most of the articles, the real time of execution of the study is not clear, which compromises the reader's understanding. According to von Elm et al. (2007), the author should describe the context in which the study is inserted, in addition to locations and relevant dates, including periods of recruitment, exposure, follow-up (if any), and data collection. Thus, an adequate description assists in the analysis of the results of the study so that they can be incorporated into public policies and/or large interventions, if necessary.

In this review, there were a large number of cross-sectional studies. The cross-sectional study can be used as an analytical study to evaluate hypotheses of association between exposure/characteristics and an event; they are cost-effective, easy, and fast to perform. In addition, they describe what happens to a particular group, at a particular time, and are thus important guides for decision making in the health-planning sector (Lima-Costa and Barreto, 2003). However, there are limitations when trying to identify the nature of the relationships between exposure and event in these situations. Therefore, confounding factors must be considered in this type of study, which emphasizes the need for clinical trials to evaluate the effect of PIDT in the elderly population (Hanlon et al., 2000). Approximately 42% of the studies included in the analysis either lacked methodological rigor in the description of the study design or did not mention it at all. Methodological rigor is necessary to provide sufficient detail so that the reader can understand and duplicate the methodology if they wish (Holmes et al., 2009).

Among the practice scenarios, there was a higher prevalence of studies performed with institutionalized elderly people in comparison to studies with non-institutionalized elderly. However, this prevalence exists because the criteria used for these studies have been primarily developed for evaluating the pharmacotherapy of non-institutionalized elderly patients who have different socio-demographic and clinical characteristics from institutionalized patients (Hanlon et al., 2011). Moreover, it was observed that some tools developed a priori for non-institutionalized elderly patients were used in

Table 1. Compliance of the Items Proposed by STROBE.

Item	Percentage of articles that completed the item
Indicate the study's design with a commonly used term in the title or the abstract	62.5
Provide in the abstract an informative and balanced summary of what was done and what was found	98.2
Explain the scientific background and rationale for the investigation being reported	99.1
State specific objectives, including any prespecified hypotheses	66
Present key elements of study design early in the paper	60.7
Describe the setting, locations, and relevant dates, including periods of recruitment, exposure, follow-up, and data collection	90.1
Cohort study—Give the eligibility criteria, and the sources and methods of selection of participants. Describe methods of follow-up. Case-control study—Give the eligibility criteria, and the sources and methods of case ascertainment and control selection. Give the rationale for the choice of cases and controls. Cross-sectional study—Give the eligibility criteria, and the sources and methods of selection of participants	93.7
Cohort study—For matched studies, give matching criteria and number of exposed and unexposed. Case-control study—For matched studies, give matching criteria and the number of controls per case	0
Clearly define all outcomes, exposures, predictors, potential confounders, and effect modifiers. Give diagnostic criteria, if applicable	91
For each variable of interest, give sources of data and details of methods of assessment (measurement). Describe comparability of assessment methods if there is more than one group	73.2
Describe any efforts to address potential sources of bias	6.2
Explain how the study size was arrived at	27.6
Explain how quantitative variables were handled in the analyses. If applicable, describe which groupings were chosen, and why	88.3
Describe all statistical methods, including those used to control for confounding	81.2
Describe any methods used to examine subgroups and interactions	81.2
Explain how missing data were addressed	7.1
Cohort study—If applicable, explain how loss to follow-up was addressed. Case-control study—If applicable, explain how matching of cases and controls was addressed .Cross-sectional study—If applicable, describe analytical methods taking account of sampling strategy	76.2
Describe any sensitivity analyses	26.7
Report the numbers of individuals at each stage of the study—e.g., numbers potentially eligible, examined for eligibility, confirmed eligible, included in the study, completing follow-up, and analyzed	83
Give reasons for non-participation at each stage	83
Consider use of a flow diagram	93.7
Give characteristics of study participants (e.g., demographic, clinical, social) and information on exposures and potential confounders	88.3
Indicate the number of participants with missing data for each variable of interest	7.1
Cohort study—Summarize follow-up time (e.g., average and total amount)	13.3
Cohort study—Report numbers of outcome events or summary measures over time. Case-control study—Report numbers in each exposure category, or summary measures of exposure. Cross-sectional study—Report numbers of outcome events or summary measures	96.4
Give unadjusted estimates and, if applicable, confounder-adjusted estimates and their precision (e.g., 95% confidence interval). Make clear which confounders were adjusted for and why they were included	74.1
Report category boundaries when continuous variables were categorized	76.7

Table 1. Cont'd.

If relevant, consider translating estimates of relative risk into absolute risk for a meaningful time period	14.2
Report other analyses done—e.g., analyses of subgroups and interactions, and sensitivity analyses.	86.6
Summarize key results with reference to study objectives.	100
Discuss limitations of the study, taking into account sources of potential bias or imprecision. Discuss both direction and magnitude of any potential bias	83
Give a cautious overall interpretation of results considering objectives, limitations, multiplicity of analyses, results from similar studies, and other relevant evidence	97.3
Discuss the generalizability (external validity) of the study results	43.7
Give the source of funding and the role of the funders for the present study and, if applicable, for the original study on which the present article is based	50.8

institutions. According to Bakken et al. (2012), the application of these criteria should be carefully applied, because they can be affected by differences in study population and data source. The institutionalization of patients can facilitate the collection and evaluation of data, justifying the high number of hospital-based studies. In this sense, the applicability and reliability of these tools should be evaluated carefully through the analysis of the results obtained in their respective studies to avoid reproducing the erroneous selection of criteria.

Regarding the citation of research limitations in the text, most of the studies were in agreement with Malta and colleagues who advocate that the manuscript should describe its limitations and consider potential sources of inaccuracy (Malta et al., 2010). Further, the study should discuss the magnitude and direction of potential bias, which is essential for the reader's understanding, as well as evaluations by the article reviewers (Holmes et al., 2009; Malta et al., 2010). Fewer than half of the observational articles included in the review fulfilled 60% or more of the items proposed by STROBE. Overall, the studies included in this review had no good methodological consistency. This may be related to lack of standardization of studies and the fact that discussion on the use of PIDT has been recent. The intention of the STROBE initiative is to offer a recommendation on how to report observational studies more accurately, without making recommendations or prescriptions to the design or conduct of these studies. However, adherence to the items contributes to a more accurate report of such studies, and consequently facilitates the review of these publications by editors, reviewers, and readers (Malta et al., 2010).

In general, the results of the studies included in this review indicated high levels of PIDT. Strategies to reduce unnecessary prescriptions should be implemented to promote more appropriate use of these medications among this age group. The careful use of PIDT lists can assist with the detection of these drugs and prevent problems related to their use (Gallagher et al., 2007). In addition to identification of PIDT, it is necessary to undertake practical interventions. A study that aimed to systematically review the effects of interventions to optimize prescription found that, of the 16 studies assessed, 8 reviewed the impact of educational interventions, and of those, six showed statistically significant improvements in prescription quality. A multi-faceted approach and clearer policy guidelines are required to improve prescriptions for these vulnerable patients (Loganathan et al., 2011). Moreover, strategies shown to be effective for improving prescription outcomes include educational outreach visits (academic detailing), and interventions involving a pharmacist. Pharmacist services, such as conducting medication reviews or providing advice to general practitioners, may lead to improvements in prescription outcomes (Clyne et al., 2013).

Strengths and limitations

The study's strength is that it was the first review to assess the methodological rigor of studies evaluating PIDT. Its limitations include the use of English, Portuguese, and Spanish keywords, which can omit important publications in different languages; this limitation is common to systematic review articles. Other keywords, such as "potential inappropriate drug therapy," were not used. Furthermore, database restriction and the search strategy may have excluded important studies not published in the data sources used. The exclusion criteria used in this study may have also excluded relevant studies; however, it was necessary to adopt such measures, as the review's purpose was to evaluate studies focusing on various diseases and medications. Moreover, no studies were analyzed that evaluated the omission or sub-use of medication, and studies that obtained negative results may not have been published.

Agenda for future studies

Current PIDT studies are potentially valuable

because, in general, their objective is to verify PIDT prevalence in various scenarios, as well as serve as a warning to health care professionals who work with elderly patients. However, more research is needed in this area, particularly in developing countries, as it is necessary to evaluate the morbidity and mortality related to PIDT use. To reduce the limitations of PIDT studies, an active data collection search is needed, through which the reported prevalence of PIDT will be more reliable. Moreover, studies that relate the use of PIDT with outcomes such as adverse effects, hospitalizations, and deaths are rare, but are required to verify the real problems associated with using PIDTs. As noted in this review, studies evaluating interventions, such as education, have shown positive results. Thus, more studies, especially randomized clinical trials, are needed to conclude whether the interventions are indeed effective.

Conclusion

A discussion of the methodological rigor of studies evaluating PIDT is critical and can contribute to the wider health care discussion. This review showed that PIDT is studied mainly in developed countries, which reinforces the need for more research in developing countries. The articles included in this study focused on observing the prevalence of PIDT in various practice scenarios. Most studies were observational and fulfilled at least 40% of the items proposed by the STROBE initiative. Our results have highlighted the potential for more detailed studies about PIDT with practical implications for patient safety.

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Conflict of interest

The authors declare that they have no conflicts of interest.

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Full Length Research Paper

Suspending properties of natural gums extracted from *Abelmuscus esculentus* pod and *Chrysophyllum albidium* fruit

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The aim of this study was to extend the application of gum extracted from *Abelmuscus esculentus* pods (AEG), ripped *Chrysophyllum albidium* fruit (RCAG) and unripped *C. albidium* fruit (UCAG) to pharmaceutical suspensions. The extracted gums, gelatin and compound tragacanth were used to formulate Paracetamol suspension in concentrations of 0.5 to 4.0% w/v. The sedimentation rates, sedimentation volume, ease of re-dispersibility and viscosity of the suspension were studied as assessment parameters. The rank order of the suspending ability of the suspendants as evaluated by the sedimentation volume was AEG > gelatin > compound tragacanth > RCAG. Suspensions formulated with RCAG has comparative viscosity with those containing gelatin and compound tragacanth; however, Paracetamol suspensions having AEG has significantly higher viscosity ($p<0.05$) when compared with those containing RCAG, gelatin and compound tragacanth. The flow rate decreases with increase in the concentration of the suspending agent and increase in the viscosity. Paracetamol suspensions containing RCAG were easily redispersible with minimum agitation and are stable enough for adequate dose withdrawal. The viscosity of formulations containing AEG decreases with increased speed of agitation. On the basis of these findings, pharmaceutical suspension containing *A. esculentus* and *C. albidium* gums as suspending agents may be applied as liquid drug delivery system for pediatric and geriatric patients.

Key words: Paracetamol suspension, suspending agents, okra gum, *Chrysophyllum albidium* gum, sedimentation volume, viscosity.

INTRODUCTION

Suspensions are an important pharmaceutical dosage form that is still widely in use. Owing to their versatility they are often used in situations where the patients are unable to swallow tablets or capsules (Marriot et al.,

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2010). Pharmaceutical suspensions are dispersions of an insoluble drug in an aqueous or non-aqueous continuous phase and like other disperse systems they are thermodynamically unstable. Thus, it is necessary to include in the dosage form a stabilizer or suspending agent. A well-formulated suspension should easily be re-suspended when moderately agitated and should allow uniform and accurate doses of the medicament to be withdrawn throughout the period of medication. Suspending agents are used in formulations to help the dispersed phase to remain suspended long enough when shaken and assist in easy re-dispersion of settled particles on standing (Ogaji, 2011). These have the benefit that consistent withdrawal of uniform doses is possible throughout the medication period. Natural gums from *Irvingia gabonnsis, Albizia zygia, Grewia mollis* and *Khaya grandifolia* have been reported to provide the needed platform for some of the quality attributes of a suspension due to their ability to swell when in contact with water and their viscous nature (Ndjouenkeu et al., 1996; Femi-Oyewo et al., 2004; Isimi et al., 2000; Ogaji, 2011; Nep and Conway, 2010). Natural gums are generally biodegradable, cheap, easily available, effective, and ecofriendly as compared to synthetic and semi-synthetic materials as pharmaceutical excipients (Prasad et al., 1998; Rana et al., 2011; Bakre and Abimbola, 2013).

Okra (*Abelmuscus esculentus*) is an annual or perennial herbaceous plant, growing up to 2 m tall straight up with very little phototropism. The pod could be green, red or purple, long, slender or chunky with numerous ridges running along the length of the pod. The pod varies in length from a few to about 7 cm in length and 1 to 4 cm in width. Okra plant grows very fast; therefore, it must be harvested every two days. Although, the crop can be grown on all soil types; sandy loam soils high in organic matter are the most desirable. Okra is among the most heat-and drought-tolerant vegetables in the world. Once established, it can survive severe drought conditions. The edible pods are used in soups and as a vegetable (Smith et al., 2002).

Chrysophyllum albidium fruit is almost spherical, with a slight point at the tip. There are 3 to 5 seeds brown, shiny seeds (1-1.5 × 2 cm), arranged in a star-shaped pattern in the yellow pulp. The seeds have a hard seed coat and the fruit turns from greenish grey when immature to orange, pink or yellow when ripe (Smith et al., 2002)

Although some works (Boyinbode and Iranloye, 1986; Odeku and Akinlosotu, 1997) had been carried out on the gums extracted from okra and *C. albidium* gums as excipients in pharmaceutical formulations, it appears that no work has been done to assess the suitability of these gums as suspending agents in paracetamol suspension as compared to the relatively common natural agents like gelatin and compound tragacanth gum. Paracetamol was chosen for this investigation because it is a practically insoluble drug which would require a suspending agent to be prepared as a liquid dosage form.

METHODOLOGY

Materials

The materials used were paracetamol powder (Spectum chemicals, USA), benzoic acid, acetone (BDH Chemicals, UK), compound tragacanth gum (Searl Co., England), and gelatin (Merck, Germany). Water was double distilled and every other chemical was of analytical grade.

Extraction of gums

Okra gum was extracted from the pods of *A. esculentus* fruit using the method of Onunkwo and Mba (1996). The fruits were cleaned, washed, sliced, crushed and then macerated in distilled water for 10 h with intermittent stirring. The mucilage was filtered through a white muslin cloth to extract the gum and acetone was added to precipitate the extracted gum. The gum obtained labeled as AEG was then filtered under vacuum to remove acetone and dried in a dessicator. The same procedure was used for the extraction of gum from ripped (RCAG) and unripped (UCAG) *C. albidium* fruit.

Formulation of paracetamol suspension

A 0.5 g quantity of compound tragacanth powder and 5 g of paracetamol were triturated together with 50 ml of water to form a smooth paste. The mixture was transferred into a 100 ml of measuring cylinder made up to volume with distilled water and then shaken vigorously for 2 min (thus making 0.5% w/v of the gum in the preparation). The suspension contains 0.1% w/v benzoic acid as preservative. The procedure was repeated using 1.5, 2.5, 3.0, and 4.0 g of compound tragacanth powder. The aforementioned procedure was repeated with gelatin, okra and *C. albidium* gums

Phytochemical analysis

Phytochemical analysis was carried out following established procedures in the British Pharmacopoiea (1998).

Determination of sedimentation volume and rate

Each suspension (50 ml) was stored in a 50 ml measuring cylinder for 7 days at 35°C. Observations were made at every hour for 7 h and then every 24 h for 7 days. The sedimentation volume, F (%), was then calculated using the following equation.

$$F = 100\ Vu/Vo \qquad (1)$$

where Vu is the ultimate volume of the sediment and Vo is the original volume of the suspension.

Rheological assessment using Brookfield viscometer

Viscosities of the prepared suspensions were determined using a Brookfield Synchro-electric viscometer; model LVF (Brookfield Laboratories, Massachusetts). Different concentrations of the

prepared suspensions were put separately in a 600 ml beaker, appropriate enough to immerse the spindle groove in the fluid. Viscosity values at rotational speeds of 10, 20, 50, and 100 rpm were determined at room temperature. All determinations were made in at least triplicate and the results obtained are expressed as the mean values.

Determination of flow rate

The time required for each suspension sample to flow through a 10 ml pipette was determined and the apparent viscosity (Ƞ) was calculated using the equation:

Flow rate Ƞ = Volume of pipette (ml) / Flow time (s) (2)

Ease of re-dispersibility of formulated suspensions

Fifty milliliters quantities of the formulated suspensions were poured into bottles, stoppered and kept on a vibration free platform. The suspensions were shaken 3 times, manually by hand after 7 days to find out how much of it was re-dispersed.

RESULTS AND DISCUSSION

Phytochemical analysis of gums

Phytochemical tests carried out on RCAG and AEG gums confirmed the absence of alkaloids, anthraquinones and carbohydrates in accordance with the belief that gums do not contain carbohydrates, but complex acids built up of less common sugar (Femi-Oyewo et al., 2004).

Effects of various suspending agents on the sedimentation volume of paracetamol suspension

Table 1 shows the sedimentation volume of the paracetamol suspensions at 0 to 4.0% w/v suspending agents for 7 days. The internal phase settled rapidly within the first 1 h of preparation for suspensions containing RCAG and compound tragacanth and settled constantly over the next 7 days. Paracetamol suspension formulated with AEG exhibited the highest sedimentation volume while suspensions containing UCAG had the lowest sedimentation volume. High sedimentation volume is an indication that although the internal phase particles have settled, as would be expected with suspensions, the inter particle attraction and bonding were loose and not strong enough to form hard cake during the study period. The result suggested that differences in the sedimentation profiles was probably due more to the suspending agent used than the properties of the internal phase. The rank order of the suspending ability of the suspendants as evaluated by the sedimentation volume was AEG > Gelatin > Compound Tragacanth > RCAG > UCAG.

Most pharmaceutically useful polymers contain polar functional groups that are separated by a hydrocarbon backbone. This structure provides the polymer molecule with many active centres that permit interaction with a particle surface. At very low concentration of polymer, a large number of sites on the surface of the dispersed solids are available for adsorption of the polymer. The simultaneous adsorption of the polymer molecule on to the surfaces of different particles creates a bridge. At a high concentration of polymer, there is complete coverage of the particles by the polymer and insufficient binding sites remain on the particles to form interparticulate bridges. This consequently leads to deflocculation due to formation of adsorbed layers of polymer on different particles (Gennero, 2000). Generally, at higher gum concentration of 3.0 and 4.0% w/v, it was observed that the suspensions showed low sedimentation volume.

Effects of types of suspending agents and concentrations on the viscosity, flow rate and re-dispersibility of paracetamol suspension

The viscosity of suspensions is a factor of great importance for stability and pourability of suspensions. Suspensions are the least stable dosage form due to sedimentation and cake formation. The viscosity of different concentrations of the test gums are as shown in Table 2.

Suspensions formulated with RCAG have comparative viscosity with those containing gelatin and compound tragacanth. However, paracetamol suspensions containing AEG have significantly higher viscosity ($p<0.05$) than those containing RCAG, gelatin and compound tragacanth. This suggests that paracetamol suspensions formulated with AEG have a low terminal settling velocity; thus, the dispersed phase settles at a slower rate and remains dispersed for a longer time yielding higher stability to the formulated suspension. As the concentration of the gum increases, the viscosity of the paracetamol suspension increases. This suggests that paracetamol suspension with higher gum concentration is expected to give a suspension that settles slowly. The flow rate decreases with increase in the concentration of the suspending agent and increase in the viscosity. Paracetamol suspension containing RCAG and UCAG were easily re-dispersible with minimum agitation and are stable enough for adequate dose withdrawal.

Effect of speed of rotation on the viscosity of gums

Figure 1 shows the effect of speed of rotation on the

Table 1. Values of the sedimentation volume of paracetamol suspension using various suspending agents.

Concentration (g/ml)		Sedimentation volume (%)														
		Time (h)								Days						
		0	1	2	3	4	5	6	7	1	2	3	4	5	6	7
	0.0	100	22	22	21	21	21	21	21	20	19	18	18	18	17	17
	0.5	100	16	14	14	13	13	13	13	12	12	12	12	12	12	12
	1.5	100	22	22	22	22	22	22	22	21	20	20	20	20	20	20
RCAG	2.5	100	20	20	20	20	20	20	20	19	19	18	18	18	18	18
	3.0	100	18	18	18	18	18	18	18	18	18	16	16	16	16	16
	4.0	100	28	28	27	27	27	27	26	26	25	25	25	24	24	24
	0.5	100	18	18	18	18	18	18	18	18	18	17	16	16	16	16
	1.5	100	20	20	20	20	20	20	20	20	20	18	18	18	18	18
UCAG	2.5	100	06	06	06	06	06	06	06	06	05	04	04	04	04	04
	3.0	100	12	12	10	10	09	09	09	08	08	08	08	08	08	08
	4.0	100	10	10	10	10	10	10	10	09	06	06	06	06	06	06
	0.5	100	100	100	100	99	98	98	97	96	96	93	80	74	74	74
	1.5	100	100	100	100	98	98	98	96	95	94	92	90	84	80	80
AEG	2.5	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100
	3.0	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100
	4.0	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100
	0.5	100	60	60	58	58	57	56	55	55	54	54	53	50	50	50
	1.5	100	60	60	60	60	60	60	60	60	60	60	60	60	60	60
Gelatin	2.5	100	80	80	80	80	80	80	80	80	80	80	80	80	80	80
	3.0	100	10	10	10	10	10	10	10	10	10	09	08	08	08	08
	4.0	100	12	12	12	12	10	10	10	10	10	10	10	10	10	10
	0.5	100	22	22	21	20	20	20	20	20	19	19	19	18	17	17
	1.5	100	32	32	30	30	30	30	30	28	24	24	24	23	23	23
Compound tragacanth	2.5	100	34	34	32	32	30	30	30	30	28	28	27	27	26	26
	3.0	100	40	40	32	32	30	30	30	29	29	28	28	28	28	27
	4.0	100	48	47	36	34	34	34	34	33	33	33	32	32	31	31

Table 2. Effects of the type and concentration of suspending agents on the flow rate (ml/s) and viscosity at 50 rpm (centipoise) of Paracetamol suspensions.

Suspending agent	Concentration (% w/v)	Flow rate (ml/s)	Viscosity (Centi poise)	Re-dispersibility
	0.5	1.00	6.00	+ + +
	1.5	0.95	4.00	+ + +
RCAG	2.5	0.91	4.00	+ + +
	3.0	0.89	6.00	+ + +
	4.0	0.92	6.00	+ +
UCAG	0.5	0.73	4.00	+ + +

Table 2. Cont'd

	1.5	0.75	6.00	+ + +
	2.5	0.65	6.00	+ + +
	3.0	0.57	6.00	+ + +
	4.0	0.47	6.00	+ + +
AEG	0.5	0.07	6.70	+ + +
	1.5	0.06	8.38	+ + +
	2.5	***	20.00	------
	3.0	***	***	------
	4.0	***	***	------
Gelatin	0.5	0.94	6.00	+ + +
	1.5	0.89	6.00	+ + +
	2.5	0.87	6.00	+ + +
	3.0	0.80	8.00	+ + +
	4.0	0.74	8.00	+ + +
Compound tragacanth	0.5	1.00	6.00	+ + +
	1.5	1.00	6.00	+ + +
	2.5	0.83	8.00	+ + +
	3.0	0.83	8.00	+ + +
	4.0	0.77	20.00	+ +

***: Too viscous to be determined. +++: Easily re dispersible with minimum agitation and stable enough for adequate dose withdrawal. ++: Re-dispersible with vigorous agitation and stable enough for adequate dose withdrawal. ---: Not re dispersible, formed hard cake.

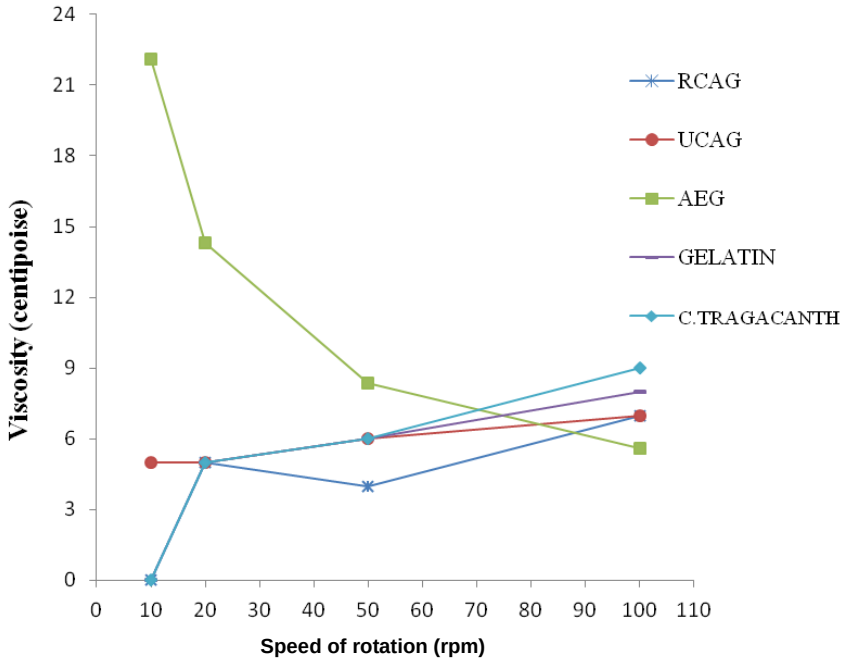

Figure 1. Effect of speed of rotation on the viscosity of paracetamol suspension formulated with 1.5% w/v concentrations of test gums.

viscosity of paracetamol suspension formulated with 1.5% w/v concentrations of test gums.

The decreased viscosity values observed with increasing speed of rotation for formulations containing AEG could be attributed to the nature of the mixture which may likely be pseudoplastic. This implies that with minimum agitation the suspension will be easily re-dispersed and a stable dose can be withdrawn. However, the viscosity of formulations containing RCAG, UCAG, gelatin and compound tragacanth was proportional to the speed of agitation

Conclusions

On the basis of these findings, *A. esculentus* and *C. albidium* may find application as suspending agents in pharmaceutical suspensions for pediatric and geriatric patients

Conflict of interest

The authors declare that they have no conflict of interests.

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Full Length Research Paper

Potential antimicrobial and antiproliferative activity of the crude extract of the endophytic fungus *Rhizoctonia sp.* from *Annona crassiflora*

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One hundred and twelve (112) endophytic fungi was isolated from leaf fragments of *Annona crassiflora*. Extracts were prepared by fermentation in Czapek broth. The extract of endophytic fungus *Rhizoctonia sp.* was selected as the most promising from an antimicrobial screening and its ability to produce metabolites with antimicrobial and antiproliferative activity was evaluated. Antimicrobial activity was evaluated through agar diffusion and broth microdilution. In the screening, the extract was able to inhibit *Staphylococcus aureus, Escherichia coli* and *Candida albicans* with inhibition zone diameters ranging from 27 to 40, but its antimicrobial activity was most prominent against *S. aureus*, with a minimum inhibitory concentration (MIC) of 500 to 1000 µg/ml. Regarding anti-proliferative activity, the results were promising. The extract displayed potent anti-proliferative activity against all tumor cell lines tested, suggesting the potential capacity to diminish the growth of human tumor cells, while also demonstrating selectivity by displaying reduced activity against non-tumor cell lines.

Key words: Endophytic fungi, *Annona crassiflora*, fermentation process, bioactive compounds, biological assays.

INTRODUCTION

Natural products constitute a strategy for the discovery of new drugs (Borges, 2008). A crucial aspect of obtaining new drugs derived from natural products is the selection of the source to be studied. According to Guimarães et al. (2008), it is important to consider unexplored sources, as they are often associated with an innovative chemical. In this respect, endophytic fungi comprise an enormously diverse species that have been minimally studied but may contain many novel compounds that may be of interest to the pharmaceutical industry (Borges, 2008;

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Mendes, 2008; Lawal et al., 2010; Newman and Cragg, 2012).

The plants of the family Annonaceae have chemical and pharmacological properties that are well described in the literature. The species *Anonna crassiflora* is popularly known as “marolo”, and shows wide use in popular medicine and is usually found in savanna areas in Brazil (Mendonça et al., 1998). The discovery of new compounds is essential principally for the development of new antimicrobial agents, as many microorganisms have developed resistance to conventional drugs. Therefore, studies are needed for the discovery of safe drugs from sources found in nature that are effective against resistant bacteria (Maciel et al., 2002).

In addition to infectious diseases caused by microorganisms, other pathologies for which treatment medications are nonspecific are becoming increasingly common, such as in the case of cancer. There is evidence that compounds produced by endophytes can be a source for new substances (Pimentel et al., 2011). Taxol (Paclitaxel trade name), a substance isolated from the plant genus *Taxus* that contains proven anticancer activity, is also produced by species of endophytic fungi such as *Taxomyces andreana* and *Pestalotiopsis microspora*. Taxol and its analogues today are already being produced by the pharmaceutical industry (Viegas et al., 2006).

In order to help address the burgeoning need for new, more effective and less toxic compounds, the aim of the present study was to perform an *in vitro* analysis of the antimicrobial and anti-proliferative potential of the crude extract from endophytic fungi from *A. crassiflora*.

MATERIALS AND METHODS

Isolation of the endophytic fungi and obtention of bioactive metabolite extracts from *A. crassiflora*

We collected branches along with the reproductive part of *A. crassiflora* in the district of Divino Espírito Santo (21° 10' 08.7" S and 46° 05' 57.9" W Gr), in the municipality of Alterosa- MG. The samples were identified and deposited in the herbarium of the Universidade Federal de Alfenas under the number 2412 UALF. The collected leaves were selected according to their apparently healthy features, packed in paper bags and sent to the Bioprocess Laboratory where they were processed within 24 h. The process of isolation of endophytic fungi according to their morphological characteristics was conducted in accordance with guidelines described by Petrini et al. (1992). The fungi was inoculated on potato dextrose agar medium (HIMEDIA, Mumbai, India) and incubated in a BOD incubator at 28°C for 20 days. The fresh mycelium grown on potato dextrose agar medium at 28°C for 20 days, under static conditions, was aseptically inoculated to 500 ml Erlenmeyer flasks containing 200 ml Czapek broth. This medium has the following composition g/L Glucose: 30.0; $NaNO_3$: 2.0; K_2HPO_4: 1.0; $MgSO_4$ $7H_2O$: 0.5; KCl: 0.5; $FeSO_4$ $7H_2O$: 0.01; Yeast Extract: 1.0 (Raper and Fennell, 1965). After incubation, the mycelium of the culture was separated by vacuum filtration. The liquid filtrate obtained was extracted four times with ethyl acetate 1:1 (v/v) (VETEC, Duque de Caxias-RJ, Brazil). The organic phase was collected and the solvent was completely removed using a rotary vacuum evaporator (801, FISATOM, Perdizes-SP, Brazil). As a negative control, un-inoculated Czapek broth extract, prepared using the same methodology, was applied.

Strain fungal selection by antimicrobial activity

Antimicrobial activity of the fermented fungal extracts generated was determined by applying the extracts to three target microorganisms: *Staphylococcus aureus* ATCC 6538 (Gram-positive bacteria), *Escherichia coli* 25922 (Gram negative bacteria) and *Candida albicans* ATCC 10231 (fungus). A pre-determined microbial suspension volume for each of the three target microbes, standardized in order to obtain a growth in agar of 3×10^8 CFU/ml per microbe, was inoculated by the pour plate method onto nutrient agar medium for bacteria and onto Sabouraud agar for fungi. The mixture was gently homogenized. After homogenization, 25 ml of medium inoculated with the target microorganisms was immediately poured into previously sterilized petri plates. After solidification of the plates, 25 µl aliquots of the fungal fermentation extracts were placed into 8 mm diameter wells that were created in the culture plates. The extracts were diluted with 500 µl of dimethyl sulphoxide (DMSO) for each extract 0.1 g, and was inoculated a rate of 25 µl of the extract in the wells. For negative control was used a rate of 25 µl of the solvent. The plates were incubated for 24 h at 37°C. The fungus with the best activity was determined by visual inspection of the formation of a halo around the wells.

Microorganisms and susceptibility test

The minimum inhibitory concentration (MIC) of the fungal extracts against target microorganisms was determined by the methodology of broth microdilution using microplates with 96 compartments, as described by the Manual Clinical and Laboratory Standards Institute (CLSI) (2005), Sarker et al. (2007) and Mann and Markham (1998), with modifications. A determined volume of microbial suspension of *S. aureus* ATCC 6538 (Gram-positive), *E. coli 25922* (Gram negative) and *C. albicans* ATCC 10231 was standardized in order to obtain a growth in agar of 3×10^8 CFU/ml. A final mixture of 100 µl composed of Mueller Hinton broth, extract test solution (Czapek broth and fungi) and a suspension of the target microorganism were added to sterile microplates. An initial addition of 50 µl of the extract sample solution compartment was dispersed in the culture broth, comprising the concentration range from 1.95 to 1000 mg/ml in Mueller Hinton broth. The suspension was also homogenized in Mueller Hinton broth and distributed by 50 µl of this suspension was added to the microplate chamber and then incubated at 37°C for 24 h. After this incubation, 50 µl of resazurin developer was added to each compartment, and plates were then read. Minimum inhibitory concentration (MIC) values and minimum bacterial concentration (MBC) above 1000 mg/ml were considered not active. All samples were tested in triplicate, and the reading of the assay was done by visual method (colorimetry). For the determination of MBC, an aliquot (50 µl) from each well with concentrations greater than the MIC was sub-cultured on Mueller-Hinton agar and incubated at 37°C for 24 h. MBC were defined as the lowest concentration that inhibited visible growth on the solid medium. All assays were performed in triplicate.

Anti-proliferative test

The proliferative study involved human keratinocytes (HaCaT) and

eight human tumor lines: NCI-ADR/RES (ovary with phenotype resistance to multiple drugs), 786-O (kidney), NCI-H460 (lung), OVCAR-3 (ovary), HT-29 (colon), U251 (glioma), MCF- 7 (breast) and PC- 3 (prostate). Cultures were conducted in 5 ml of Roswell Park Memorial Institute (RPMI) 1640 medium (GIBCO BRL, EUA) supplemented with 5% of fetal bovine serum (GIBCO, EUA). Penicillin: Streptomycin (1000 IU/ml: 1000 µg/ml, 1 ml/L) was added to experimental cultures. Cells in 96-well plates (100 µl cells/well) were exposed to various concentrations in DMSO/RPMI (0.25, 2.5, 25 and 250 µg/ml) in 37°C incubators with 5% CO_2 for 48 h. Final DMSO concentration did not affect cell viability. Doxorubicin (Eurofarma, Brazil) (0.025, 0.25, 2.5 and 25 µg/ml) was used as a positive control. Before (plate T0) and after (plate T1) the addition of the sample, the cells were fixed with 50% trichloroacetic acid and cell proliferation was determined by the quantification of proteins in a spectrophotometer at 540 nm, using sulforhodamine B. Based on the concentration-response curve for each cell line, the GI50 (concentration of raw extract needed to inhibit growth by 50%) was determined through a linear regression analysis using the Origin 7.5 program (OriginLab Corporation).

RESULTS AND DISCUSSION

Isolation of the endophytic fungi and obtention of bioactive metabolites from *A. crassiflora*

In order to isolate potentially novel compounds with pharmaceutical activity, we isolated 112 fungi from the leaves of *A. crassiflora* and tested these extracts for antimicrobial activity, making this the first report of isolation of bioactive metabolites from the endophytic fungi extracted from leaves of *A. crassiflora*. The Annonaceae family shows about 110 genera and 2,150 species. In Brazil, there have been 29 genera, with about 260 species (Barroso, 1978). According Ocampo and Ocampo (2006), this family presents an interesting by product as polyphenols, essential oils, terpenes, aromatics, particularly active acetogenins molecules anticancer activity with a broad spectrum antiparasitic and insecticidal.

Using the inhibition halo method, we determined that 73.22% of the extracts (82/112 extracts) displayed antimicrobial activity against at least one of the three target microorganisms. By analyzing the activities separately, it was found that *S. aureus* was inhibited by 45.54% (51) of the fungi extracts, *E. coli* was inhibited by 54.46% (61) extracts and *C. albicans* was inhibited by 31.25% (35) of the extracts. Based on these results, we selected fungal extract of *Rhizoctonia sp.*, which showed substantial haloes against *C. albicans* (27 mm), *E. coli* (40 mm) and *S. aureus* (35 mm).

Similarly, Souza et al. (2002) reported that *A. crassiflora* extracts harvested from the Brazilian Cerrado ecoregion have antimicrobial activity *in vitro* against the growth of *Candida* species and *Cryptococus neoformans*. According to Silva et al., (2014), extracts from the bark of fruit *A. crassiflora* showed superior results as compared to the standard (chlorhexidine) in a screening for antibacterial activity by agar diffusion.

The analysis of the antimicrobial activity of the fungal fermentation extracts of *A. crassiflora* that were tested in the present study showed that many fungal metabolites are capable of inhibiting micro-organisms of both Gram-negative and Gram-positive classifications, highlighting its importance as an alternative source for the production of new antimicrobial agents. Notably, Gram-negative bacteria have a complex cell wall with a lipid barrier that confers a greater resistance to antibiotics. The difficulties in finding drugs that can effectively penetrate the lipid barrier highlights the importance of the present study, in which we found that metabolites produced by many endophytic fungi are able to sufficiently cross this barrier and inhibit the growth of Gram negative-bacteria (Pinto et al., 2001).

Microorganisms and susceptibility test

The antimicrobial activity for the extract of the fungus *Rhizoctonia sp.* ethyl acetate against *S. aureus* showed a MIC of 500 to 1000 µg/ml but against *C. albicans* and *E. coli* showed no activity within the tested concentrations. Regarding the MBC which did not obtain positive results in the tested concentrations; it was thus classified as bacteriostatic against *S. aureus*. The evaluation of the minimum inhibitory concentration (MIC) was performed over a defined range of concentrations, from 1000 to 0.97 µg/ml. The value of 1000 µg/ml was chosen as the maximum threshold because, according to Rios and Recio (2005), seeking natural product extracts that have higher antimicrobial concentrations of 1000 µg/ml should be avoided. The activity of endophytic fungi was also confirmed by Fernandes et al. (2009) who studied a total of 22 endophytic fungi isolated from coffee (*Coffea arabica* L.), and determined the MIC against *S. aureus, E. coli* and *C. albicans*, for which the MIC ranged between 50 to 100 µg/ml for *S. aureus* and 400 to 800 µg/ml for *E. coli.*

Anti-proliferative test

The anti-proliferative activity exerted by Doxorubicin used as the standard and the ethyl acetate extract of the endophytic fungus *Rhizoctonia sp.* are shown in Figures 1 and 2, respectively. For analysis and interpretation of results of GI50, we utilized criterion from the National Cancer Institute (NCI, USA) (Fouche et al., 2008), which classifies the cytostatic effect of the extracts as inactive (mean > 1.5), weak (1.1 < mean < 1.5), moderate (1.1 > mean > 0) and potent (mean < 0) as a result of the average log GI_{50}.

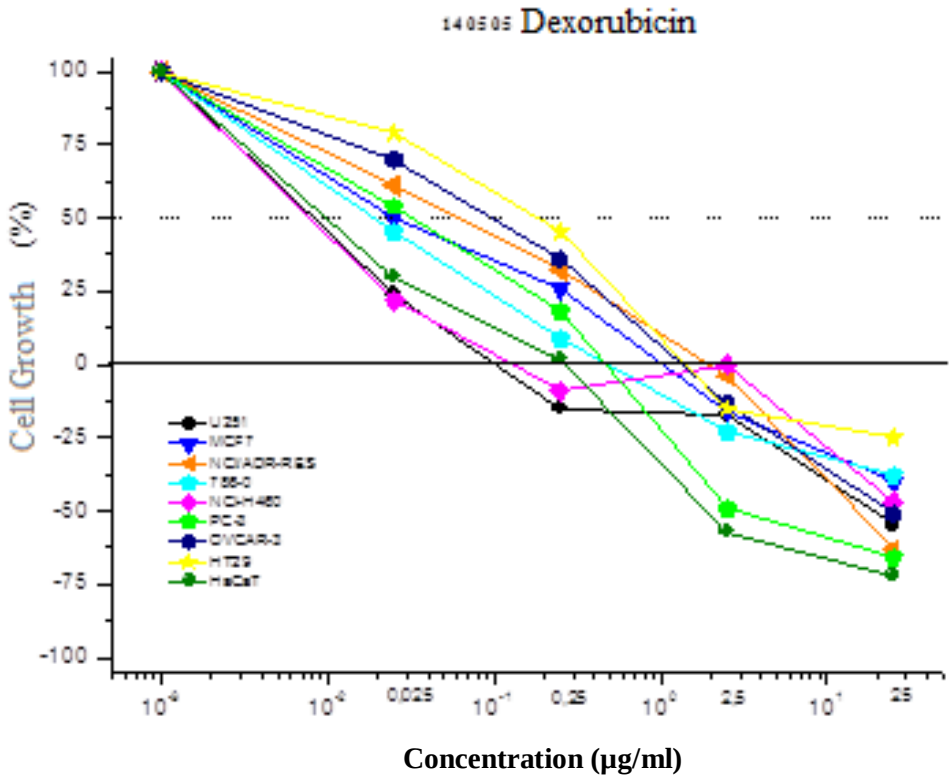

Figure 1. Activity of Doxorubicin in the anti-proliferative test.

To evaluate the anti-proliferative activities, we employed eight human tumor cell lines. Figures 1 and 2 displayed the capacity of the extract from *Rhizoctonia sp.* to diminish tumor growth in all cell lines at an approximate concentration of 2.50 µg/ml and a mean Log GI50 of 0.60 µg/ml. Already the doxorubicin at the same concentration showed LOG GI50 of -1.40 µg/ml, demonstrating the potential of fungal extract evaluated.

The extract of *Rhizoctonia sp.* stood out because it was able to inhibit tumor cell growth even at very low concentrations, according to the NCI criteria. In addition to its good activity against all tested tumor cell lines, it showed a low activity against the non-tumor cell line, demonstrating its capacity to selectively inhibit tumor cells. The extract's GI50 values were also similar in potency to the Doxorubicin positive control. The search for endophyte-derived substances with anticancer potential is a promising, emerging field, having been brought to the forefront of attention previously with the discovery of taxol, a metabolite with anti-tumoral activity derived from the endophytic fungus *Taxomyces andreanae* and isolated from *Taxus brevifolia*, as reported by Stierle et al. (1993). Paclitaxel (taxol) and its derivatives represent the first and largest group of anticancer agents produced by endophytes. Several fungi such as *T. andreanae* and *T. brevifolia* are able to produce them, amongst other fungi of various plant species (Strobel and Daisy, 2003). Taxol, whose active ingredient is paclitaxel, has been used to cure many malignant tumors, such as breast cancer, ovarian cancer and lung (Pandi et al., 2010). Another example is cajanol, a substance extracted from the roots of plant species *Cajanus cajan*, that has inhibitory activity against prostate-specific antigen. Cajanol is also being considering in breast cancer treatment, due to its ability to inhibit apoptosis of tumor cells (Duker-Eshun et al., 2004; Liu et al., 2011).

The fact that the anti-proliferative activity of the fungal extracts against tumoral cell lines displayed similar curves as compared to the doxorubicin standard suggested that the fungal extracts were capable of inhibiting the growth of all tested tumor cell lines. Importantly, the fungal extracts displayed decreased activity against the non-tumor cell line (HaCaT), suggesting that the extracts were at least partly selective.

Despite the wide array of drugs that have already been discovered or approved for use in cancer therapy, these treatments are not effective for many tumors and/or are plagued by a number of side effects. Thus, new therapeutic agents are necessary, which will require the discovery of new molecules with potential anti-tumor activity (Costa-Lotufo et al., 2010). The results obtained

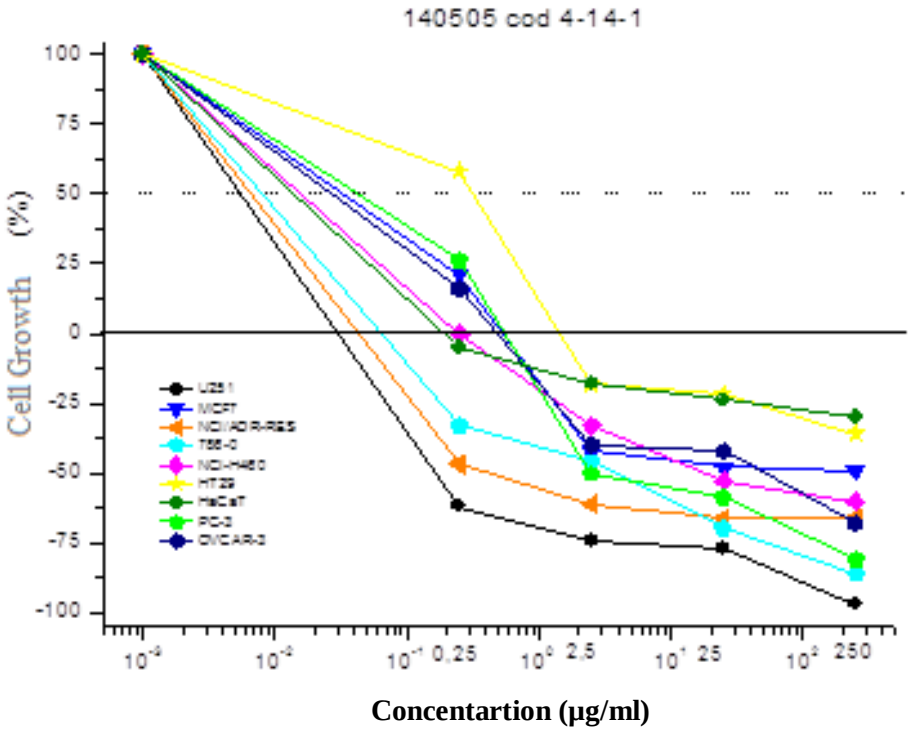

Figure 2. Activity of *Rhizoctonia sp.* extract in the anti-proliferative assay.

in this study confirm that endophytic fungi are a rich resource for defining new compounds with anti-proliferative activity, and furthermore should encourage the search for deeper knowledge on the action mechanisms, effectiveness and application of endophytic metabolites for a variety of disease states.

Conclusion

The results obtained highlight the endophytic fungus *Rhizoctonia sp.*, whose crude extract stood out as a result of its potent antimicrobial activity, demonstrated by its significant inhibition halo zone size and low MIC. This fungal extract showed promising results in anti-proliferative tests against all tumor cell lines analyzed and displayed selectivity against a non-tumor cell line. The extract from this isolated fungi of *A. crassiflora*, consisting of a potentially promising source for the production of bioactive compounds of interest, should encourage the search for deeper knowledge of the mechanisms of action of the compounds and the verification of their activity against various diseases.

Conflict of interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

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Possible cardio-protective effect of ginger and lipoic acid on normal senile female rats

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Cardiovascular disease and oxidative stress are involved in aging. Aging causes many alterations in myochardial functions and metabolism. The aim of this study was to investigate the effect of aging on heart functions, total antioxidant capacity and membrane lipid composition and the effects of oral administration of ginger and lipoic acid for 30 days. The levels of serum lactate dehydrogenase, creatine kinase and heart antioxidant capacity exhibited marked alterations in normal senile female rats. The treatment of ginger and lipoic acid for 30 days improved these alterations. Furthermore, the relationship between membrane lipid composition and aging was detected. There were significant increases in lauric and myristic acids and significant decrease in arachidic acid in senile rats. On the other hand, the unsaturated fatty acids (olic, linoleic, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) were significantly decreased. Ginger treatment showed significant decreases in saturated fatty acids. In lipoic acid treated group, there was a significant improvement in the level of unsaturated fatty acids (EPA and DHA) as compared with normal senile female rats. In conclusion, ginger and lipoic acid may have ameliorative effect of heart functions, total antioxidant capacity and membrane free fatty acids in old female rats.

Key words: Ginger, lipoic acid, senile, heart, antioxidant, free fatty acids.

INTRODUCTION

Aging is a major factor for the development of oxidative damage and heart disease (Gao et al., 2013) and associated with fibrosis and inflammation that leads to cellular senescence (Li et al., 2014). Shih et al. (2011) stated that myocardial infarction and chronic heart failure increased with age. Heart is provided with antioxidant systems to scavenge excess reactive oxygen species (ROS), repair oxidative stress and maintain sulfhydryl homeostasis (Meyer et al., 2009). Nageswari et al. (1999) demonstrated that myochardial antioxidant status decreased and lipid peroxidation increased with age. Antioxidant reduced the reactive oxygen species which causes carcinogenesis, DNA damage, heart disease and other health problems revealed to advancing age (Yeh et al., 2014).

Ginger is a traditional medicine which processes antiinflamatory, anticancer, antioxidant, antipyretic and antibacterial properties (Kabuto et al., 2005; Chung et al., 2009; Kim et al., 2010; Durak et al., 2015; Hosseini and Mirazi, 2014; Lee et al., 2014; Yeh et al., 2014 and Yudthavorasit et al., 2014). Also, ginger acts as a hypolipidimic factor in which it stimulates the conversion

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of cholesterol to bile acids leading to the excretion of cholesterol from the body (Afshari et al., 2007). The biological activities of ginger come from its active chemical component such as gingerols and gingerol related compounds (Chang et al., 2011; Lee et al., 2012; Jafarzadeh et al., 2014; Yudthavorasit et al., 2014). On the other hand, lipoic acid is a natural dithiol compound with an antioxidant activity in which it raises the level of sulfane sulfur and rodanese activity in the tissues (Dudek et al., 2008, 2014). In addition, it plays a role in lipid and carbohydrate metabolism by enhancing glucose transport in muscle cells (Paker et al., 2001; De-Oliveira et al., 2011).

The present study aimed to evaluate the age-related changes on heart tissue in normal female senile rats and the effect of oral administration of two antioxidants ginger and lipoic acid administered for 30 days on the heart functions, antioxidant activity and membrane free fatty acids.

MATERIAL AND METHODS

Experimental animals

Adult female albino rats weighing approximately 130 to 150 g (3 to 4 months old) and senile (24 months old) weighing 280 to 300 g were used. Rats were maintained in iron mesh cages, each cage contained six rats and housed for 10 days prior to the initiation of the experiments, for adaptation to laboratory conditions. Animals were fed with commercial standard rat-pellet and tap water provided *ad libitum.* Handling and usage of animals agreed strictly with the regulations and guidelines set by the research Ethics Committee of the Ain Shams University authorities and followed Egyptian rules for animal protection, which was performed according to the UK Animals (Scientific Procedures) Act, 1986.

Chemicals

Ginger was purchased from Arab Company for Pharmaceuticals and Medicinal Plants, Egypt (MEPACO) and alpha lipoic acid was purchased from EVA Company, Egypt. All other chemicals and solvents used were of the high performance liquid chromatography (HPLC) and analytical grade. Saturated fatty acids (SFAs), lauric (C12:0), myristic (C14:0), palmitic (C16:0), stearic (C18:0) and arachidic (C20:0). Unsaturated fatty acids (USFAs), oleic (C18:1 ω–9), linoleic (C18: 2 ω–6),), *cis*-5.8.11.14.17-eicosapentaenoic (C20:5ω–3) (EPA) and*cis*-4.7.10.13.16.19-docosahexaenoic acid (DHA) (22:6 ω-3). All FAs purchased from Sigma-Aldrich Co. (St. Louis, USA). All other chemicals and solvents used were of the HPLC and analytical grade.

Experimental design

The animals were divided into four groups each containing six rats as follows: the first group was the control adult rats and received orally 0.1 ml/100 g b.wt. carboxy-methyl cellulose sodium salt (0.5% CMC). The second group was the control senile rats and received the same amount of CMC. The third group, senile rats administrated ginger at a dose of 250 mg/kg body weight and dissolved in CMC vehicle. The fourth group was senile rats administrated alpha-lipoic acid (ALA) at a dose of 65 mg/kg body weight and dissolved in CMC. All groups received treatments for 30 days. Doses were calculated in relation to the human therapeutic dose according to Reagan-Shaw et al. (2008).

Biochemical assay

At the end of the experiments, the rats were sacrificed after 12 h from the last dose by rapid decapitation. Heart was excised for the determination of free fatty acids by GC according to the method of Firlag et al. (2013). Serum was collected for determination of creatine kinase MB (CKMB) according to the method of Young (1997), lactate dehydrogenase (LDH) according to the method of Van der heiden et al. (1994), and total antioxidant capacity according to the method of Koracevic et al. (2001).

Statistical analysis

Reported values represent means ± standard error (SE). Statistical analysis was evaluated by one-way analysis of variance (ANOVA). Once a significant F-test was obtained, least significant difference (LSD) comparisons was performed to assess the significance of differences among various treatment groups. Statistical processor system support "SPSS" for Windows software, Release 12.0 (SPSS, Chicago,IL) was used.

RESULTS

Table 1 shows the effects of ginger and lipoic acid administration on serum lactate dehydrogenase, serum creatine kinase-MB, total antioxidant capacity, saturated fatty acids and unsaturated fatty acids levels in heart tissue of adult and senile female rat groups. The data demonstrated significant decrease in serum lactate dehydrogenase and heart tissue total antioxidant activity in senile female rats compared with that of the adult group. These levels increased significantly in ginger and lipoic acid treated senile group in comparison with senile rats. Significant increases was observed in serum creatine kinase-MB level in senile groups and this increase diminished after the treatment with ginger or lipoic acid as compared with adult control and ginger treated groups.

The results of saturated fatty acids (lauric acid, myristic acid, stearic acid and arachidic acid) and unsaturated fatty acids (palmatic, olic, linoleic, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA)) represented in Table 1 showed significant increase in lauric and myristic acids and a significant decrease reported in arachidic acid in senile group. While in ginger treated rats, there were significant decrease in lauric acid and arachidic acid as compared with normal senile group. Generally, there were a significant decrease in all saturated fatty acids in ginger treated rats as compared with adult control, senile and lipoic acid treated groups.

After 4 weeks of treatment, unsaturated fatty acids (olic, linoleic, EPA and DHA acids) and total unsaturated free fatty acids decreased significantly in senile group. The content of all unsaturated fatty acids

Table 1. Effect of ginger and lipoic acid on lactate dehydrogenase (LDH U/L), creatine kinase-MB (CKMB U/L), total antioxidant activity (AOA mMol/L), polyunsaturated fatty acids and saturated fatty acids content (mg/g wet heart tissue) in all groups.

Parameter	Control	Aged	Ginger	Lipoic acid
LDH	1471.59±58.91	1150.148±60.78[a]	1552.48±43.80[b]	1493.14± 26.36[b]
CKMB	904.35±38.36	1111.83±39.63[a]	936.018±37.375[b]	960.74±47.97[b]
AOA	2.9795±0.0879	1.9035±0.05199[a]	2.4298±0.0906[ab]	2.7047±0.11597[ab]
(C12:0) lauric acid	0.165±01422	0.3492±0.1975[a]	0.221±0.0213[b]	0.3407±0.523[ac]
(C14:0) myristic acid	0.20217±0.246	0.322±0.0338[a]	0.273±0.0166	0.017±0.0319[abc]
(C18:0) stearic acid	0.561±0.043	0.456±0.0149	0.516±0.0410	1.0197±0.0615[abc]
(C16:0) palmitic acid	8.491±8.491	11.479±0.646[a]	5.798±0.442[ab]	11.280±0.323[ac]
(C20:0) arachidic acid-mg/g	1.106±0.223	0.384±0.0423[a]	0.0996±0.0114[a]	.6799±0.0394[ac]
Saturated fatty acids	10.526±0.727	12.989±0.715[a]	6.908±0.475[ab]	13.828±0.407[ac]
(C18:1 ω–9) olic acid	9.618±1.187	2.373±0.360[a]	0.948±0.064[a]	2.438±0.282[a]
(C18: 2 ω–6) linoleic acid	15.548±0.257	12.084±0.347[a]	8.066±0.699[ab]	12.784±0.432[ac]
(C20:5ω–3) eicosapentanoic acid	19.069±0.536	9.939±0.303[a]	11.441±0.454[a]	17.896±0.723[bc]
(22:6 ω-3) docosahexaenoic acid	250.212±10.723	212.771±7.169[a]	224.958±10.103	266.418±10.816[bc]
Polyunsaturated fatty acids	294.448±10.766	237.167±7.135[a]	245.413±10.748[a]	299.535±10.738[bc]

Values are means of 6 rats± SE. a = significant change from control, b = significant change from senile rats and c = significant change from senile rats treated with ginger at $p \leq 0.05$.

decreased after the treatment with ginger except in EPA and DHA which increased insignificantly as compared with adult control group. In lipoic acid treated group, there were significant improvements in the levels of EPA and DHA as compared to senile and ginger treated group and the results reached to the values of the control group.

DISCUSSION

Aging is a major risk factor of heart failure that is associated with an increment myocardial rigidity, impaired ventricular filling, coronary artery disease and oxidative dysfunction (Sample et al., 2006). Lactate dehydrogenase (LDH) and creatine kinase (CK) are important enzymes participating in transferring the lactic acid to pyruvate and formation of adenosine tryphosphate (ATP) from adenosine diphosphate (ADP) in anaerobic systems and they are also known as indicators of oxidative stress (Nikbakht et al., 2014). The experimental results recorded a significant decline in serum lactate dehydrogenase and a significant increase in creatine kinase-MB (CK-MB) levels in normal senile female rats. Sample et al. (2006) investigated the effects of aging on the profile of myocardial substrate utilization and cardiac function and stated that there was a marked decline in cardiac function and efficiency. Also, Anitha and Asha Devi (1996) and Prathima and Asha Devi (1999) reported significant decreases in total myocardial LDH activity and LDH isoenzyme profile in aged rats. Aging heart is thought to have many features in terms of remodeling and adaptation such as changes in the myocardial energy substrate profile, a decline in fatty acid oxidation and an increase in carbohydrate metabolism, oxidative function disturbance, metabolic reconstruction, mitochondrial dysfunction, oxidative damage and alteration in the plasma membrane integrity (Chuffa and Seiva, 2013; Sample et al., 2006; Cordero-Reyes et al., 2014; Chitra et al., 2013).

Oxidative stress is associated with several pathological conditions including aging (Bonnefont-Rousselot and Collin, 2010; Guney et al., 2013). Also, the increment of mitochondrial reactive oxygen species (ROS) in aged heart has been reported by Dröge (2002) and Turrens (2003) and the endogenous mitochondrial antioxidant defenses may be diminished with age. Current study recorded a significant decrease in total antioxidant activity in normal senile female rats. Our result is in agreement with Youdim and Deans (1999a) and Asha-Devi et al. (2003). Youdim and Deans (1999b) indicated that total myocardial antioxidant status decreased significantly in old rats. Free radicals induced lipid peroxidation proposed as an etiologic factor in cell membrane damage, atherosclerosis, cancer and aging (Nageswari et al., 1999). Aging results in changes related to molecular and functional alteration in the properties of biological membranes (Vazquez-Memije et al., 2005). Esterbauer et al. (1991) found that the release of polyunsaturated fatty acids from membrane phospholipids undergo lipid peroxidation by reacting with ROS to produce various aldehydes, alkenals and alkenes. The present study represents the relationship between membrane lipid composition and aging.

Data analysis reveals a distinct age dependent decrease in the total polyunsaturated fatty acids

(especially EPA and DHA) accompanied by an increase in saturated fatty acids content in senile female group. The increment in the level of saturated fatty acids may be due to increased lipolysis (Nageswari et al., 1999). Herrero et al. (2001) showed that long lived animals have lower fatty acid double bond content in their mitochondrial membranes than short-lived ones. This is due to a decrease in the process of unstauration. The degree of in vitro lipid peroxidation increases as the process of unstauration of fatty acid substrates increase (Bondy and Marwah, 1995). Therefore, we can say that, the decrease in fatty acid double bound content of the mitochondria of long-lived animals will protect them against lipid peroxidation (Herrero et al., 2001). Linoleic acid levels were inversely associated with age and decreasing about 3% per year up until age 70 (Harris et al., 2013). The n-3 class EPA and DHA accumulated in the phospholipids in our membranes especially in brain, heart and testes (Leaf et al., 2003).

Harris et al. (2013) mentioned that, n-3 fatty acids EPA and DHA decreases in red blood cell (RBC) membranes have been associated with cardiovascular disease, neuropsychiatric diseases and cellular aging. The reduction in unsaturated fatty acids may be attributed to a reduction in the number of myocytes, reduced efficiency in mechanisms to detoxify ROS, alteration of metabolic, ionic and electrical properties of myocytes which characterize aged myocardium (Anversa and Sonnenbikk, 1990; Olivetti et al., 1991; Lakatta, 1992; Lakatta, 1993; Walker et al., 1993). Since oxidative stress in myocardium is a factor that contributes to aging, antioxidant treatment is considered to be a potential strategy for prevention of heart aging. Ginger plant and its single constituent such as 6-gingerol, 6- paradol and zingerone plays an effective role against lipid peroxidation (Ippoushi et al., 2003; Chrubasik et al., 2005). Our results revealed that ginger administration for 30 days have an amelioration effect on heart functions, total antioxidant activity and free fatty acids regarding to aged control group. Ahmed et al. (2000) and Liu et al. (2003) reported that rats which received a diet with ginger showed an increase in glutathione and decrease in plasma lipid peroxide levels. Also, mice fed ginger oil by gavage for 14 days showed significant elevation in glutathione s- transferase and aryl hydrocarbon hydroxylase (Chrubasik et al., 2005).

Moreover, many studies showed that ginger causes an increase in plasma antioxidant level and decrease lipid peroxidation (Afshari et al., 2007; Nicoll and Henein, 2007). Free radicals and oxidative stress causes oxidation of polyunsaturated fatty acids (PUFA) which embedded in the cell membrane (Afshari et al., 2007). Oxidized arachidonic acid metabolites such as the cyclooxygenase and lipoxygenase products cause arterial inflammation and heart disease. Ginger constitutes inhibit the production of arachidonate -5-lipooxygenase, pro-staglandins and leukotrienes from the cyclooxygenase and lipooxygenase, respectively (Grzanna et al., 2005; Nicoll and Henein, 2007; Mozaffari-Khosravi et al., 2014). Also, ginger plant and its constituents protects against linoleic acid peroxidation and reduced atherosclerotic lesions (Chrubasik et al., 2005; Nicoll and Henein, 2007).

The present study evidenced that the lipoic acid treatment improved heart functions, total antioxidant activity and unsaturated fatty acids (EPA and DHA) as compared to normal senile female rats. Many studies have reported the antioxidant and cardioprotective properties of lipoic acid (Smith et al., 2004; Ghibu et al., 2009). Sokolowska et al. (2014) returned the antioxidant activity of lipoic acid to its normalizing effect on the antioxidant status in cardiomyocytes, restoration of normal catalase activity, increase the level of cysteine, cystathionase, mercaptopyrovate sulfurtransferase activation and activation of aldehyde dehydrogenase 2. Moreover, Dudek et al. (2014) reported that lipoic acid elevated the level of sulfane sulfur which played an important role in the release of hydrogen sulfide. Hydrogen sulfide is an endogenous signaling molecule which activates potassium ATP-sensitive channels of cardiovascular system. It can affect blood pressure, vasodialation, protects heart against ischemia-reperfusion injury and has antioxidant activity (Dudek et al., 2014; Ji et al., 2008; Bian et al., 2006; Szabo et al., 2011).

Conclusion

From the present results, ginger and lipoic acid showed ameliorative effect in ageing that induced significant changes in heart functions and total antioxidant deficiency in heart tissues of female aged rats through decreasing oxidation of membrane free fatty acids and reduction of oxidative stress.

Conflict of interest

The authors have declared that there is no conflict of interest.

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