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*Full Length Research Paper*

## **Incorporation of tannic acid in formulations for topical use in wound healing: A technological prospecting**

**Antonia de Sousa Leal<sup>1\*</sup>, Layne Hellen de Carvalho Leal<sup>2</sup>, Davi da Silva<sup>3</sup>, Livio Cesar Cunha Nunes<sup>1</sup> and José Arimateia Dantas Lopes<sup>1</sup>**

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**The tannic acid is a polyphenol with several properties that give it different applications, including leather and pharmaceutical industry. It belongs to the group of water soluble metabolites, and has the ability to complex macromolecules and metal ions, which gives it antioxidant, antimicrobial and healing activity. Therefore, such properties can be used to develop technologies related to wound healing systems. This prospective study summarizes bibliographic information about the use of tannic acid in topical formulations for healing skin wounds. The data were collected through the search tool available in patent databases (INPI, USPTO, EPO, WIPO, DERWENT) and articles (Web of Science) about the application of tannic acid in skin wound healing system. In recent decades, there has been growth in number of searches for elucidation of antimicrobial and antioxidant activities of tannic acid, and its incorporation in biomaterials in the pharmaceutical field. The United States, Japan and China stood out in inventions and publishing articles. The Brazilian biodiversity is immense, although underexplored and poorly study about extraction and properties of tannins in topical formulations, which lacks encourages to research technologies for healing system more effective and cheap.**

**Key words:** Tannic acid, cutaneous healing system, technological prospecting.

### **INTRODUCTION**

Natural products are raw materials of great interest for different industrial sectors, due to the fact that it presents in its composition a variety of active ingredients. The tannins, for example, have applications inecological agriculture, preparation of flocculants for water and effluent treatment, obtaining natural polymers, wine clarification, biomaterials, etc. In almost all botanical families there are species that contain tannins. When they occur in large quantities, the tannins are usually located in certain parts of the plant as the roots, bark,

leaves, fruits, seeds and sap (Claus and Tyler, 1965).

Tannins belong to a group of phenolic compounds from the secondary metabolism of plants. They have a high molecular weight (500 to 3000 Da) and contain phenolic hydroxyl groups in sufficient amounts to allow crosslinking to macromolecules (Min et al., 2003). They are classified into two groups: hydrolysable tannins and condensed tannins.

The tannic acid is water-soluble tannin, comprising a central glucose molecule attached with five chains formed

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by two esterified gallic acid molecules. In an acid medium, in mild alkaline conditions or under the action of enzymes (such as tannase), the tannic acid can undergo hydrolysis obtaining carbohydrates (glucose) and phenolic acids (gallic acid, especially) (Bruneton, 2001).

The tannic acid is considered a safe food additive (Akiyama et al., 2001). Moreover, the tannin has astringent and antibacterial properties, and it can be used as a medicament against diarrhea, hemostatic, anti-hemorrhoid formulations, in the treatment of skin ulcers, wounds and toothache (Aelenei et al., 2009). However, this compound should not be used in large quantities because it inhibits the absorption of iron in the body (Chung et al., 1998).

In previous studies, the tannic acid showed antimicrobial activity against pathogens (Chung et al., 1998; Scalbert, 1991). Research with the seed extract of *Vitis rotundifolia* species showed that tannic acid was the main chemical constituent responsible for its antimicrobial properties (Kim et al., 2008). The pure tannic acid when subjected to heat treatment, its antimicrobial activity is significantly increased (Kim et al., 2010). Among the polyphenols, the tannic acid and quercetin were considered the constituents of higher antioxidant capacity (Pulido et al., 2000).

Plants rich in tannins are used in the traditional medicine as drugs for the treatment of various organic diseases, including the healing process of wounds, burns, and inflammation (Haslam, 1996). This is explained because of its property of complexing proteins at the injury site. The tannins form complex with macromolecules (proteins and/or polysaccharides) providing a protective layer on the epithelial tissues injured; the healing process of the wound occurs naturally just below this layer (Monteiro et al., 2005).

In Brazil, there is an increasing public spending on treatment of skin lesions, especially the wounds originating from chronic diseases such as *Diabetes mellitus*, considered difficult to heal. Investigations on the pharmacological and biological activities of tannins, more specifically the tannic acid, can contribute to the technological development of cheaper and effective curative systems for skin lesions. In this context, this study aimed to perform a technological prospecting on the incorporation of tannic acid in formulations for topical use in wound healing process.

## MATERIALS AND METHODS

The prospecting was conducted based on the patent applications filed at the National Institute Database of Industrial Property (INPI), European Patent Office (EPO), United States Patent and Trademark Office (USPTO), World Intellectual Property Organization (WIPO) and Derwent Innovations Index. Also, the study investigated the publication of articles on the basis of the Web of Science. The key words used in the search are listed in Table 1. In this research, were considered valid documents that submitted key words in the "title" and/or "abstract". This prospective study was conducted in March, 2015 and included the years

available at the databases until now. With regard to the articles, the discussion was based on the technological field, country, year of publication and the bibliographic review.

## RESULTS AND DISCUSSION

### National institute of industrial property (INPI) database

The patent search in the INPI using the term "tannic acid" resulted in 15,025 patents. In the analyze of the abstracts noted that the search results generated with patents that only included the word "acid", and that did not match the tannic acid. So the study chose to use only the term "tannic" to refine the search, culminating in 18 patents. The deposit period of these patents was between the years 1991 to 2014. Brazil and the United States were the countries with the highest depositors numbers with 7 and 5 patents, respectively. Countries like Japan, Italy, Mexico, Argentina and Belgium also had depositors patents in INPI.

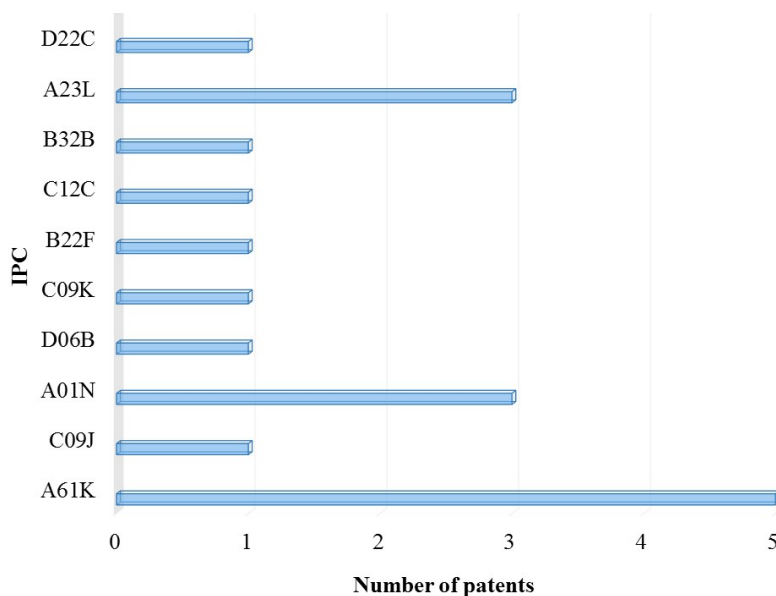
The international patent classification (IPC) classifies the invention according to different technological fields to which the invention pertains. The A61K code had the highest number of patent applications (Figure 1). This IPC is related to the area of preparations for medical purposes, dental or hygienic purposes in Human Needs section. For example, the patent CI 0400241-5 (Marques, 2005) have A61K classification and describes a formulation for tattoo removal, stretch marks, scars, keloid, vitiligo, sunburn and any visible mark characterizing a skin lesion. It consists of tannic acid and water, may be present as liquid, creamy, paste or gel forms. When applied to the skin, this product causes local inflammation that causes pigments that migrate to form a scab that detached part of the dermis caring 50 to 100% of the tattoo pigment.

The PI 9811478-6 (Lener et al., 1998), invention, also A61K IPC, refers to the controlled or sustained release compositions of pharmaceutical agents that include one or more cellulosic polymers (hydroxyethyl cellulose, hydroxypropyl cellulose or hydroxypropyl methylcellulose) or proteinaceous (gelatin or collagen), and tannic acid or other tannin. The agent's field of action is topical, local or systemic. The tannic acid when combined with these polymers can make it more resistant to proteolytic enzymes, and when applied to living tissue have astringent action, which is the therapeutic application base of tannins.

Falcon (1998), in his PI 9811875-7 invention, discloses a pharmaceutical composition based in tannic acid which can be used to treat any cancer condition. The formulation is composed of tannic acid, sorbitol, castor bean oil, potassium sorbate, methylparaben and propylparaben, and can be used in the form of tablets, capsules, solutions or suspensions. Degenerative diseases such as cancer, are associated with high intercellular concentrations of reactive oxygen species or

**Table 1.** Terms used in the study of scientific documents in databases.

Key words	Number of patent/scientific papers					
	INPI	EPO	USPTO	WIPO	DERWENTS	Web of Science
Tannic acid	15025	2319	85	1074	2926	3460
Tannic acid and healing	0	05	0	05	31	15
Tannic acid and wound	0	17	0	03	46	26
Tannic acid and carboxymethyl cellulose	0	13	01	01	60	06

**Figure 1.** IPC by number of patents.

free radicals. It is then suggested that the tannic acid acts as a free radical scavenger, which intercepts the active oxygen to form stable free radicals. It has the ability of inhibiting lipid oxidation by removing free radicals (Sanchez-Moreno et al., 1999; Gulçin et al., 2010).

The A01N and A23L classifications have around 18 patent applications, which also comprise human needs. The A01N IPC comprises biocidal formulations as disinfectants, herbicides, pesticides, repellents, and plant growth regulators. The field of preparing, processing and storage of food and non-alcoholic beverages is represented by A23L IPC.

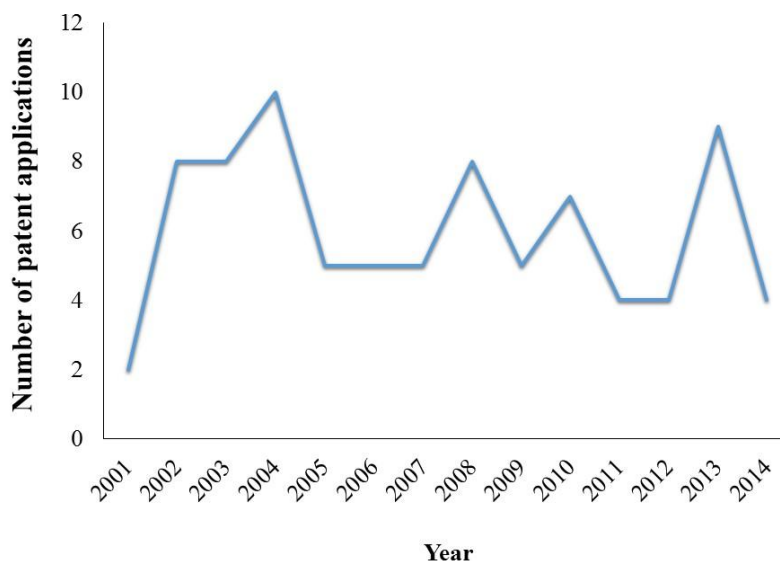
#### United States patent and trademark office (USPTO) database

On the databases of the USPTO were found 85 patent (Table 1) for the term "tannic acid". Among the countries of the depositors' origins, Canada stood out with 16 patents, followed by Gabon and Japan with 12 and 10 patents, respectively. The publication period included the years between 2001 to 2014 (Figure 2), in 2004 were 10

patents and in 2013, totaled 9 published patent applications. The patent application filed in 2015 included compositions to increase the selenium and lithium content in vegetables and processed products. The abstracts revealed that 62% of patents found were related to medical sciences or veterinary and hygiene.

The refined patent search using the terms "wound and healing" did not obtain any patent result that had tannic acid involved in the wound healing process. Although the two terms have not appeared in the title or abstract, some patents made use of tannic acid with dermatological purposes. Murard (2006), proposed a pharmaceutical composition for cleaning the skin to facilitate the prevention, treatment, and management of skin diseases, such as seborrheic dermatitis, psoriasis, folliculitis, rosacea, perioral dermatitis, acne and other inflammatory skin conditions, including a sufficient amount of a hydroxy acid or tannic acid, or a pharmaceutically acceptable salt thereof. Mitts et al. (2014), have formulated compositions containing polyphenol of ellagic acid and/or tannic acid for skin protection against degradation of elastic fibers by skin enzymes that act to promote total deposition of elastic fibers in skin cells. Hamtini (2004), developed a





**Figure 2.** Evolution of patent applications period.

rash treatment product consisting of salicylic acid, boric acid, tannic acid, zinc oxide, calamine and lanolin, castor oil or cod liver oil. In this case, the exposed tissue proteins precipitate and form an antiseptic protective layer promoting the tissue regeneration.

The only patent found with a combination of tannic acid and carboxymethylcellulose terms, discussed about an invention for the treatment of soil (Ballard, 2007). The use of organic acids have great interference in sorption/desorption processes of cations and anions, in the microbial activity, and in the metabolic disposition of carbonaceous substrate with a direct effect on soil fertility.

### **World intellectual property organization (WIPO) database**

In the WIPO database, 1074 patents were found with the use of the "tannic acid" term in the title and/or abstract. China is the country with the largest number of applications filed (287), followed by Japan (214) and United States (205) (Figure 3). The inventions stood out in the category of human needs with the A61K, A61P and A23L IPC (Figure 4). As mentioned before, the rating A61K covers the sub-area of medical science or dental and hygiene; A61P is the sub-area of specific therapeutic activity of chemical compounds or medicinal preparations, and A23L refers to processes, treating and storage of food products and non-alcoholic beverages. The publication period was between the years 1990 to 2014. The largest number of patent applications occurred in the years 2014 and 2008 with 68 cases, followed by 2000, 2007 and 2009 with 67, 58 and 54 patents, in that order. It was observed in the last two decades an

increasing in research that explore the physical, chemical, biological and pharmacological properties of tannic acid. Its application versatility can be attributed to three general characteristics of tannins: complexation with metal ions, oxidant activity and scavenging of free radicals, and the ability to form complexes with macromolecules (Bruneton, 2001). The search resulted in 3 patents with combination of the words "tannic acid and wound" in which contemplated formulations for: wound healing in horses, second degree burns and hemorrhoids treatment. Using the term "healing", 5 patents were obtained as a result of the search. The inventions have applications in wound healing in the form of ointment, chewing gum (oral ulcer) and the biomaterial (membrane) made by other chemical constituents, and tannic acid. The use of the term "carboxymethylcellulose" resulted in a patent, and that one is the same as the patent found in the USPTO database, that described the use of tannic acid in the treatment of soil.

### **European patent office (EPO) database**

The patent informations in the EPO were obtained using the ESPACENET search tool, taking the Worldwide collection of patents as a reference. The search resulted in 2319 patents using the term "tannic acid" (Table 1). The five patents found with the combination of the term "tannic acid" and "healing" are the same ones available in WIPO database. This is because the World wide database provides access to applications from more than 90 countries. Using the words "tannic acid" and "wound" the study got a number of 17 patents. Among these, nine patents were related to the human needs and hygiene. For example, the preparation of a biomaterial (in the form

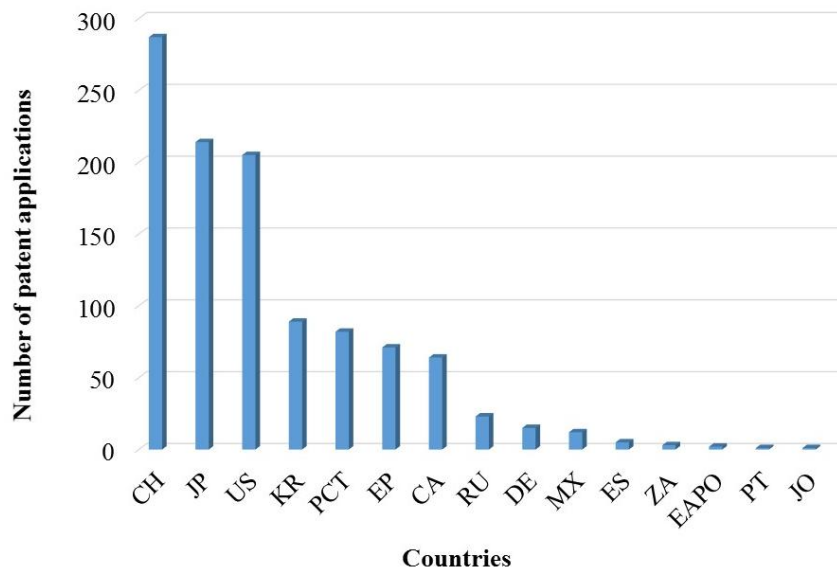


Figure 3. Number of patents per countries.

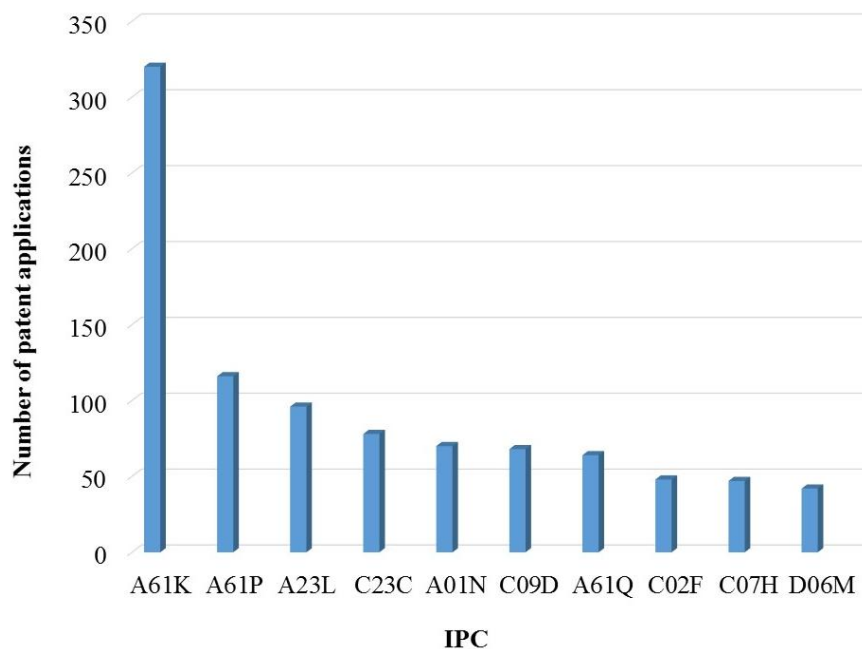


Figure 4. Number of patents per IPC.

of powder or film) for wound treatment (George, 1970). The term "wound" was used in the abstract of four patents with no topical wound healing purposes. These patents included inventions related to textile and paper industry. The search for tannic acid and carboxymethylcellulose compositions resulted in 13 patents with different application areas, like anti-rust coating, hair dye, explosives, cigarette filter and drug

entrapment.

**DERWENTS database**

In this database, 2926 patents were found using only the term "tannic acid" (Table 1). In addition to A61K classification, the C09D IPC also exceeded in number of

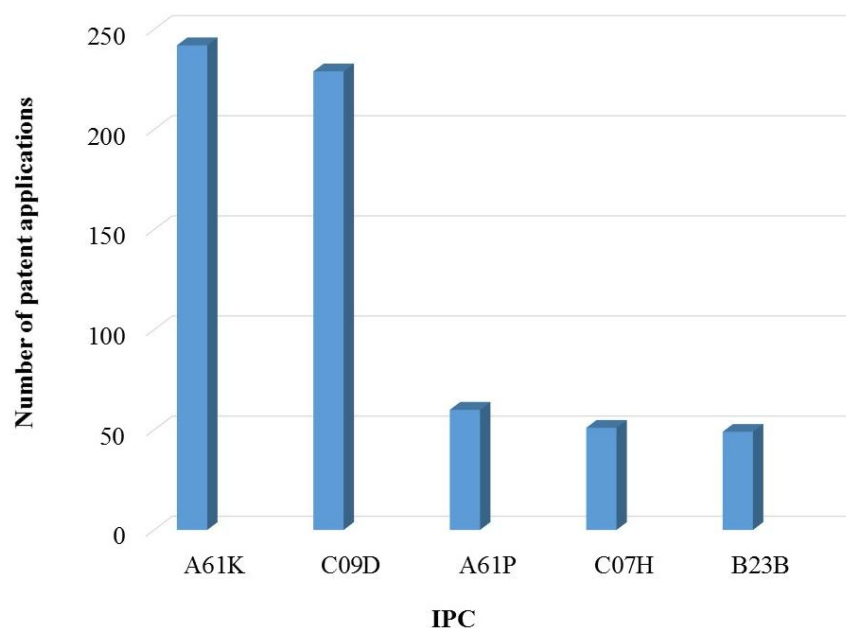


Figure 5. Number of patents per IPC.

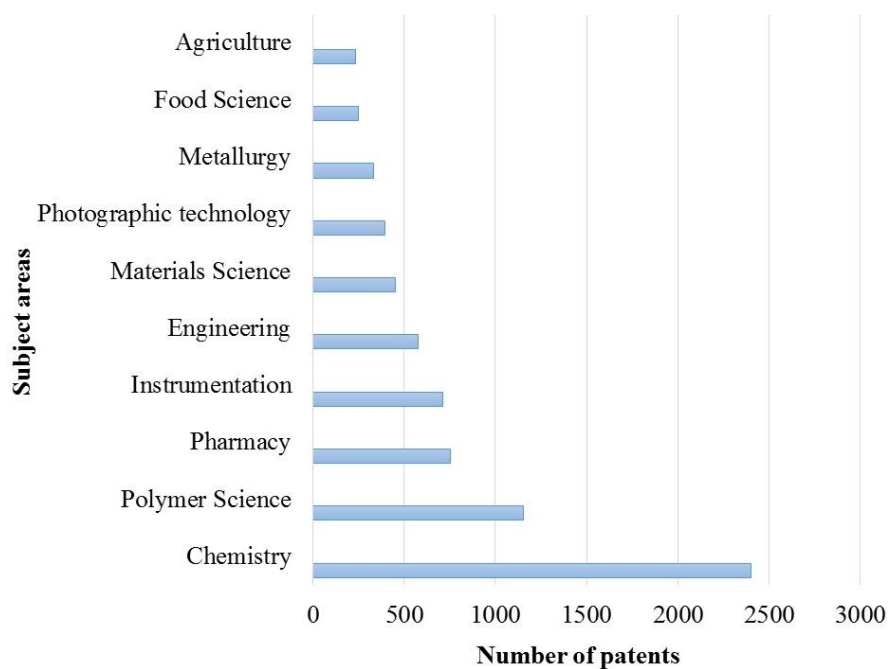


Figure 6. Number of patents for subject areas.

patents (Figure 5). The C09D category belongs to the Chemistry and Metallurgy section, but specifically, to the class of coatings, inks, dyes and removers. The chemical field, showed the greatest number of patents, followed by Polymer Sciences and Pharmacy (Figure 6). After analyzing the patent abstracts with the key words "tannic

acid and healing", it was found that four patents used the term healing as a cure synonymous, causing results of curing agents, known as hardeners and used in Chemical, Polymer and Material Science sectors. Thus, only 27 patents were directly related to wound healing. Chen and Zhou (2014), proposed a formulation based on

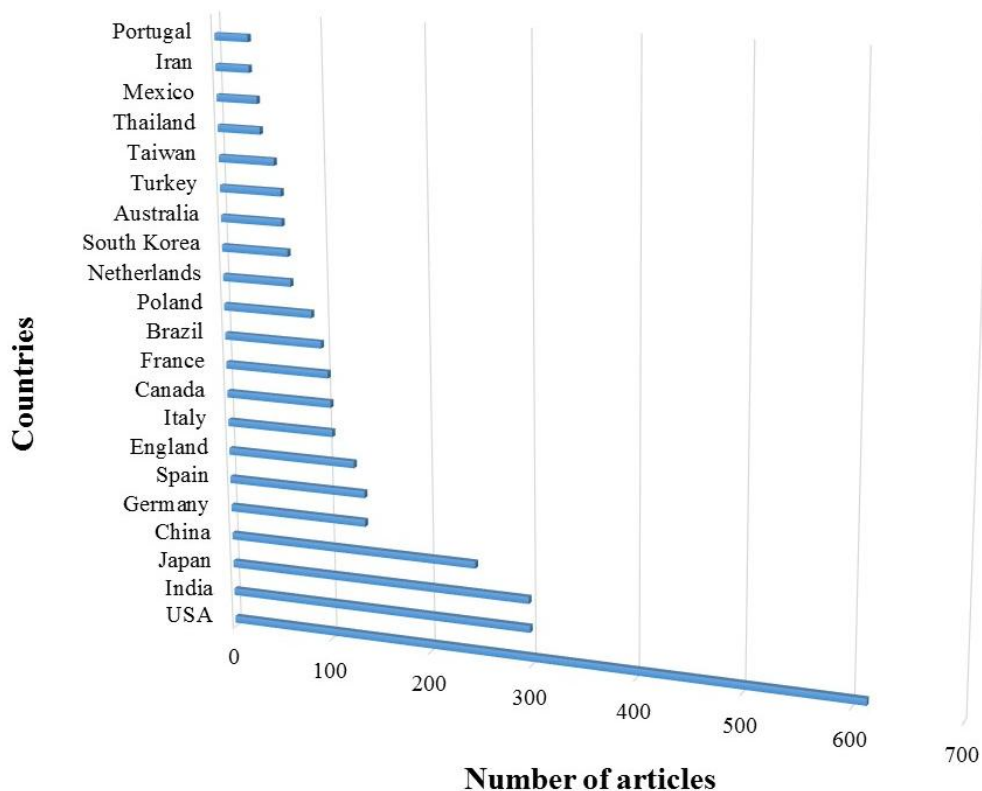


Figure 7. Number of articles per country.

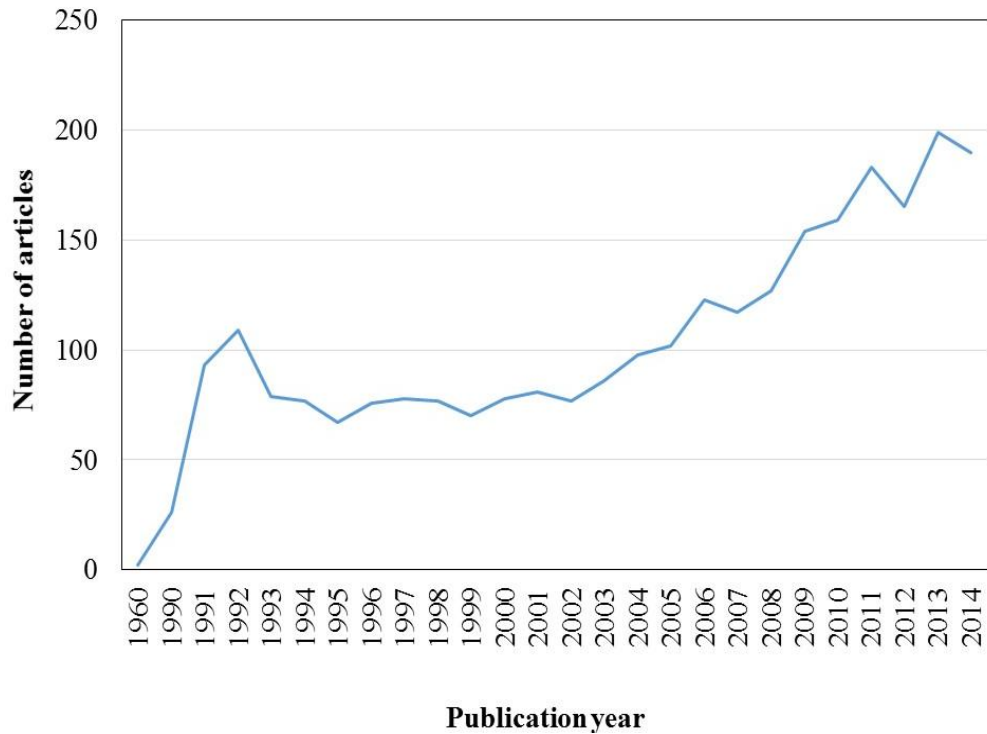
protease enzyme isolated from the terrestrial fungus *Aspergillus* and tannic acid, for the treatment of wounds originating from burns, chronic ulcers and diabetic foot. McClure (2001), has developed a pharmaceutical mixture which included sulfur, zinc oxide, tannic acid and olive oil to heal horses' wounds. According to the author, the product can be used in dogs, human beings, cows and administered topically as an ointment. The combination of the terms "tannic acid" and "carboxymethyl cellulose" resulted in 60 patents with different applications, as in the paint sector and drug delivery systems. Among the patents related to wound healing, there was a composition to treat burns which included menthol, thymol, camphor, vitamin E, tannic acid, niacin, *Salvia* extract and other constituents (Li and Zhang, 2014). Zhao (2013), developed a product constituted by seed extracts, fruit or bark, alcohol, glycerol, Carbopol, tannic acid, carboxymethyl cellulose and others. The paste obtained was indicated for the treatment of exudative ulcers, burns, eczema, bedsores and haemorrhoids.

### Web of science database

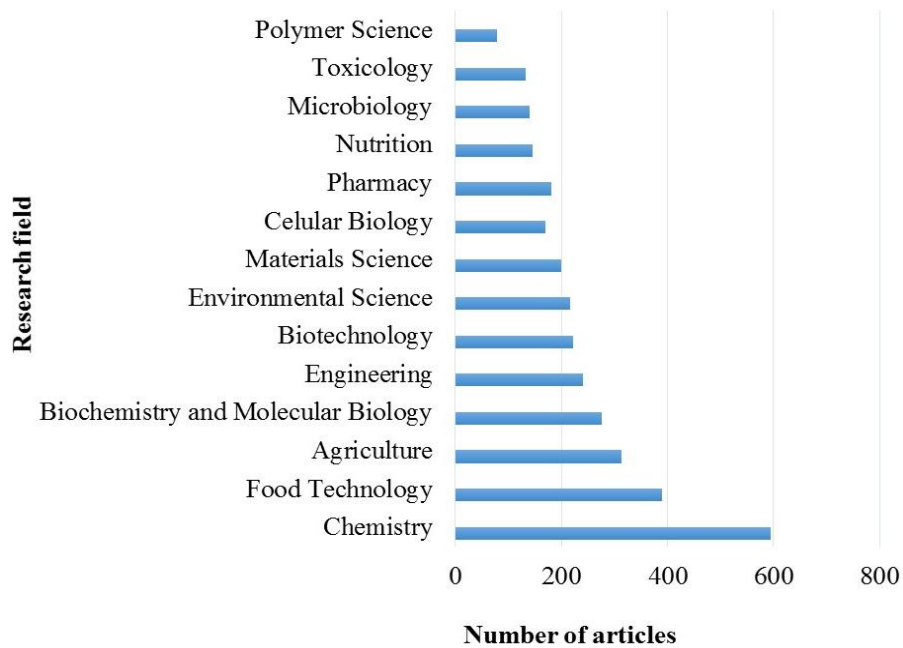
The main collection of web of science is a tool that lets you search for various types of scientific papers (articles, patents, book chapters, etc.). The search resulted in on

included 3460 documents that had "tannic acid" as a keyword, among which, 3080 were articles and two were book chapters. The United States was the country that had the largest number of research results developed with tannic acid (614 published articles), followed by India, Japan and China with 299, 298 and 246 articles, in that order (Figure 7). Brazil appears on the list with 97 publications, representing a significant number compared with patent applications. The publication period comprised the years 1960 (two posts) and 2014 (190 articles). Until the research data, it has published 35 articles in 2015. There is increasing interest in research that include to unravel the tannic acid properties aimed at different technological applications. Figure 8 shows the evolution of the number of publications per year. The chemical field presented the greatest number of publications with 595 articles (Figure 9). Among these articles, there is a study of processing and characterization of chitosan nanocomposites reinforced with chitin, using tannic acid as a crosslinking agent. The addition of chitin improved mechanical properties of the chitosan film and, tannic acid reduced the moisture content and solubility in water of these nanocomposites (Rubentheren et al., 2015).

The search for data with the combination of the terms "tannic acid" and "healing" resulted in 15 documents. Among these are 11 articles, six of which publications



**Figure 8.** Number of articles per publication year.



**Figure 9.** Number of articles per research field

belong to the areas of Pharmacy and Chemistry, with three items each. Japan stood out with three published articles Table 2. One of these articles had a study of

calcium phosphates composites which included tannic acid in the composition. The material was intended to repair bone defects (Yoshikawa and Toda, 2000). The tannic

**Table 2.** Published articles about the tannic acid incorporation in the healing process of skin wounds (Web of Science).

Periodic	Authors	Title	Innovation
Biomaterials	Heijmen et al. (1997)	Cross-linking of dermal sheep collagen with tannic acid	Characterization of biomaterials developed from the dermal sheep collagen matrix, tannic acid and metallic ions with possible application as a therapeutic agent in the burn treatment.
Journal of Biomedical Materials Research	Yoshikawa and Toda (2000)	Reconstruction of alveolar bone defect by calcium phosphate compounds.	Use of tannic acid in the cement liquid phase calcium phosphate based in bone alveolar reconstruction process.
Wounds-A Compendium of Clinical Research and Practice	Halkes et al. (2001)	The use of tannic acid in the local treatment of burn wounds: Intriguing old and new perspectives	Review study of the tannic acid use in the burn treatment. A critical appraisal of the literature showed that liver toxicity is not exclusively related to the use of tannic acid. Furthermore, in the studies reported adverse effects, tannic acid preparations used in high concentrations, or impurities. The survey showed the use of this tannin with potential in treating burns in safe dosages.
Free Radical Biology and Medicine	Khanna et al. (2002)	Dermal wound healing properties of redox-active grape seed proanthocyanindins	The tannin extract use obtained from the grape seed for topical application in dermal healing <i>in vivo</i> processes.
Burns	Halkes et al. (2002)	Transaminase and alkaline phosphatase activity in the serum of burn patients treated with highly purified tannic acid	Treatment of burns with highly purified tannic acid. The tests indicate that tannic acid does not cause hepatotoxicity when applied by at least 10% of the total body surface area.
Bioorganic and Medicinal Chemistry Letters	Halkes et al. (2002)	Synthesis and biological activity of polygalloyl-dendrimers as stable tannic acid mimics	Synthesis of dendrimer derivatives of tannic acid with stability, antioxidant activity and collagen crosslinking capacity improved. These compounds showed potential application in the burn treatment.
Journal of Materials Science: Materials in Medicine	Silva et al. (2003)	In vitro degradation and cytocompatibility evaluation of novel soy and sodium caseinate-based membrane biomaterials	Biomaterials consisting of crosslinked casein with tannic acid did not exert cytotoxic effects on cells and promoted their proliferation. The membranes demonstrated potential application in drug delivery system and astopicals curatives.
Revista de Chimie	Albu et al. (2009)	Spectral Characteristics and Antioxidant Properties of Tannic Acid Immobilized on Collagen Drug-Delivery Systems	Obtaining biomaterials compounds with antioxidant activity made up of collagen and tannic acid for use in both topical formulations as biativos systems in the wound healing process.
Molecular crystals and liquid crystals	Albu et al. (2010)	Doxycycline Delivery From Collagen Matrices Crosslinked With Tannic Acid	Collagen matrices containing doxycycline and tannic acid showed activity against gram-positive and gram-negative bacteria, fungi or yeasts. The material may be suitable for wound treatment.

Table 2. Contd.

Letters in Microbiology	Applied	Chusrin and Voravuthikunchai (2011)	Damage of staphylococcal cytoplasmic membrane by <i>Quercus infectoria</i> G. Olivier and its componentes	Establishment of the mechanism of action of thr ethanol extract of <i>Q. infectoria</i> , as well asits major constituent chemicals for their application in skin infections and wounds. The study provides scientific information to support its traditional use, suggesting antibacterial mechanisms.
International Journal	Wound	Hemmati et al. (2011).	Topical grape ( <i>Vitis vinifera</i> ) seed extract promotes repair of full thickness wound in rabbit	Evaluation of healing activity of hydroalcoholic extracts of grape seed in rabbits. The results demonstrated that the topical use of the extracts promoted wound healing.
Surgery Today		Hachiro et al. (2011)	Aluminum potassium sulfate and tannic acid (ALTA) injection as the mainstay of treatment for internal hemorrhoids	The therapy for symptomatic hemorrhoids with the use of potassium sulphate and aluminum and tannic acid. The therapy proved to be simple and safe, with few complications.
Digest Journal of Nanomaterials and Biostructures		Jinga et al. (2013)	Silver green synthesis on bacterial celulose membranes using tannic acid	Silver nanoparticles were added to the bacterial cellulose membrane using tannic acid as crosslinking agent. The films showed good antibacterial activity against <i>E. coli</i> K12 MG1655, and can be used as wound curative.
Journal of Biomedical Materials Research Part B-Applied Biomaterials.		Natarajan et al. (2013)	Preparation and properties of tannic acid cross-linked collagen scaffold and its application in wound healing.	Development of cross-linked collagen films with tannic acid. The material has potential for use as a curative on skin lesions.
Digest Journal of Nanomaterials and Biostructures		Popa et al. (2013)	Hysteresis of contact angle. Dynamic wettability studies of collagen and doxycycline porous matrices crosslinked with tannic acid	Porous membranes of collagen crosslinked with doxycycline and tannic acid showed good wetting properties and hydrophobicity for use in drug delivery systems.
Chemical Communications		Krogsgaa et al. (2014)	Gels and threads: mussel-inspired one-pot route to advanced responsive materials	Obtaining hydrogels for biomedical use from the reaction with tannic acid, trivalent metal ions and polyallyamine.
International Pharmacology		Upadhyay et al. (2014)	A review on the pharmacological aspects of <i>Terminalia chebula</i> .	A review study of the pharmacological effects of <i>Terminalia chebula</i> and the relation of isolated chemical constituents in its biological activities.
Journal of Bioactive and Compatible Polymers: Biomedical Applications		Xu et al. (2014)	Large-scale production of a ternary composite nanofiber membrane for wound dressing applications	The development of membranes from chitosan, tannic acid and polyvinyl alcohol. The membranes showed potential for use as wound curative with antimicrobial activity against <i>E. coli</i> , adhesion and development of fibroblast cells.
Diseases of the colon and rectum		Abe et al. (2014)	Combined aluminum potassium sulfate and tannic acid sclerosing therapy and anal encirclement using an elastic artificial ligament for rectal prolapse	Evaluation of clinical data and recurrence rates in patients with rectal prolapse treatment based on the potassium sulfate, tannic acid and aluminum. The procedure is easy to perform, inexpensive, low recurrence rate and appears to be a reasonable alternative to rectal prolapse in elderly patients, debilitated and high risk.

Table 2. Contd.

RSC Adv.	Zhou et al. (2014)	Vacuum-assisted layer-by-layer electrospun membranes: antibacterial and antioxidative applications	The LBL type of film prepared with tannic acid and AGNPS-Lis showed antioxidant and antibacterial activity and potential for application in food packaging, wound healing and tissue engineering.
Carbohydrate Polymer	Xu et al. (2015)	Development of tannic acid/chitosan/pullulan composite nanofibers from aqueous solution for potential applications as wound dressing	Development of curative systems for wounds from nanocomposites of tannic acid/chitosan/pullulan. The material showed antibacterial properties and promoted the regeneration of skin on wound healing assays.
European Journal of Medicinal Chemistry	Moulaoui et al. (2015)	Identification and nanoentrapment of polyphenolic phytocomplex from <i>Fraxinus angustifolia</i> : In vitro and in vivo wound healing potencial	The quercetin, rutin, catechin and tannic acid were identified in the extracts of the leaves and bark of <i>Fraxinus angustifolia</i> species. The application of the extracts in wound healing promoted the cure of the wounds.
Journal of Applied Polymer Science	Sahiner et al. (2015)	p(AAm/TA)-based hydrogel films with antimicrobial and antioxidant properties for biomedical applications	Crosslinked polyacrylamide hydrogel preparation with tannic acid and evaluation of antibacterial and antioxidant properties. The hydrogel has the potential for application in the biomedical field.

acid can be applied in the dentistry field for the complex dentin-pulp protection.

In the area of wound healing, the development of tannic acid/chitosan/pullulan composite showed potential applications as a curative, with antibacterial properties against Gram-negative bacteria (*Escherichia coli*), and favor the attachment of fibroblast cells and intercalary growth on the membrane, regenerating the skin (Xu et al., 2015). The healing properties of tannins is attributed to its astringent action, promoting the elimination of water into cells, leading to contraction of the fibers by decreasing hemorrhage and facilitating healing.

Akiyama et al. (2001), investigated the tannins antibacterial action against *Staphylococcus aureus* and the effect of standard chemotherapy in combination with tannic acid. The results indicate that tannic acid can be a useful adjunctive agent for treating skin infections caused by *S. aureus*, because this tannin seems to act on the cell membranes of *S. aureus*. The  $\beta$ -lactam antibiotics had their antistaphylococcal activity enhanced by the presence of tannic acid.

Park et al. (2006) and Jung et al. (2010), examined the effect of tannic acid on skin inflammation with a cell line of human keratinocytes (HaCaT). Topical application of tannins in mouse skin treated with UVB irradiation demonstrated inhibition of erythema formation. The results showed that the tannic acid presented significant anti-inflammatory activity in response induced by UVB radiation on the skin and can be a natural candidate

compound for regulating skin inflammation.

The combination of the words "tannic acid" and "wound" were found in 26 documents, including 23 articles. The United States published five articles, followed by Romania with 4 articles. Four of the 23 articles belong to the dermatological field, one of which evaluated the effects of burn treatment with highly purified tannic acid. The results indicate that purified tannic acid can provide a valuable tool for improving wound healing (Halkes et al., 2002). In another study, tannic acid promoted the stabilization of the collagen matrix against collagenolytic degradation, achieving significant results on wound healing in rats. The collagen-based material and tannic acid were considered biodegradable, and may have applications in tissue engineering and drug entrapment (Natarajan et al., 2013). The tannic acid has important properties that gives it a variety of technological applications, especially in the Pharmaceutical field as curative in the wound healing process.

Using the "carboxymethylcellulose" and "tannic acid" terms, six articles were obtained of which covered different areas, such as quantification of heavy metals in water (Sui et al., 2013), substrates for isolated yeasts from the rotten wood (Middelhoven, 2006), wax additives (Tascioglu and Akar, 2003) and reduced astringency of phenolic compounds (Troszynska et al., 2010). Two articles have dealt with the use of tannic acid and carboxymethylcellulose as biomaterials in the medical



field. Shutava et al. (2009), research involved the development of nanocapsules with polyelectrolyte polymer for entrainment of natural polyphenols, in order to assess its anti-cancer potential. The second article encompassed the studies by Aelenei et al. (2009), which reviewed the addition of tannic acid in biodegradable polyelectrolyte complex for medical and pharmaceutical applications. The results confirmed the possibility of adding tannic acid in microparticles based on chitosan, sodium alginate and carboxymethylcellulose. These complexes have shown potential for use in the treatment of dental pain and skin lesions. Table 2 includes the searches with tannic acid, pure or extract, in the development of biomaterials for the treatment of dermal healing.

## Conclusion

In pure form or in extracts, the tannic acid introduced itself as a versatile material with applications in different industrial sectors. Regarding the healing subject, the United States, Japan and China are the countries that invested the most in the elucidation of mechanisms and inventions that contemplated the different properties of tannic acid in wound healing, burns and inflammations. Tannins are found in nearly all plant families, and Brazil has a large plant diversity, which makes it a potential field to develop biomaterials with extracts rich in tannic acid. The public spending on curative for skin ulcers is growing, which requires urgent researches to develop more effective healing systems at lower cost. Within this scenario, the tannic acid appears with potential application in topical formulations, although there are controversial ideas about its cytotoxic effects. Also, in Brazil the development of this economic sector is still lacking in research and technology.

## Conflict of Interest

The authors have not declared any conflict of interest.

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Full Length Research Paper

## Effects of rice straw burning products on guinea pig lungs

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This study aims to investigate rice open field burning and their deleterious effects which has become an obvious problem in Egypt, which apparently affects the Egyptians' lungs and increased hospital admission for pulmonary complaints. Therefore, this study was designed to investigate this phenomenon by using guinea pigs which were subjected to rice straws burning products (RSBP). The effects of RSBP on differential leukocytic count in lung lavages, histopathological examination, malondialdehyde (MDA) content in lung tissues (marker of lipid peroxidation) and nitric oxide (NO) content in lung tissues were studied. Results of this study demonstrated that RSBP induced pulmonary emphysematous lesions are progressive with subsequent smoke exposures together with the sensitization of the lung in the present model. Changes in the count of macrophages, neutrophils and eosinophils in the bronchoalveolar lavage (BAL) of guinea pigs lungs compared to normal lavages. RSBP exposure has a potent potential capacity for being a source of reactive oxygen species and possibly oxidizing species which can lead to decrease nitric oxide content in lung tissue.

**Key words:** Rice, straws, smoke, guinea pigs, total leukocytic count, neutrophil and eosinophils count, macrophages count, nitric oxide content, malondialdehyde content, lung, histopathological examination.

### INTRODUCTION

Over the last few years, Egyptian farmers in the middle delta tend to burn the rice straw in the rice field as an easy and cheap method for its disposal. At the same time, farmers burn the rice straws to kill insects and provide minerals to the soil (Estrellan et al., 2010). Agricultural field burning activities are linked to elevated air pollution levels in Asia, for example, Taiwan (Yang et al., 2006), Thailand (Tipayarom et al., 2007), USA (Jiminez et al., 2007) and Europe (Viana et al., 2008). Clinicians working in agricultural areas in delta counties

are aware of an abrupt increase of patients suffering from asthmatic attacks after the harvesting is completed, and the remaining paddy straws have dried up. The patients themselves are aware that rice smokes aggravate their airway symptoms after the harvesting season (from September to November) every year. This can result in adverse health effects (Regalado et al., 2006; Yang et al., 2007; Ryu et al., 2007).

In recent years, it has been observed that open burning of crop residues also contributes to emissions of harmful

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air pollutants, which can cause severe impacts on human health, including polycyclic aromatic hydrocarbons (PAHs) (Korenaga et al., 2001), as well as polychlorinated dibenzo-p-dioxins (PCDDs), and polychlorinated dibenzofurans (PCDFs), referred to as dioxins (Gullett and Touati, 2003; Lin et al., 2007). These air pollutants have significant toxicological properties and are notably potential carcinogens. Air pollution not only affects human health and the environment, but also indirectly the economy of a country. Air pollutants and emission factors are considered for rice straw open burning. Open field burning is an uncontrolled combustion process during which species such as CO<sub>2</sub>, nitrous oxide (N<sub>2</sub>O), CH<sub>4</sub>, CO, non-methane hydrocarbons (NMHC), NO<sub>x</sub>, SO<sub>2</sub>, particulate matter (PM) and few others are being emitted as mentioned in Gadde et al. (2009) study. Among these, the greenhouse gases (GHGs) of importance are N<sub>2</sub>O and CH<sub>4</sub> which contribute to global warming and climate change. CO<sub>2</sub> emitted from biomass burning is considered to have a neutral effect due to its photosynthetic uptake during plant growth. Particulate matter (PM), because of their impacts on human health and the environment, can be further categorized as PM less than 2.5 micron (PM<sub>2.5</sub>) and PM less than 10 micron (PM<sub>10</sub>). PAHs and PCDD/F are also of importance due to their toxicity and carcinogenic nature. Emission factors specific to air pollutant species emitted from open field burning of agricultural residues are presented in Gadde et al. (2009) study. This emission factors (EFs), which is the fraction of the mass combusted during the course of a fire, were collected from the literature and are mostly specific to rice straw burning.

A larger number of people can be subject to rice straws burning products (RSBP) effects in the event that the dominant winds are directed towards more densely populated areas since many urban areas are typically located next to rice cultivated areas. Therefore, although rice cultivation has assumed great importance for the national economy, especially because of the growing demand for food in the current decade, it presents a high potential for environmental impact. This burning practice is likely to remain an important issue until effective measures are taken to eliminate this practice. The rice smoke is an important PAH emission source. The rice straw burning primarily contributes fine particulate PAHs. During the rice straw burning periods, PAH size distribution shows that 70.9% of PAH mass is distributed in the sizes smaller than 2.5 μm, which can easily penetrate the pulmonary alveoli and damage the health of humans (Hsi-Hsien et al., 2006; Chia-Hsiang et al., 2009).

Particles from wood smoke have been associated with various indicators of inflammation, including pulmonary influx of inflammatory cells such as neutrophils, lymphocytes and alveolar macrophages (Larsson et al., 2007). An increase of neutrophils in the airway lumen has

been documented by bronchoalveolar lavage (BAL) fluid and sputum analysis in smokers with mild to moderate chronic airway obstruction (Bohadana et al., 2004). The guinea pig cigarette smoke (CS) models have contributed to the understanding of histological and physiological aspects of smoke-associated lung disease. Guinea pig models are considered to be adequate for further investigations of chronic obstructive pulmonary disease (COPD) because of the anatomical and pathophysiological similarities to human COPD. The guinea pig develops morphologic and physiologic alterations after exposure to cigarette smoke at roughly the same concentrations as humans. In contrast, rats and mice require respectively greater and shorter exposure concentrations before developing disease than humans do (Kubo et al., 2005; Ricciardolo et al., 2008).

In this study, we used guinea pig model for exposure to RSBP, and sensitization with egg albumin for simulation of hypersensitivity reaction, in order to investigate various histopathological changes and bronchoalveolar lavages to see what possible cells are responsible for lung damage. The suspected effects of the RSBP on the content of malondialdehyde (MDA) in lung tissues as a marker of lipid peroxidation, and oxidative stress have also been investigated. Nitric oxide (NO) content in lung tissues was also studied.

## MATERIALS AND METHODS

Normal healthy male guinea pigs (Egyptian breeding) weighing 400 to 500 g were used. The animals were kept in cages, in a standard animal laboratory room. They had free access to water and food at room temperature. At the moment of experiment, guinea pigs were all of the same age, and had approximately the same body weight in order to minimize biological variations. Rice straws were brought from nearby country area in Gharbiya County, Tanta, Egypt. The burning rate was adjusted to burn about 10 g of rice straws in about 20 min on an electric heater (Gallenkamp Magnetic Stirrer Hotplate 400, UK). It was adjusted at the middle heat which was about 200°C. The burnings were continued for 4 h per day in a burning chamber. The animal exposure was performed by direct inhalation of the smokes for 4 h. Smokes come out from burning chamber by inlet pipe to exposure chamber made of wood of the following diameters 90×50×45 cm with inlet opening for air atmosphere to enter. The area of the pipe of the inlet is about (1 cm<sup>2</sup>) (Six animals per each exposure chamber).

### Egg albumin suspension (EA)

A suspension of egg albumin (Sigma Chemical Co. Ltd., Egypt) was prepared in normal saline to give a final concentration of 100 mg/ml and 10 mg/ml for sensitization of guinea pigs.

### Induction of sensitization

Guinea pigs sensitization was induced according to a previously described procedure (Mccraig, 1987). The animals were injected subcutaneously and intraperitoneally I.P. with two equal doses of egg albumin and 1 ml of suspension containing 100 mg egg albumin (EA) on day one, and a further 10 mg I.P. on day eight. At

day 14, sensitized animals were exposed to an aerosol of 4% EA for 18±1 days, 4 min daily. The aerosol was administered in a closed chamber, dimensions 30x20x20 cm. Control group was treated identically, except that 0.9% saline vehicle alone was used. The animals were ready for exposure to the RSBP.

### The experimental design

The animals were divided into six groups. Six guinea pigs were used in each group:

- A. Control group: Non-exposed normal guinea pig.
- B. Egg albumin sensitization group: Guinea pigs with sensitized lungs.
- C. Acute RSBP exposed group: Normal guinea pigs exposed to RSBP for two weeks.
- D. Subchronic RSBP exposed group: Normal guinea pigsexposed to RSBP for four weeks.
- E. Sensitizedacute RSBP exposed group: Sensitized guinea pig exposed to RSBP for two weeks directly after sensitization period.
- F. Sensitized Subchronic RSBP exposed group: Sensitized guinea pig exposed to RSBP for four weeks directly after sensitization period.

### Inflammatory cell analysis in bronchoalveolar lavage (BAL)

Guinea pigs were anaesthetized with pentobarbital sodium (60 mg/kg i.p.). The thorax was opened and the aorta and inferior vena cava were cut. The trachea was then cannulated and 10 ml of phosphate buffered saline at 37°C was gently introduced into the lung and then gently withdrawn. Three further washes with 10 ml of the saline were carried out. The combined lavage fluid was kept in an ice bath. The collected BAL was centrifuged at 1,500 rpm for 10 min, (GIBCO, Grand Island, NY). Total cell counts were determined by using (Burkerhemocytometer, NY). Differential leukocyte counts were then performed on cytospin slides. A minimum of 300 cells were identified and differentiated as mononuclear cells, neutrophils or eosinophils using the standard morphological criteria (Kubo et al., 2005). Points falling on a specific cell type must be counted, and then divided by the total number of points falling on tissue area in each microscopic field, as previously described (Gundersen et al., 1988)

### Determination of lung lipid peroxides contents measured as malondialdehyde (MDA)

MDA lung tissue content was assayed as an indirect indicator of *in situ* lipid peroxidation (Yoshioka et al., 1979). Briefly, lung tissues were homogenized in 10 volumes ice-cold 1.15% (w/v) potassium chloride solution using polytron homogenizer (PT 3100). To 0.5 ml of homogenate, 3 ml of 0.5% (w/v) trichloroacetic acid and 1 ml of 0.6% (w/v) thiobarbituric acid were added; the entire solution was then mixed and heated for 45 min in a boiling water bath. After cooling, 4 ml *n*-butanol was added and the sample vigorously shaken. The *n*-butanol layer was separated by centrifugation at 3000 rpm for 15 min. The absorbance of the pink colored product was measured at 535 nm against blank containing water instead of the sample, using double-beam spectrophotometer (Shimadzu UV-PC 1601, Japan).

### Determination of lung nitric oxide contents

The lung nitric oxide content was determined by measuring its

stable metabolites nitrite and nitrate (Miranda et al., 2001) briefly, lung tissues ( $\approx 0.25\text{g/guinea pig}$ ) were homogenized in 10 volumes of ice cold saline (0.9% NaCl) using polytron homogenizer (PT3100). 1 ml absolute ethanol was added to 0.5 ml of the homogenate toprecipitate proteins. Samples were centrifuged at 3000 rpm for 10 min. Addition of 0.5 ml saturated solution of vanadium (III) chloride (8 mgVCl<sub>3</sub>/ml) to 0.5 ml of the clear supernatant was rapidly done, followed by addition of 0.5 ml freshly prepared griess reagent. The mixture was vortexed, and incubated at 37°C for 30 min in a water bath. The absorbance of samples was measured at 540 nm using double-beam spectrophotometer (Shimadzu UV-PC 1601, Japan).

### Histopathological examination of lung sections

At the end of the rice smoke exposure, the lungs were immediately removed, washed with saline, and prepared for histopathological examination. The lungs was immediately fixed in 10% buffered formalin solution (pH 7.4) for 24 h, and then routinely processed in ascending grades of alcohol, then xylene. The tissues were then embedded in paraffin wax, serially-sectioned to  $\approx 4 \mu\text{m}$  thickness, and stained with Hematoxylin and Eosin (H&E; Sigma). Ultimately, each stained tissue section was examined using a light microscope (Olympus BX 51, Olympus America, Melville, NY) and photographed with a digital camera (Olympus DP11) connected to the microscope.

### Statistical analysis

The results represented as the mean  $\pm$  SD of cell count change. The collected data were organized, tabulated and statistically analyzed using SPSS software (Statistical Package for the Social Sciences, version 16, SPSS Inc. Chicago, IL, USA). For comparison between more than two means of parametric data, F value of ANOVA test was calculated, where scheffe test was performed to compare between each two means if F value was significant. Significance was adopted at  $p < 0.05$  for interpretation of results of tests of significance (Dawson and Trapp, 2001) (Tables 1 and 2, Figures 15 to 18)

## RESULTS AND DISCUSSION

The resulting hazards of smoke exposure arise from burning of rice straws and its deleterious effects on the lungs of exposed persons in Egypt. Fine particles, one of the major pollutants emitted from the field burning of rice straws, are of major concern due to their harmful effects on human health (Pope et al., 2009). Crop rice burning is a serious environmental health hazard, and children are more sensitive to air pollution, as RSBP poses some unrecoverable influence on their pulmonary function test (PFT) (Awasthi et al., 2010), early small airway obstruction (Ravinder et al., 2012). PAHs compounds have also been reported to induce a release of pro-inflammatory mediators, supporting a role for PAHs in the pro-inflammatory response (Lecureur et al., 2005). Substituted PAHs, such as nitro- and oxy-PAHs, have been suggested to affect biological systems by induction of oxidative stress, mitochondrial damage, necrosis and apoptosis (Xia et al., 2004; Kubatova et al., 2006; Landvik et al., 2007).

**Table 1.** Mean values of blood cells in lung lavage of the study groups of guinea pigs (exposed and not exposed to rice straw burning products (RSBPs) (n=36).

Findings of lung lavage	The study guinea pigs (n=36)						F-value (P)	
	Unexposed to RSBPs (n=12)			Exposed to RSBPs (n=24)				
	Group (A) Control (n=6)	Group (B) (Sensitized) (n=6)	Group (C) Normal acute exposed (n=6)	Group (D) Normal subchronic exposed (n=6)	Group (E) Sensitized acute exposed (n=6)	Group (F) Sensitized subchronic exposed (n=6)		
<b>Total white blood cells (TWBC) (cell/um<sup>2</sup>)</b>								
Range	715-975	2756-3320	975-1350	2058-2836	3900-4550	3900-4550		
Mean±SD	845±92.73	2975±207.04	1124±157.49	2440±318.89	4115±235.79	4146±223.20	256.90 (0.0001*)	
Median	838	2913	1089	2439	4040	4097		
<b>Scheffe test (P)</b>	GA vs GB, P=0.0001*, GA vs GD, P=0.0001*, GA vs GE, P=0.0001*, GA vs GF, P=0.0001*		GB vs GC, P=0.0001*, GB vs GD, P=0.011*, GB vs GE, P=0.0001*, GB vs GF, P=0.0001*	GC vs GD, P=0.0001*, GC vs GE, P=0.0001*, GC vs GF, P=0.0001*	GD vs GE, P=0.0001*, GD vs GF, P=0.0001*	-	-	-
<b>Macrophages (cell/um<sup>2</sup>)</b>								
Range	81-98	38-59	59-77	69-91	36-49	36-49		
Mean±SD	91±5.90	46±8.71	70±6.86	80±7.89	40±4.59	42±4.88	64.020 (0.0001*)	
Median	91	43	70	80	39	41		
<b>Scheffe test (P)</b>	GA vs GB, P=0.0001*, GA vs GC, P=0.0001*, GA vs GE, P=0.0001*, GA vs GF, P=0.0001*		GB vs GC, P=0.0001*, GB vs GD, P=0.0001*	GC vs GE, P=0.0001*, GC vs GF, P=0.0001*	GD vs GE, P=0.0001*, GD vs GF, P=0.0001*	-	-	-
<b>Neutrophils (cell/um<sup>2</sup>)</b>								
Range	5-9	38-59	9-18	23-39	68-86	62-81		
Mean±SD	7±1.41	45±7.73	13±3.16	30±6.20	74±6.48	71±7.92	136.04 (0.0001*)	
Median	7	44	13	30	73	70		
<b>Scheffe test P</b>	GA vs GB, P=0.0001*, GA vs GD, P=0.0001*, GA vs GE, P=0.0001*, GA vs GF, P=0.0001*		GB vs GC, P=0.0001*, GB vs GD, P=0.008*, GB vs GE, P=0.0001*, GB vs GF, P=0.0001*	GC vs GD, P=0.002*, GC vs GE, P=0.0001*, GC vs GF, P=0.0001*	GD vs GE, P=0.0001*, GD vs GF, P=0.0001*	-	-	-
<b>Eosinophils (cell/um<sup>2</sup>)</b>								
Range	0	3-8	1-3	1-2	5-9	6-9		
Mean±SD	0	6±1.75	2±0.75	1±0.55	7±1.47	8±1.26	52.126 (0.0001*)	
Median	0	6	2	1	7	8		
<b>Scheffe test P</b>	GA vs GB, P=0.0001*, GA vs GE, P=0.0001*, GA vs GF, P=0.0001*		GB vs GC, P=0.0001*, GB vs GD, P=0.0001*, GB vs GF, P=0.049*	-	-	-	-	-
<b>Basophils (cell/um<sup>2</sup>)</b>								
Range	0	0	0	0	1-3	1-3	-	
Mean±SD	0	0	0	0	2±0.82	2±0.89	-	
Median	0	0	0	0	2	2	-	

\*Significant (P&lt;0.05)

**Table 2.** Mean values of malondialdehyde (MDA) and nitric oxide content (NO) of the study groups of guinea pigs (exposed and not exposed to rice straw burning products (RSBPs) (n=36).

Oxidative stress indicators	The study guinea pigs (n=36)						F-value (P)
	Unexposed to RSBPs (n=12)		Exposed to RSBPs (n=24)				
	Group (A) Control (n=6)	Group (B) (Sensitized) (n=6)	Group (C) Normal acute exposed (n=6)	Group (D) Normal subchronic exposed (n=6)	Group (E) Sensitized acute exposed (n=6)	Group (F) Sensitized subchronic exposed (n=6)	
<b>MDA (uM/g tissue)</b>							
Range	43-59	43-59	58-78	82-99	84-95	83-110	
Mean±SD	50.50±6.06	53.17±6.58	64.33±7.23	91.50±6.83	89.00±4.86	93.50±9.95	47.499 (0.0001*)
Median	50.50	56.00	62.00	91.50	88.50	93.00	
<b>Scheffe test (P)</b>	GA vs GD, P=0.0001*, GA vs GE, P=0.0001*, GA vs GF, P=0.0001*	GB vs GD, P=0.0001*, GB vs GE, P=0.0001*, GB vs GF, P=0.0001*	GC vs GD, P=0.0001*, GC vs GE, P=0.0001*, GC vs GF, P=0.0001*	-	-	-	-
<b>NO (uM/g tissue)</b>							
Range	52-68	39-59	20-35	24-39	24.39	24-39	
Mean±SD	60.67±6.38	46.83±8.11	27.00±5.62	30.33±5.99	30.67±4.93	31.17±5.98	26.831 (0.0001*)
Median	61.50	43.00	26.00	29.50	30.00	31.50	
<b>Scheffe test (P)</b>	GA vs GB, P=0.028*, GA vs GC, P=0.0001*, GA vs GD, P=0.0001*, GA vs GE, P=0.0001*, GA vs GF, P=0.0001*	GB vs GC, P=0.001*, GB vs GD, P=0.005*, GB vs GE, P=0.007*, GB vs GF, P=0.009*	-	-	-	-	-

\*Significant (P<0.05)

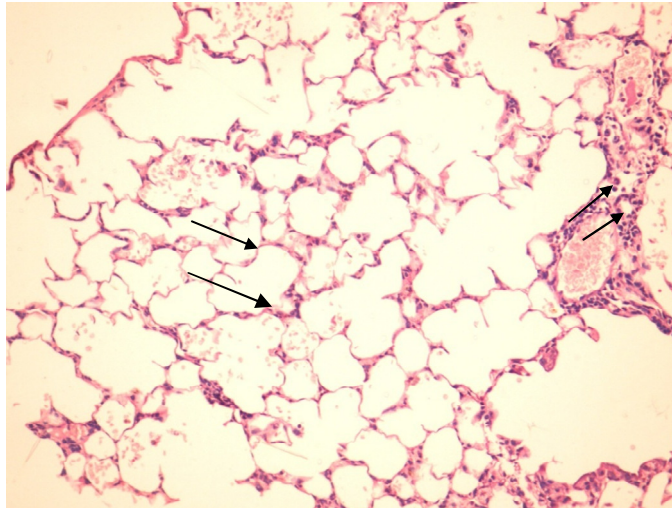
Total WBC significantly increased in subchronic exposed groups with normal or sensitized lungs, also in sensitized acute exposed lungs which suggest that RSBP had the potential to alter host pulmonary immune defense mechanisms. However, gp B, which was sensitized and not exposed to RSBP, also had an increase of WBC, once sensitization is related to inflammation. So, cell recruitment observed in groups E and F could have happened in response to both sensitization and RSBP exposure.

In the present study, non exposed Egg albumin sensitized lungs (gp B), acute normal RSBP exposed (gp C), (gpE) acute RSBP sensitized and

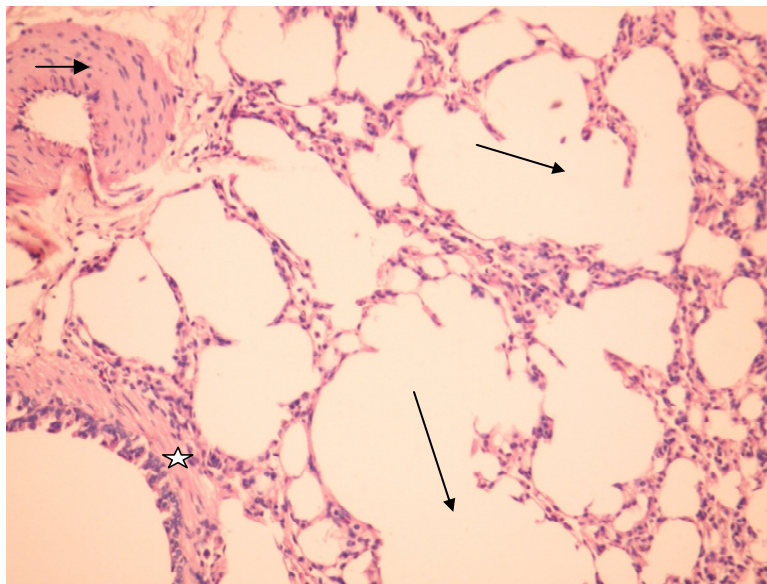
subchronic RSBP sensitized (gp F) exposed lungs show significant decrease in macrophages count compared to control non exposed group. This may be explained by another study of Nieuwenhuizen et al. (2012) who demonstrated that macrophages are not necessary for allergic airway disease, and may only be a consequence of the elevated T helper t-lymphocytes (Th2) response. They studied the contribution of macrophages to acute, chronic and house-dust-mite-induced allergic airway inflammation by using mice with abrogated IL-4Rα signaling on macrophages. It was demonstrated that airway hyperreactivity, Th2 responses, mucus hypersecretion, number of

eosinophils, and collagen deposition were not significantly affected by decreased development of macrophages. Also, macrophages seem to be beneficial to the resolution of asthma through production of IL-10 but are not present or not functional in asthma, and therefore allergic inflammation can progress (Fitzpatrick et al., 2010). This of course is in line with the above-cited finding by Shaykhiev et al. (2009) that macrophages genes are down regulated in alveolar macrophages of healthy smokers and smoking COPD patients as compared to nonsmokers.

It has been noticed that the increase of Neutro-



**Figure 1.** Lung section of guinea pig with normal lung (gp A) aspirated normal air showing normal lung architecture except some minimal perivascular and peribronchial mononuclear cellular infiltration (single arrow head) with normal interalveolar septa (double arrow head) and alveolar spaces (in five guinea pigs out of six) (H& E  $\times 125$ ).

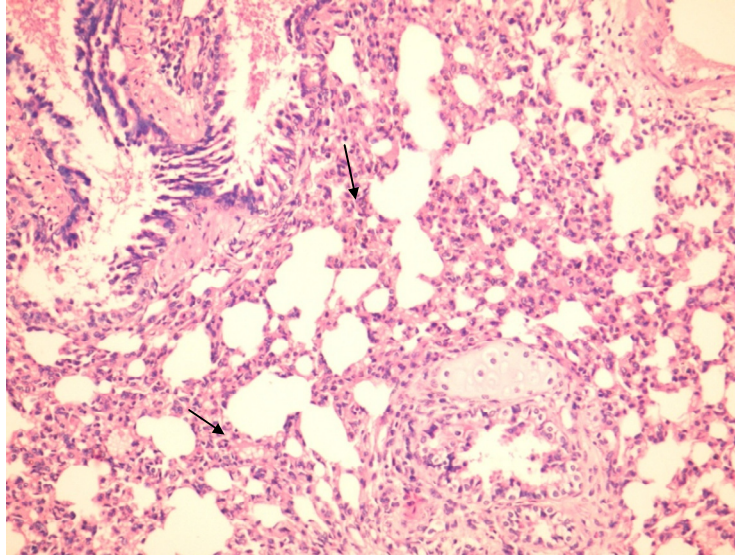


**Figure 2.** Lung section of guinea pig with sensitized lung aspirated air atmosphere (gp B) showing centrilobular emphysematous changes (single arrow head) with thickened bronchial walls (star) (three guinea pigs out of six) (H& E  $\times 125$ ).

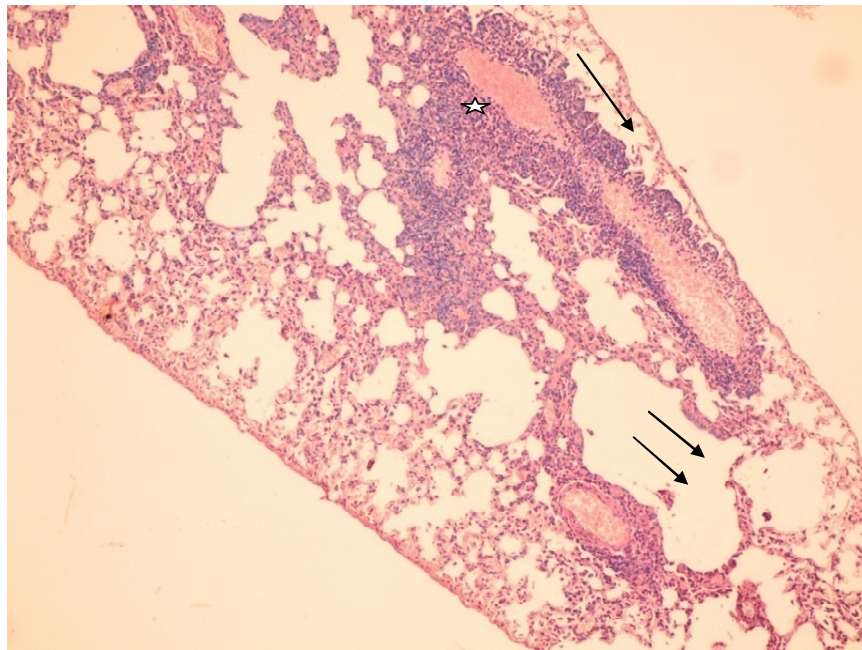
phils count occurred not only in RSBP groups (D, E, F), but also in Group B (Sensitized only) compared to control non exposed (gp A). This suggests that the sensitization of the lungs and exposure of RSBP for two or four weeks induce proliferation of neutrophils and infiltration in the

lung. Also, we found a close association of neutrophil infiltration with emphysematous changes, and destruction of alveolar walls (Figures 1- 9). These findings indicate that the protease-antiprotease imbalance had already occurred in exposure model. In





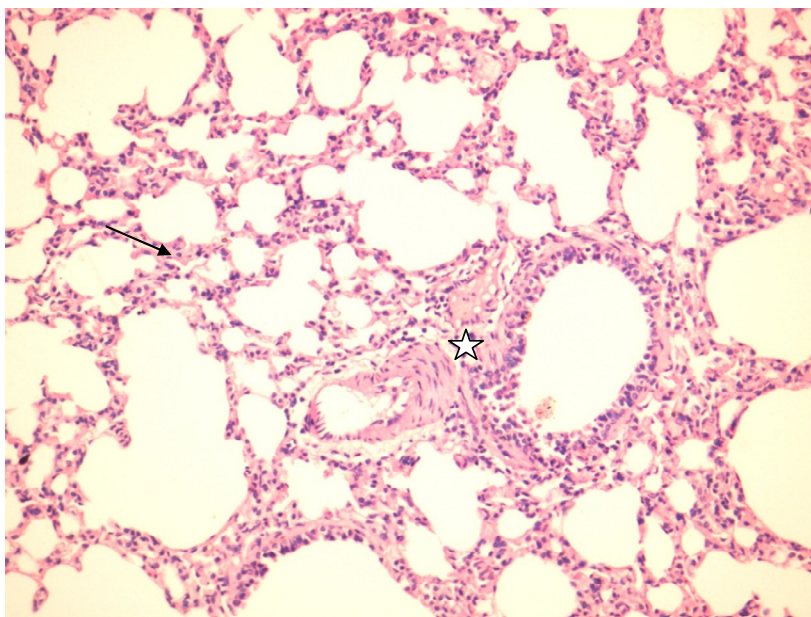
**Figure 3.** Lung section of guinea pig with sensitized lung aspirated air atmosphere (gp B) showing diffuse thickening of interalveolar spaces with emphysematous changes and focal hyalinization (star) of bronchial walls (in two guinea pigs out of six) (H& E  $\times 125$ ).



**Figure 4.** Lung section of guinea pig with sensitized lung aspirated air atmosphere (gp B) showing thickened interalveolar spaces with mononuclear cellular infiltration (star) appeared as micronodular accumulates with diffuse emphysematous changes (double arrow head) and subpleural emphysematous bullae (single arrow head) (in one guinea pigs out of six) (H& E  $\times 125$ ).

other studies, an increase of neutrophils in the airway lumen has been documented by bronchoalveolar (BAL) fluid and sputum analysis in smokers with mild to

moderate chronic airway obstruction (Bohadana et al., 2004), and a good correlation was observed between the number of neutrophils and the annual decline in forced



**Figure 5.** Lung section of guinea pig with normal lung aspirated RSBP for two weeks (gp C) showing mild to moderate thickened interalveolar septa (single arrow head) (mild eodema & minimal infiltrated) together with mild to moderate peribronchial mononuclear cellular infiltrations (star) with normal empty alveolar spaces (in four guinea pigs out of six) (H& E  $\times 125$ ).

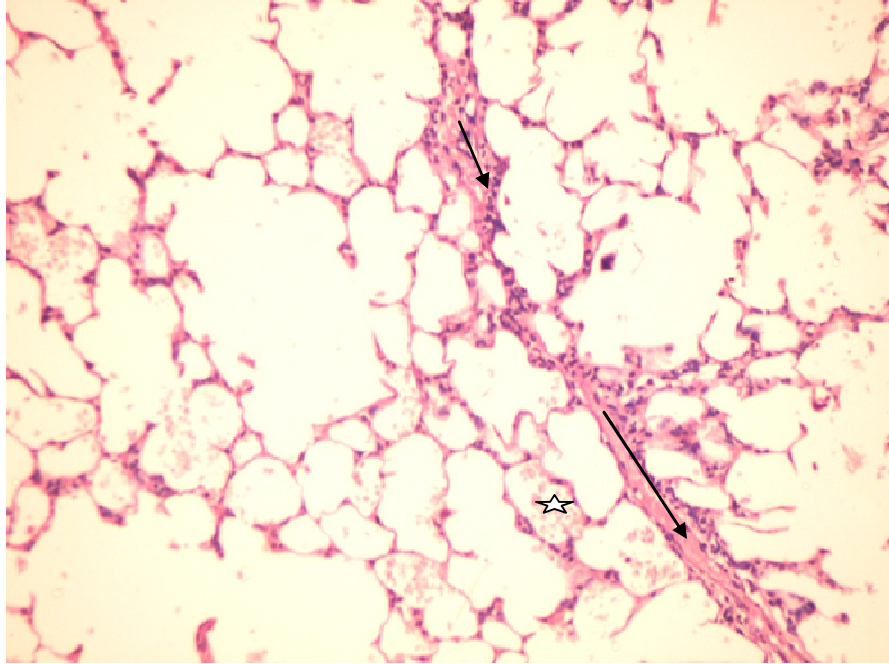
expiratory volume in the first second (FEV1) (Bohadana et al., 2004; Ravinder et al., 2012).

It has been noticed that eosinophils was not present in the BAL of control (gpA). It has been noticed that the increase of eosinophils count occurred in sensitized groups exposed or not. The significant increase was only present in sub chronic exposure of RSBP (gpF) compared to nonexposed sensitized group (gpB). Airway inflammation with eosinophilic infiltration of the bronchial mucosa is a characteristic feature of atopic asthma (Akuthota et al., 2011). It has been found that stimulation of macrophages by ovalbumin uptake induced increased production of IL-10 by these macrophages which could play an important role in the resolution of asthma (Fitzpatrick et al., 2010), and this resulted in lower levels of IL-5 and ovalbumin-specific IgE, and a lower number of eosinophils in a mouse model of asthma (Vissers et al., 2004).

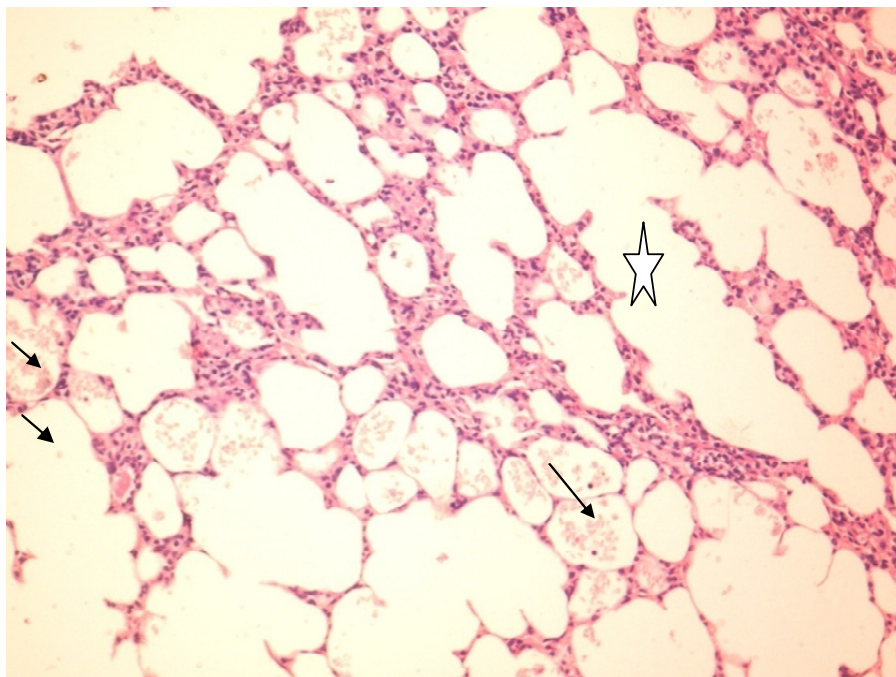
It has been reported that rice straws, and its smoke up regulate the expression of intercellular adhesion molecule-1 (ICAM-1) and human leucocyte antigen (HLA) on Eosinophils which play an important role in Eosinophils function, migration and degranulation (Kayaba et al., 2004). Eosinophils may also contribute to the induction of airway remodelling by synthesizing a variety of profibrotic mediators. Eosinophils are thought to be an important source of the potent pro-fibrotic cytokine TGF- $\beta$  (Cho et al., 2004). TGF- $\beta$  is able to induce extracellular matrix (ECM) protein production (Kenyon et

al., 2003), and also contributes to the accumulation of fibroblasts below the reticular basement membrane by stimulating fibroblast proliferation. It further contributes to airway remodelling by promoting the differentiation of myofibroblasts from resident fibroblasts, and also from circulating precursor cells known as fibrocytes (Mori et al., 2005). The differentiation of myofibroblasts into smooth muscle cells (Wicks et al., 2006), and their proliferation (McMillan et al., 2005) may also be governed by TGF- $\beta$ . This was confirmed by histopathological examination of lungs sample of subchronic sensitized RSBP exposed group (gpF) showed pericatricial emphysema with interstitial fibrosis in three animals out of six (Figure 12). In the present study, it has been noticed that basophiles wasn't present in the BAL of all groups except in acute and subchronic RSBP exposed sensitized lungs with no significant changes. Histopathological examination of lungs sample of acute RSBP exposed group (gp C) showed mild to moderate thickened interalveolar septa (mild eodema and minimal infiltrated) together with mild to moderate peribronchial mononuclear cellular infiltrations with normal empty alveolar spaces in four animals (Figure 5), and the two remaining animal showed the same findings associated with intraalveolar cellular exudates (Figure 6). These findings are compatible with previous study (Li et al., 2003).

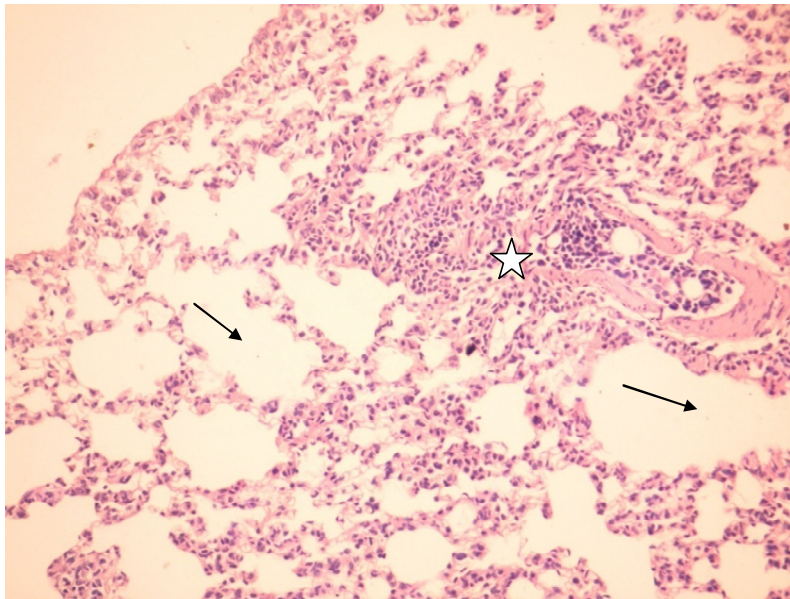
Histopathological examination of lungs sample of subchronic RSBP showed the result of exposed group (gp D). Another two animals showed diffuse thickening of



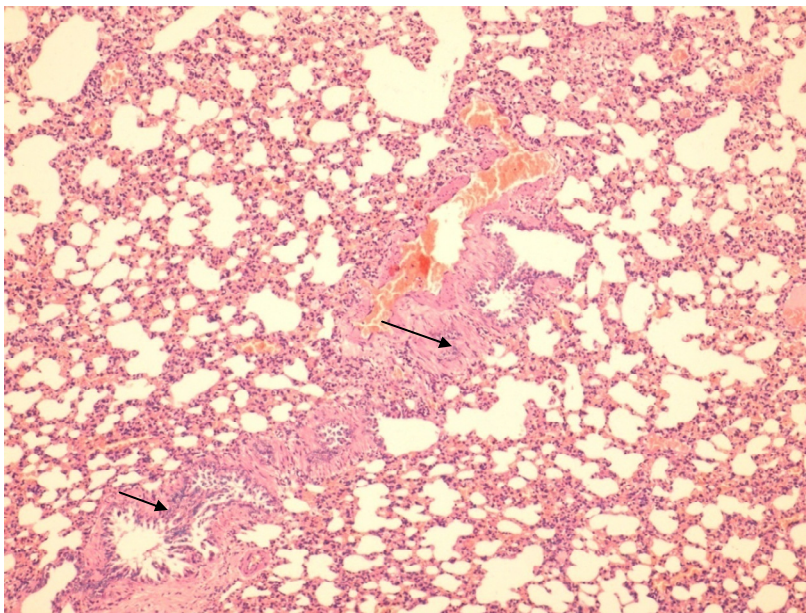
**Figure 6.** Lung section of guinea pig with normal lung aspirated RSBP for two weeks (gp C) showing mild to moderate thickened interalveolar septa (arrow head) (mild edema & minimal infiltrated) together with mild to moderate peribronchial mononuclear cellular infiltrations with normal empty alveolar spaces with intraalveolar cellular exudates (star) (in two guinea pigs out of six) (H& E  $\times 125$ ).



**Figure 7.** Lung section of guinea pig with normal lung aspirated RSBP for four weeks (gp D) showing moderate mononuclear interstitial & prebronchial mononuclear cellular infiltration associated with interalveolar cellular debris (arrow head) with focal areas of destroyed interalveolar septa (emphysematous spaces) (star) (in two guinea pigs out of six) (H& E  $\times 125$ ).



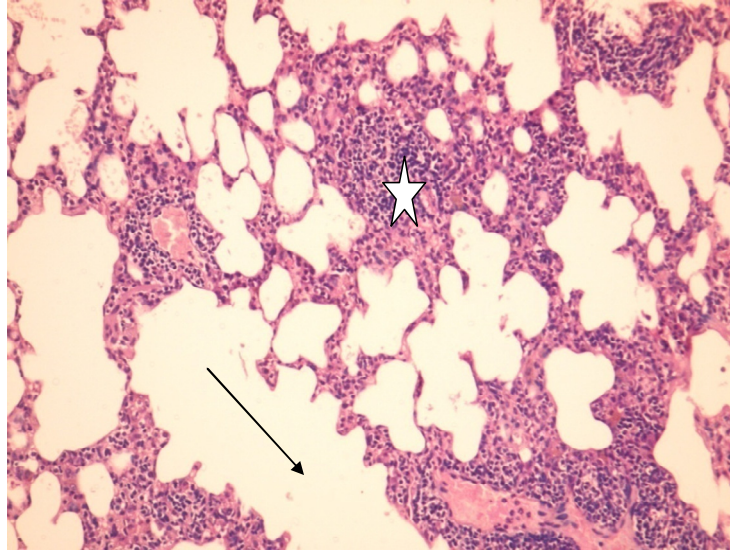
**Figure 8.** Lung section of guinea pig with normal lung aspirated RSBP for four weeks (gp D) showing showed diffuse thickening of interalveolar septa with mononuclear cellular infiltration around blood vessels and bronchioles (star) with patchy emphysematous spaces (arrw head) (in two guinea pigs out of six) (H& E  $\times 125$ ).



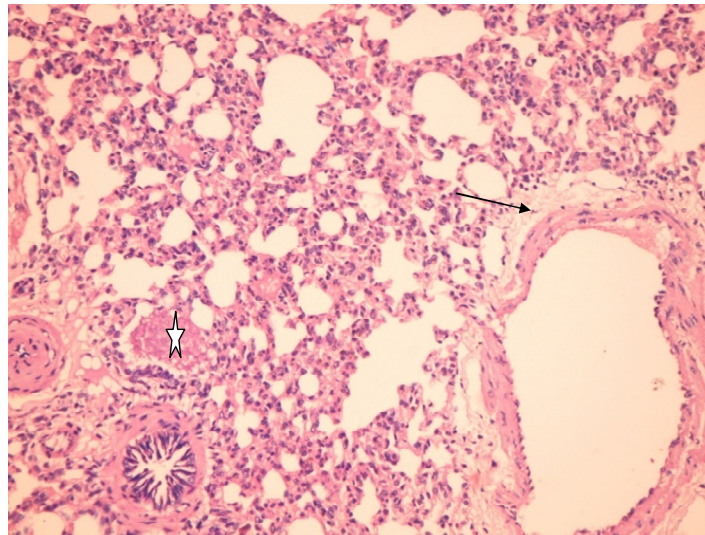
**Figure 9.** Lung section of guinea pig with normal lung aspirated RSBP for four weeks (gp D) showing peribronchial focal micronodular mononuclear cellular accumulates (arrow head) (in two guinea pigs out of six) (H&E  $\times 125$ ).

interalveolar septa with mononuclear cellular infiltrations around blood vessels and bronchioles with patchy emphysematous spaces (Figure 8), and the last two

animals showed peribronchial focal micronodular mononuclear cellular accumulates (Figure 9). These findings are compatible with previous studies (Li et al.,



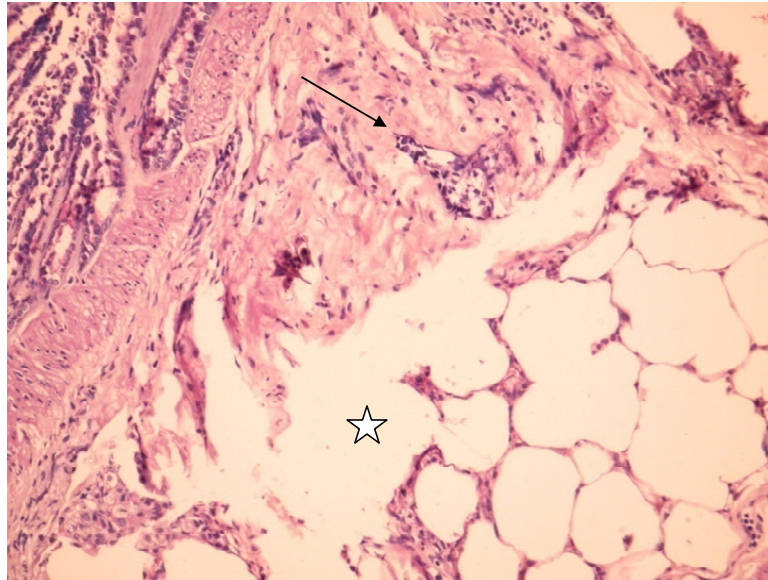
**Figure 10.** Lung section of guinea pig with sensitized lung aspirated RSBP for two weeks (gp E) showing interstitial mononuclear cellular infiltration (star) with numerous cellular nodules with large wild emphysematous spaces (arrow head) (in three guinea pigs out of six) (H&E  $\times 125$ ).



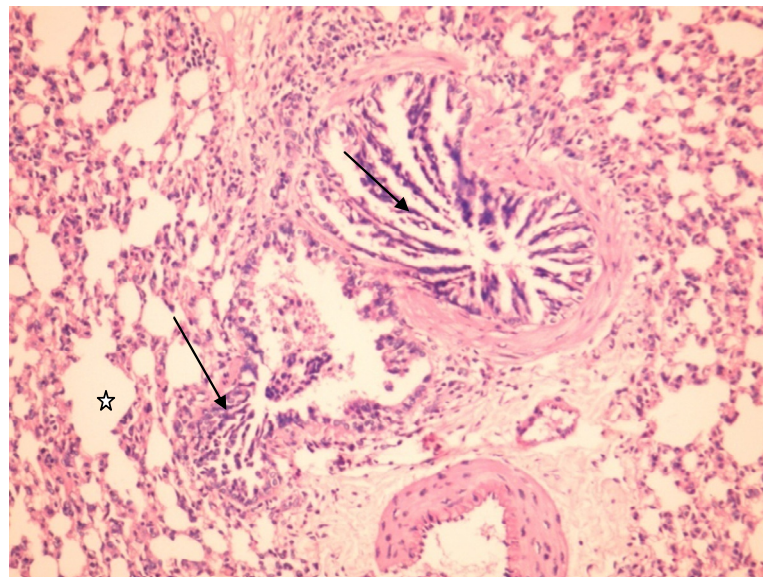
**Figure 11.** Lung section of guinea pig with sensitized lung aspirated RSBP for two weeks (gp E) showing the typical picture of interstitial pneumonia (star) (interstitial mono nuclear cellular infiltration with the presence of some hyalinized bodies), dilated arterioles and perivascular edema (arrow head) (in three guinea pigs out of six) (H&E  $\times 125$ ).

2003; Sezer et al., 2007). Histopathological examination of lungs sample of sensitized acute RSBP exposed group (gp E) showed interstitial mononuclear cellular infiltration with numerous cellular nodules with large wild emphysematous spaces in four animals (Figure 10). Histopathological examination of lungs sample of

sensitized acute RSBP showed the result of exposed group (gp E). The remaining two animals showed the typical picture of interstitial pneumonia (interstitial mono nuclear cellular infiltration with the presence of some hyalinized bodies), dilated arterioles and perivascular edema (Figure 11). Histopathological examination of



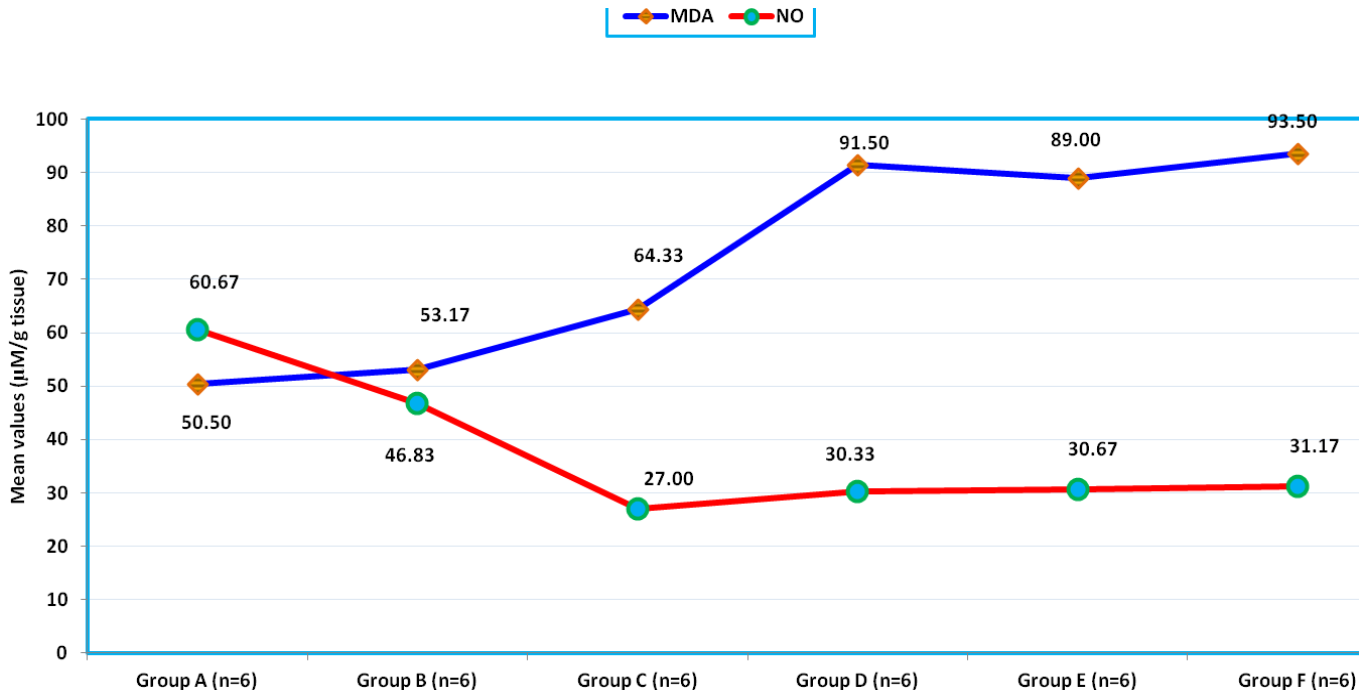
**Figure 12.** Lung section of guinea pig with sensitized lung aspirated RSBP for four weeks (gp F) showing interstitial and peribronchial fibrosis (arrow head) with emphysema (star) (in three guinea pigs out of six) (H&E  $\times 125$ ).



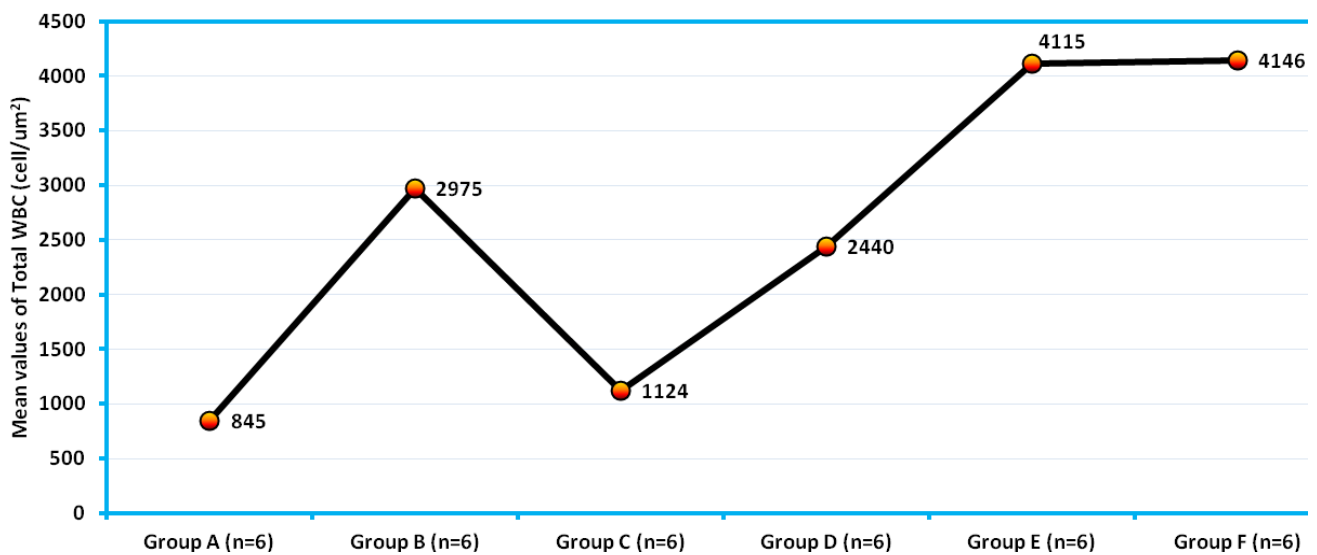
**Figure 13.** Lung section of guinea pig with sensitized lung aspirated RSBP for four weeks (gp F) showing dense interalveolar inflammatory infiltration hyperplastic (arrow head) bronchial epithelium with peribronchial inflammatory infiltration with peribronchial emphysematous bollea (star) formation (in three guinea pigs out of six) (H&E  $\times 125$ ).

lungs sample of sensitized subchronic RSBP exposed group (gp F) showed pericatricial emphysema with interstitial fibrosis in three animals (Figure 12). Histopathological examination using H&E stain of lungs sample of sensitized subchronic RSBP exposed group

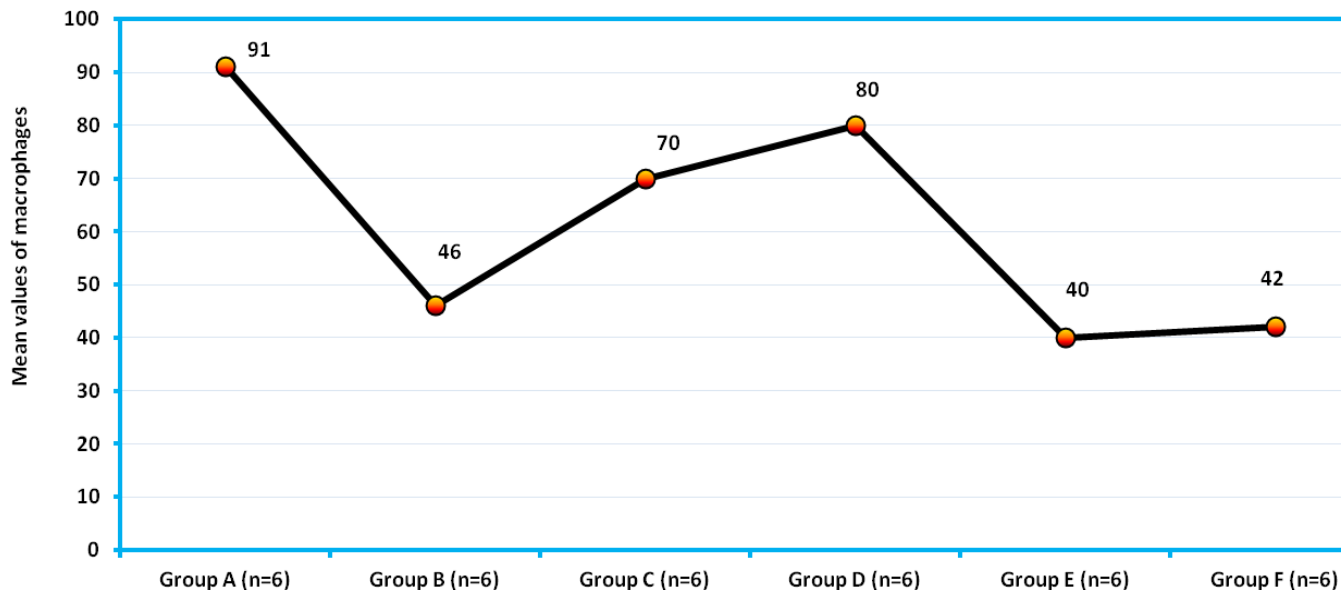
(gp F). The remaining three animals showed dense interalveolar inflammatory infiltration hyperplastic bronchial epithelium with peribronchial inflammatory infiltration with peribronchial emphysematous bolea formation (Figure 13). These findings are compatible



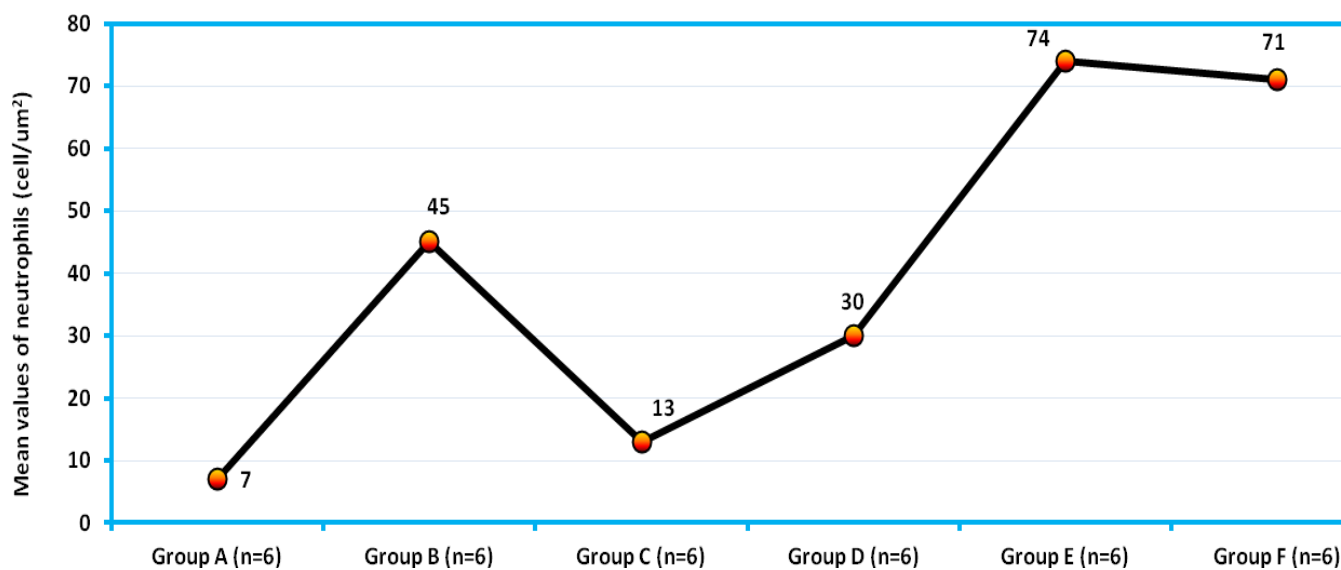
**Figure 14.** Mean values of malondialdehyde (MDA) and nitric oxide content (NO) of the study groups of guinea pigs (exposed and not exposed to rice straw burning products (RSBPs) (n=36). Group A: control group, group B: Egg albumin sensitization group, group C: Acute RSBP exposed group, group D: Subchronic RSBP exposed group, group E: Sensitized acute RSBP Exposed group, group F: Sensitized Subchronic RSBP exposed group. Results expressed as The mean of MDA content (µM/g tissue)± SD ; n = 6/group. The mean value was significantly different between GA and GD (normal lungs) at p < 0.05. The mean value was significantly different between GB and GE, GF (sensitized lungs) at p < 0.05. The mean value of NO content (µM/g tissue) ± SD; n = 6/group. The mean value was significantly different between GA and GC, GD (normal lungs) at p < 0.05. The mean value was significantly different between GB and GE, GF (sensitized lungs) at p < 0.05



**Figure 15.** Mean values of total white blood cells (TWBCs) of the study groups of guinea pigs (exposed and not exposed to rice straw burning products (RSBPs) (n=36). Group A: control group, group B: Egg albumin sensitization group, group C: Acute RSBP exposed group, group D: Subchronic RSBP exposed group, group E: Sensitized acute RSBP Exposed group, group F: Sensitized Subchronic RSBP exposed group. Results expressed as the mean of total leukocytic count (cell/µm<sup>2</sup>) ± SD; n = 6/group. The mean value was significantly different between GA and GD (normal lungs) at p < 0.05. The mean value was significantly different between GB and GE, GF (sensitized lungs) at p < 0.05.



**Figure 16.** Mean values of macrophages of the study groups of guinea pigs (exposed and not exposed to rice straw burning products (RSBPs) (n=36). Group A: control group, group B: Egg albumin sensitization group, group C: Acute RSBP exposed group, group D: Subchronic RSBP exposed group, group E: Sensitized acute RSBP Exposed group, group F: Sensitized Subchronic RSBP exposed group. Results expressed as the mean of macrophages count (cell/ $\mu\text{m}^2$ )  $\pm$  SD; n = 6/group. The mean value was significantly different between GA and GC (normal lungs) at  $p < 0.05$ .



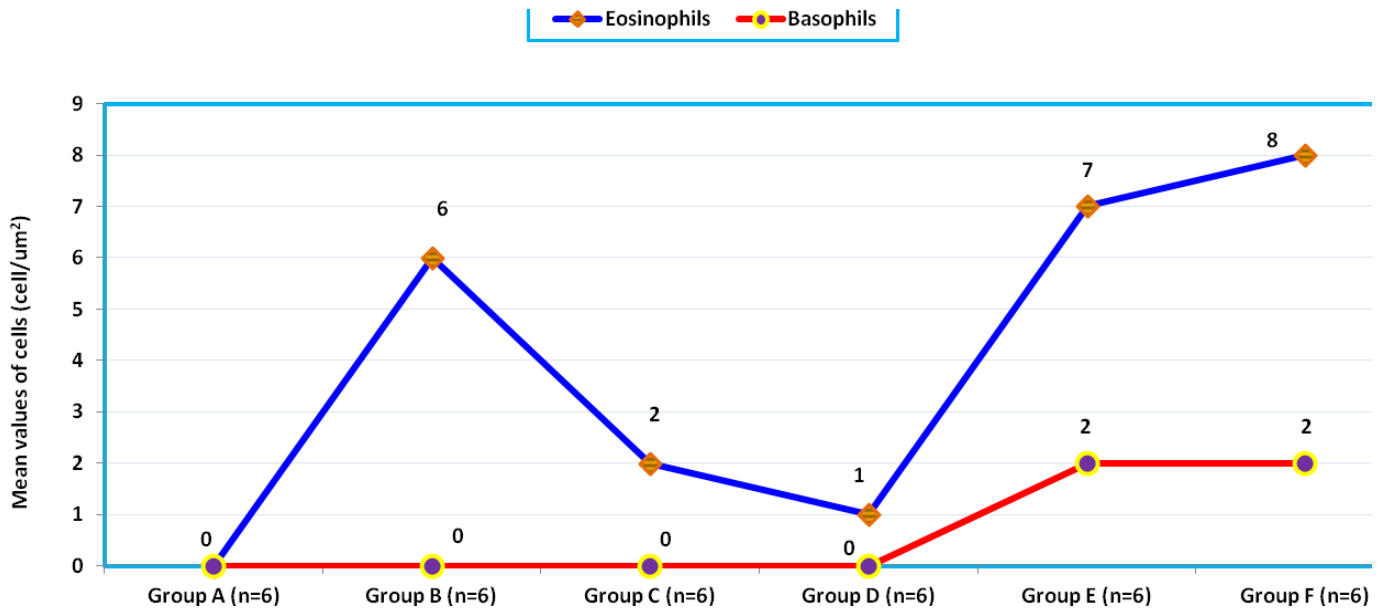
**Figure 17.** Mean values of neutrophils of the study groups of guinea pigs (exposed and not exposed to rice straw burning products (RSBPs) (n=36). Group A: control group, group B: Egg albumin sensitization group, group C: Acute RSBP exposed group, group D: Subchronic RSBP exposed group, group E: Sensitized acute RSBP Exposed group, group F: Sensitized Subchronic RSBP exposed group. Results expressed as the mean of neutrophils count (cell/ $\mu\text{m}^2$ )  $\pm$  SD; n = 6/group. The mean value was significantly different between GA and GD (normal lungs) at  $p < 0.05$ . The mean value was significantly different between GB and GE, GF (sensitized lungs) at  $p < 0.05$ .

with previous study (Beasley, 2010).

Malondialdehyde (MDA) is the end product of lipid peroxidation, and an indicator of oxidative stress (Kamal

et al., 1989). Antioxidant enzymes such as superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px) are essential for preservation of cellular balance and





**Figure 18.** Mean values of eosinophils and basophils of the study groups of guinea pigs (exposed and not exposed to rice straw burning products (RSBPs) (n=36). Group A: control group, group B: Egg albumin sensitization group, group C: Acute RSBP exposed group, group D: Subchronic RSBP exposed group, group E: Sensitized acute RSBP Exposed group, group F: Sensitized Subchronic RSBP exposed group. Results expressed as the mean of Eosinophils count (cell/um<sup>2</sup>) ± SD; n = 6/group. The mean value was significantly different between GB and GF (sensitized lungs) at p < 0.05. Results expressed as the mean of Basophils count (cell/um<sup>2</sup>) ± SD; n = 6/group.

elimination of free radicals (McLaughlin et al., 1990). The present results have shown that there was significant increase in the level of MDA in the lung tissues in (D, E, F) groups that had been exposed to the smoke of RSBP in normal or sensitized lungs. These results suggest that the RSBP exposure has a potent potential capacity for being a source of reactive oxygen species and possibly oxidizing species. Confirmatory observation by Ho and Kou (2002) who reported the increase hydroxyl radical (OH) burdens following smoke exposure which was actively involved in evoking the acute irritant effects of wood smoke. Oxidative stress has been shown to be an important contributor to the pathogenesis of COPD (Ito et al., 2009).

It has been demonstrated in the present study that the concentration of nitric oxide (NO) contents showed a significant increase in the normal group than in other RSBP exposed groups. It have been reported that NO, was significantly decreased in cigarette smoke-exposed animals compared with healthy controls (Pekmez et al., 2010). It is quite possible that RSBP exposure was interfered with the function of endothelial nitric oxide synthase (eNOS). This was supported by the observation of MacNee (2000) who reported that smokers have serum and tissue evidence of oxidative damage to many proteins like tetrahydrobiopterin (BH<sub>4</sub>), a required co-factor for eNOS activity, which can be altered to its inactive BH<sub>2</sub> form by oxidants, and in this situation switches production from NO to superoxide (O<sup>-</sup>) (Heitzer

et al., 2000; Wagner et al., 2007). Peroxynitrite, a highly reactive molecule, is produced in a near diffusion-limited rate as a reaction between O<sub>2</sub><sup>-</sup> and NO. Peroxynitrite can oxidatively inactivate eNOS, thus decreasing NO synthesis, and in addition peroxynitrite catalytically disrupts eNOS, resulting in increased O<sub>2</sub><sup>-</sup> production by the eNOS dimers (Zou et al., 2004).

Furthermore, cigarette smoke has been shown to inhibit production of tetrahydrobiopterin (Heitzer et al., 2000), disrupt the active eNOS dimers, and abnormally phosphorylate eNOS, producing an inhibitory state, all of which are alterations that would reduce NO bioavailability (Wagner et al., 2007). Smoke also exerts a number of other effects on eNOS production. TNF $\alpha$  which is increased in the sputum of human smokers with chronic obstructive pulmonary disease (COPD) and in the plasma of animal models of COPD (Churg et al., 2002), destabilizes eNOS mRNA (Neumann et al., 2004; Searles, 2006). Smoke also contains large concentrations of O<sub>2</sub><sup>-</sup> which can reduce functional levels of NO by converting it to peroxynitrite (Wright and Churg, 2008).

## CONCLUSION

This study has demonstrated that RSBP induced pulmonary emphysematous lesions are progressive with subsequent smoke exposures together with the sensi-

zation of the lung in the present model. RSBP have the ability to change the counting of macrophages, neutrophils, eosinophils in the BAL of guinea pigs lungs compared to normal lavages. RSBP exposure has a potent potential capacity for being a source of reactive oxygen species and possibly oxidizing species which can lead to decrease nitric oxide content in lung tissue. It was found out that Rice straws burning products can easily penetrate the pulmonary alveoli and damage the health of humans. It is recommended that the rice burning activities should be stopped as it affects human health, and we should find alternative ways for disposal of the rice after harvesting.

## ACKNOWLEDGMENTS

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## Conflict of Interest

The authors have not declared any conflict of interest.

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## Review

## Brazil nut allergy: A review

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**Tree nuts and their products are highly nutritive and beneficial to health. On the other hand, some of them can lead to development of allergic reactions, protein intolerance or liver diseases in some groups of consumers. Therefore, it is important to study the risks of tree nuts ingestion as a major issue for food regulators and the pharmaceutical industry safety. A review of surveillance and correlation to possible Brazil nut allergy reactions with effects on consumers and some therapeutic potential was carried out to help in understanding the mechanisms and so preventing damages to sensitive consumer's health.**

**Key words:** *Bertholletia excelsa*, aflatoxin, allergy, protein, immunoglobulin, risk.

### INTRODUCTION

#### Allergies

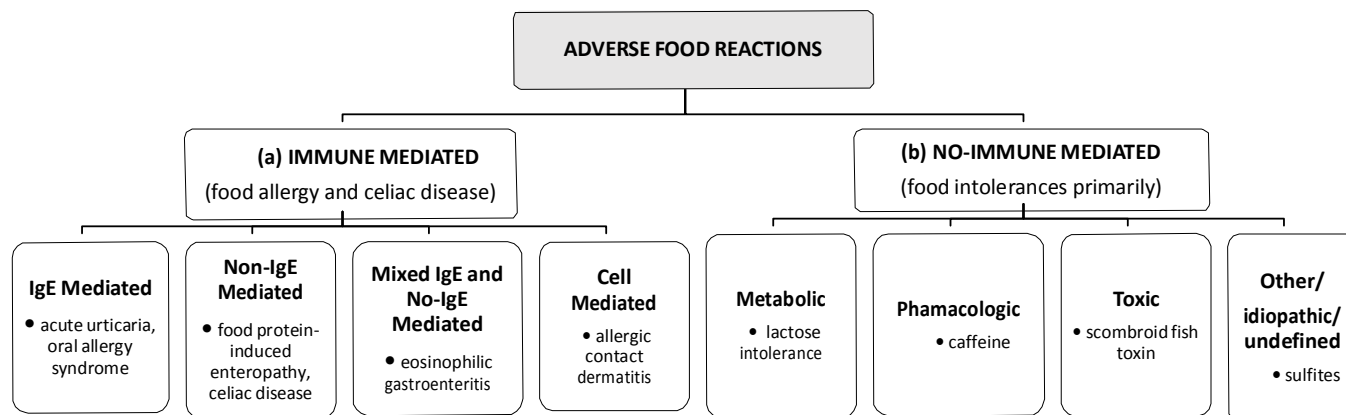
Adverse reactions caused by the consumption of some foods have been widely studied and reported since ancient times. They can occur by several mechanisms. However, the food allergies are caused by specific immune responses (immune mediated). Food allergy is defined as an immune response to food proteins disease (Cianferoni and Spergel, 2009). For example, in the urticaria (commonly referred to as hives) the symptoms and the anaphylaxis are triggered by the immunoglobulin E (IgE), which is antibody-mediated immune responses. On the other hand, the cell-mediated diseases (eosinophilic esophagitis and enterocolitis), which are also induced by food proteins, are classified as Mixed IgE and No-IgE mediated. The allergic reactions involve immunologic mechanisms that

may or may not be mediated by the IgE. They are usually associated to food allergies and hypersensitivity reactions characterized by a rapid release of mediators such as histamine (Figure 1).

On the other hand, the term food intolerance (non-immune mediated) refers to any abnormal response to (one or more than one) specific food, food substance (lactose/caffeine/fish proteins) or additive (sulfites) without the involvement of immunologic mechanisms. Allergies are characterized by the IgE synthesis against antigens that enter the body through ingestion, the inhalation (particles) or dermis contact (skin). Food allergy is an increasingly common problem in Western countries, and an effective therapeutic treatment is needed, however, it has not been

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**Figure 1.** Types of adverse reactions developed through foods ingestion (a) Immune mediated and (b) Non-immune mediated (adapted from Boyce et al., 2010).

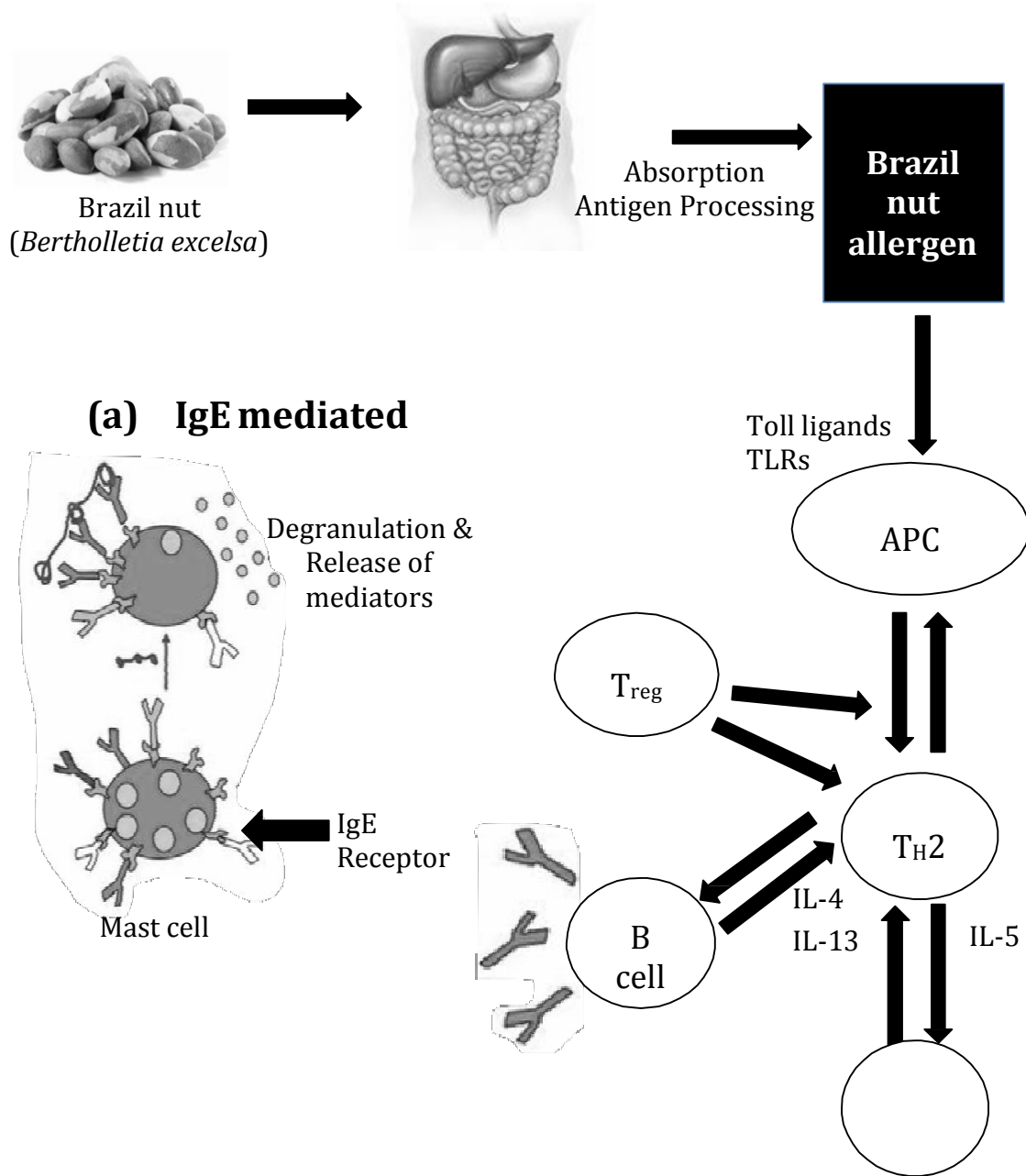
accomplished yet. Some therapeutic strategies have been investigated to identify the foods that most often cause severe anaphylactic IgE-mediated reactions, which commonly cause allergies in children (such as peanuts, walnuts, and shellfish or cow's milk and hen's egg). Boyce et al. (2010) strategies stated that an adverse reaction to food may be: (a) a mediated-immune response: food allergy (food protein-induced and urticaria reactions) or celiac disease; and (b) non mediated-immune response: primary food intolerance (lactose, caffeine, histamine, and sulfite intolerance).

Food allergy should be considered in individuals presenting anaphylaxis or any combination of symptoms that occur: within minutes to hours of ingesting food (especially in children) or after the ingestion of a specific food (on more than one occasion). The severity of allergic reactions to foods, however, is multifactorial and variable. Accordingly, the approaches being pursued are both food allergen -specific and -nonspecific. The allergen-specific approaches include oral, sublingual, and epicutaneous immunotherapy. It is carried out with native food allergens and mutated recombinant proteins. Diets containing cooked foods (milk and eggs) are an alternative approach to food *oral* immunotherapy and are already changing the paradigm of strict dietary avoidance for patients with food allergy. On the other hand, the nonspecific approaches include monoclonal anti-IgE antibodies, which might increase the threshold doses for food allergen in patients with food allergy. The variety of strategies for the treatment of food allergies increases the likelihood of success. Schreier and Wright (2014) reported that it is also necessary to consider, among the risk factors, the environmental influences on the healthy development of an immune system (during critical stages of development), particularly in the intestine. It is also important to mention that in addition to genetic predisposition, the psychological stress is known to play an important role in allergic and other inflammatory

diseases, such as asthma.

Food additives such as sulfites (asthma), monosodium-glutamate and tartrazine (hives) and other chemical substances that are added to foods are also associated with adverse reactions (ASBAI, 2008). Regarding environmental aspects, the presence of mycotoxins (carcinogens that are products of fungal metabolic processes - immune systems suppressors) in the environment can be considered as a factor that increases the immune response. A family history of atopy and the presence of atopic dermatitis is already considered risk factors for sensitization to food allergens and the development of food allergy. According to Schutze et al. (2010), epidemiological studies have shown that indoor fungi, including mycotoxin producers, are associated with increased prevalence and exacerbation of respiratory diseases. Low et al. (2011) reported that the environmental growth conditions significantly influence the allergenicity of common fungi (through the differential production of allergenic proteins), and they highlight the importance of allergenicity measurements for understanding the environmental exposure to allergenic fungi. Still considering the environmental exposure, cross-reactivity may occur due to the presence of aeroallergens.

According to Souza and Rosario Filho (2012), house dust mites are the main sensitizers of atopic individuals, followed by *cockroaches* (*Periplaneta americana* and *Blattella germanica*), which caused skin reactions in 24.1% of people with asthma. Bees (*Hymenoptera*) can also cause anaphylactic reactions. According to Kohler et al. (2014) the diagnosis of allergy by bee stings is usually based on clinical history of anaphylactic reactions. The antibodies cross-reactive to conserve structures found in allergens include homologous primary structures of protein allergens (*Hyaluronidases*, *Dipeptidyl peptidases IV* and *Vitellogenins*) and cross-reactive carbohydrate determinants that are present in the majority of *Hymenoptera* venoms. Regarding



**Figure 2.** Mechanism of Food Allergen Sensitization (adapted from Otsu and Dreskin, 2011). APC: antigen presenting cells (APCs); T cells: that are of the Th2 variety, leading to activation of B cells; IgE, FcεRI, on mast cells and basophils; MlgE/FcεRI complexes are cross linked, leading to cell activation and release of pro-inflammatory mediators; (a) IgE Mediated; (b) Non IgE mediated.

reactions to food, Figure 2 shows types of different reactions, where initial sensitization is thought to be through the GI track.

Food allergens are absorbed and taken up by the dendritic cells or other antigen presenting cells (APCs). Peptides are then presented to T cells (Th2 variety), leading to activation of B cells, that mature into IgE-producing plasma cells. Then it (IgE) binds avidly into the high affinity receptor for IgE (FcεRI) on the mast-

cells and basophils. When allergen is reintroduced, these M-IgE/FcεRI complexes are crosslinked, leading to cell activation and release of pro-inflammatory mediators (Otsu and Dreskin, 2011).

**Food allergy x tree nuts**

The allergic reactions to tree nuts are a growing global

**Table 1.** Nutrients of tree nuts and pulses including protein content

Nutrients	Tree nuts (g%)			Pulses
	Brazil nut (Freitas et al., 2012)	Cashew nut (Vicent et al., 2009)	Macadamia (Freitas and Naves, 2010)	Peanut (Freitas and Naves, 2010)
Lipid	57.94	34.95	66.1	44.57
Protein	16.30	27.31	8.40	24.03
Carbohydrate	3.36	25.39	22.18	12.01
Fiber	12.53 <sup>1</sup>	1.42 <sup>2</sup>	NI <sup>3</sup>	11.30

<sup>1</sup>Total dietary fiber; <sup>2</sup>crude fiber; <sup>3</sup>not informed.

concern, as the number of affected individuals continues to rise (Willison et al., 2014). Unlike some other food allergies, tree nut severe reactions can persist throughout life since they are usually consumed as snacks or used as ingredients in other products (by different consumer groups in various geographical locations). Among the tree nuts (and pulses) usually associated to food allergies are: cashew nuts, almonds, hazelnuts, pecans, Brazil nut, pistachio, peanuts and chestnut. The contact with the native allergenic proteins derived from tree nuts, which are generally difficult to isolate and purify, triggers the allergic reaction. The substances that cause food allergy belong to protein families with diverse biological functions: (a) Allergens: enzymes (mainly proteases), ligand-binding proteins or lipocalins, albumins, tropomyosins and calcium-binding proteins; (b) Pollen allergens: pathogenesis-related proteins, calcium-binding proteins, pectate lyases, bexpansins, and trypsin inhibitors; (c) Plant and animal food allergens: lipid transfer proteins, profilins, seed storage proteins and tropomyosins (Chapman et al., 2007). Tree nuts are in general energy dense (23.4 and 26.8 kJ/g), have high lipid content (45 to 75%), and are rich sources of proteins, unsaturated fatty acids, fibers, vitamins (E, B6, folic acid, and niacin), minerals (magnesium, potassium, and copper), phytosterols (stigmasterol, sitosterol, and campesterol), and polyphenols (catechins, resveratrol, etc.) (Vadivel et al., 2012). Table 1 summarizes the proximate composition of some tree nuts and peanut for comparison.

Some of the risk factors associated with food allergies are genetic inheritance, diet, and intestinal microbiota. Therefore, it is important to establish the relationship between clinical reports and the consumption of specific foods such as tree nuts, commonly associated with a higher prevalence of causes of food allergies. Some tree nuts have been studied for the bio accessibility of nutritional compounds and antioxidant activity and are part of the diet of some consumer groups and specific populations. They stand out especially due to their benefits to the cardiovascular system and to the facts that they reduce blood pressure and the risk of hypertension (Alexiadou and Katsilambros, 2011;

Yazdekhesti et al., 2013) and have anti-cancer effects and promote weight loss. Table 2 shows some foods including tree nuts that are associated with allergies.

The prevalence of tree nut allergy in the United States of America (USA) in children is about 0.5% (Boyce et al., 2010). In Brazil, Guimarães et al. (2015) collected information from parents of pre-school children with allergy to different foods and found the following: milk (42.7%), pork (11.6%), fruits (10.9%), chocolate (9.4%), and eggs (9.2%). Investigating the prevalence of sensitivity to food allergens in children in Brazil, Naspitz et al. (2004) reported reaction to food allergens in subjects tested for IgE sensitivity, indicating a significantly greater proportion of sensitive individuals than non-sensitive ones.

Table 3 shows the prevalence of tree nuts allergy in patients from several countries. Unfortunately, there is still little information on the prevalence and incidence of food allergy in developing and emerging economies such as China, India, and Brazil. Brazil nut (*Bertholletia excelsa* HBK), for example, a seed native to the Amazon region, has remarkable nutritional properties, especially the contents of protein and sulfur amino acids. However, the nutrient profile of tree nuts is associated to allergic reactions. Nut allergy can be dangerous and that it may occur in highly atopic individuals of any age with a strong family history of atopy (Ridout et al., 2006). The nutritional and therapeutic potential of tree nuts and the pathology and immune reactions that influence the inherent risks associated with their consumption need to be evaluated. In order to discuss the case-reports of Brazil nut allergy this review is presented.

## Brazil nut and allergy reactions

### Composition

Brazil nut is an extractive product of the Amazon region, that is, the fruits are collected from the forest when they fall freely from the trees to the ground during the rainy season and from which the seeds are removed and industrially processed. It can be used as raw material for the production of various products. Its worldwide

**Table 2.** Animal and Vegetable food allergy prevalence rates from different countries.

Protein	Food allergy prevalence (%)				
	Canada (Soller et al., 2012) <sup>3</sup>	USA (Boyce et al., 2010)	Australia (Osborne et al., 2011)	Brazil (Naspitz et al., 2004) <sup>1</sup>	Brazil (Guimarães et al., 2015) <sup>2</sup>
<b>Animal</b>					
Egg	0.8	1	16.5	24.4	9.2
Fish	0.51	0.6	NI	29.5	NI
Milk	1.97	3	5.6	23.1	42.7
Pork	NI	NI	NI	NI	11.6
Shell fish	1.6	1.2	0.9	NI	NI
<b>Vegetable</b>					
Tree nuts	1.22*	0-4.1*	NI	NI	NI
<b>Pulses</b>					
Peanut	1	0.6	8.9	14	NI
Soy	0.2	0-0.6	NI	11.8	NI
Wheat	0.77	0.2-1.3	NI	20	NI
Sesame seed	0.1	NI	2.5	NI	NI

<sup>1</sup>Atopic children; <sup>2</sup>children in pre-scholar ages, <sup>3</sup>self-reported symptoms; <sup>4</sup>NI: not informed; \*not specified.

production is of 78,000 tones (Freitas-Silva and Venâncio, 2011). It is an excellent source of proteins, carbohydrates, lipids, vitamins, and minerals. Its lipid content is over 60%, and the ratio of saturated, monounsaturated and polyunsaturated fatty acids is 25:41:34 (USDA, 2008). It is a source of vitamin E and sulfur amino acids. Among which, methionine and cysteine are part of the 2S albumin protein fraction, the most relevant protein fraction in Brazil nuts, which makes it more appealing than other nuts that lack sulfur amino acids, essential to human health. Brazil nut is also a source of micronutrients such as selenium (Se), phytosterols, tocopherols, squalene and phenolic compounds (Costa et al., 2010; Silva et al., 2010). Beta- and gamma-

tocopherols are the most abundant tocopherol isomers present in Brazil nuts. Barium (Ba) and Radio (Ra) can also be found in Brazil nuts (Martins et al., 2012).

Compared to other tree nuts, Brazil nuts are known as the best source of Se (Pacheco and Scussel, 2007; Chunhieng et al., 2008; Manfio et al., 2012). Adequate intake of Se is essential for normal activity of Se-enzymes involved in the protection against oxidative stress, maintenance of the redox system, modulating the immune system, and regulation of the thyroid. Thomson et al. (2008) demonstrated that 100 µg/day of Se (equivalent to two units of Brazil nuts) ingestion for three months was effective on increasing plasma Se concentrations and

glutathione peroxidase in healthy individuals. Stockler-Pinto et al. (2010) reported that the ingestion of a single serving of Brazil nut (5 g) a day for three months is effective in increasing the concentration of Se and glutathione peroxidase activity in patients with Se deficiency improving the antioxidant capacity of this mineral. Accordingly, the association between Se content and amino acids in Brazil nuts has been investigated. Silva et al. (2010) reported that Brazil nut showed the organic species *se-methionine* and *Se-cystine* after water extraction, but after simulated gastrointestinal digestion, only *Se-methionine* was found as bio accessible, corresponding to 74% of total Se ( $54.87 \pm 4.6$  ug.g<sup>-1</sup>). Analysis of urine samples suggested the



**Table 3.** Tree nuts and pulses allergy prevalence in population from different countries.

References	Food	Ages <sup>1</sup>		Prevalence (%)		Origin
		Group	Years old	Mean	Range	
<b>Tree nuts</b>						
Ben-Shoshan et al. (2010)	NS <sup>2</sup>	Adults and children	NI <sup>3</sup>	1.1	NI	Canada
Fleisher et al. (2005)	NS	Adults and children	NI	0.4	NI	USA <sup>4</sup>
Sicherer et al. (2010)*	NS	Adults	31-40	0.3	NI	USA
	NS	Children	0-5	0.5	NI	
Shek et al. (2010)	NS	Teenager	14-16	0.8	NI	Singapore
<b>Pulses</b>						
Nwaru et al. (2014)	Peanut	Adults and children	NI	0.4	NI	Europe
Soller et al. (2012)	Peanut	Adults	NI	0.78	0.58-0.97	Canada
		Children	NI	1.77	1.21-2.33	
Osborne et al. (2011)	Peanut	Infants <sup>5</sup>	NI	8.9	NI	Australia
Sicherer et al. (2010)	Peanut	Adults	>65	0.7	0.4-1.1	USA
Lucioli et al. (2008)	Soy	Children	1	1.4	NI	USA
Oh et al. (2004)	Peanut	Children	6-12	2.8	NI	South Korea

<sup>1</sup>It is expressed "children" when the authors did not specify the children ages; <sup>2</sup>Not specified or different tree nuts involved reported by the authors; <sup>3</sup>not informed; <sup>4</sup>United States of America; 512 months old;; \*2008 data.

presence of Se-cystine, and there were no significant differences between samples from men and women in terms of the concentration of this species after consumption of Brazil nuts (1 nut/day for 15 d). Therefore, food processing appears to influence the potential allergenicity associated with the protein fraction. According to Van Bostel et al. (2008), in Brazil nuts, for example, the protein fraction 2S albumin (denominated *Ber e1*), considered the major allergen, was stable to *in vitro* peptic digestion. Thermal denaturation (melting) can affect the digestibility of protein, since these

authors found that the denaturation temperature of 2S albumin ranged from 80 to 110°C, depending on the pH value.

Under heating at temperature higher than its denaturation temperature, at pH 7.0, there was partial formation of insoluble protein aggregates and the protein dissociated into its polypeptides, while heating at pH 5.0 did not seem to induce aggregation or dissociation of protein aggregates. The denaturation temperature of about 110°C, under pH values corresponding to the general food pH values (pH 5 to 7) is high. As

a result, it is likely that heat processing causes the denaturation of allergy protein fraction present in food products. Koppelman et al. (2005) investigated the high resistance of 2S albumin fraction, previously identified as an allergen, against proteolysis by pepsin. They found that although the protein denaturation temperature exceeds 110°C, at neutral pH, a fully reversible thermal denaturation was observed at 82°C at low pH. Chemical processing (the subsequent reduction and alkylation of the protein) was used to destabilize the globular fold. Far-UV circular

**Table 4.** Amino acid composition and total protein content in tree nuts and pulses (peanuts).

Amino acids	Tree Nuts (g%) <sup>1</sup>		Pulses
	Brazil nut	Cashew nuts	Peanut <sup>2</sup>
	(Venkatesh and Sathe, 2006)	(Latif et al., 2013)	(Latif et al., 2013)
Alanine	4.30	4.44	3.98
Arginine	12.91	9.84	11.35
Aspartic acid	7.69	8.53	11.17
Cysteine*	0.75	0.54	1.20 <sup>3</sup>
Glutamic acid	20.26	22.43	18.72
Glycine	4.75	4.55	5.49
Histidine	2.92	2.68	2.33
Isoleucine	3.21	4.15	3.12
Leucine	7.89	8.00	6.24
Lysine	2.95	4.59	3.35
Methionine*	8.98	2.27	1.09
Phenylalanine	4.06	4.83	5.04
Proline	5.21	5.37	4.32
Serine	4.00	5.21	4.92
Threonine	2.27	3.22	2.67
Tyrosine	2.47	2.43	3.65
Valine	4.71	5.65	3.83
Total protein (g%)	13.93	18.81	26.6

<sup>1</sup>g% average from crude protein; <sup>2</sup>untreated kernel residue data; <sup>3</sup>expressed by the authors as "cystine"; \* most important sulfur amino acids involved in allergy reactions.

dichroism and infrared spectroscopy showed that the reduced and alkylated form lost some structures, whereas the alpha-helix structure of protein was protected. Accordingly, studies on protein digestion are important due to the potential allergenicity of Brazil nut 2S albumin.

### Protein versus Brazil nut allergy

Since ingestion of some proteins is associated with allergic reactions in some consumers, Breiteneder and Mills (2005) summarized the common biochemical and physicochemical properties of food allergens. They reported that thermal stability, resistance to proteolysis, binding capacity, and lipids interactions are factors that promote the allergenic properties of food proteins. Although one or more of these factors are characteristic of the allergen proteins, there is no rule to predict the allergenicity of a given protein. It is well known that proteins denature under high pressure. Thus, several attempts have been made to change the structure of the protein allergens using high pressure processing in order to reduce its allergenicity. Several studies have been carried out on simple protein solutions and on complex food systems. Allergens have been investigated under or after high-pressure treatment using methods capable of detecting changes in the secondary and tertiary structures of proteins. When

considering the protein content of the Brazil nut as potentially high (13-19 g%) and its essential amino acids, the beneficial aspects should be highlighted without disregarding food allergies. Table 4 presents the contents of protein and amino acids, including the sulfur amino acids.

In Brazil nut the methionine content is higher than cashew and peanut, and the amount of sulfur fraction has been studied in allergic reactions. Sun et al. (1987) studied the protein fractions (11S, 7S, and 2S) and registered that they contain high concentrations of glutamine/glutamic acid and arginine. However, the 2S protein contains unusually large amounts of sulfur amino acids (17.9% methionine and 8.7% cysteine). The major allergens found in Brazil nut are: (a) 2S albumin and (b) 11S legumin (Ber e 2) (Crespo et al., 2006). According to Van Bilsen et al. (2013), it is not known exactly why certain food proteins are more likely to cause reactions. One of the characteristics of most food allergens is that they are stable to the acidic and proteolytic conditions in the digestive tract. This property appears to be a risk factor for allergic sensitization. Van Bilsen et al. (2013) investigated the contribution of 2S albumin protein structure to the ability to induce *in vivo* oral sensitization using an animal model of food allergy. Disulfide bridges of albumin 2S were reduced and alkylated resulting in loss of protein structure and an increased *in vitro* pepsin digestibility. The reduced and alkylated

**Table 5.** Some case reports of Brazil nut allergies reported in the literature

Reference	Number of patients	Patterns of Brazil nut contact			Patient			Symptoms
		Test	Ingestion	Skin contact	Age <sup>1</sup>	Gender	Origin	
Nordlee et al.(1996)	04	Skin <sup>2</sup>	NA	NA <sup>3</sup>	NI <sup>4</sup>	NI	USA <sup>5</sup>	Oropharyngeal swelling and itching/facial swelling/ laryngeal edema and bronchospasm with wheezing/
Ewan (1996)	04	IgE <sup>6</sup>	NA	NA	NI	NI	UK <sup>7</sup>	Edema of tongue/ laryngeal edema/urticaria
Bartolome et al.(1997)	01	NA	01 BN <sup>8</sup>	NA	NI	NI	NI	Vomiting/diarrhea and loss of consciousness/
Pastorello et al. (1998)	11	NA	02 BN	NA	NI	M& F <sup>9</sup>	Italy	Urticaria/glottis edema/anaphylactic reaction
Borja et al.(1999)	01	NA	01 BN	NA	31	F	Spain	Pharyngeal itching/lip swelling/dysphonia/ dyspnea/ wheezing/macular exanthema.
Senna et al. (2005)	01	IgE	NA	NA	18	M	Italy	Acute wheezing/dyspnea/facial angioedema/ precipitating hypotension (max= 90 mm).
Ridout et al.(2006)	56	IgE	NA	Labial	NI	M & F	UK	Irritation/discomfort of mouth or lips/swelling of lips or tongue/ throat tightening/difficulty in swallowing/ swelling/puffiness of face or eyelids/skin rash/increased heart rate/shortness of breath/sense of doom/fear/nausea/abdominal pain/vomiting/ wheezing;/stridor/collapse/unconsciousness
Bansal et al. (2007)	01	NA	NA	Contact	NI	F	England	Urticaria and dyspnea

<sup>1</sup>years old; <sup>2</sup>IgE Skin-prick tests in response to extracts of Brazil nut and positive radioallergosorbent tests; <sup>3</sup>not applicable; <sup>4</sup>Not informed; <sup>5</sup>United States of America; <sup>6</sup>IgE measurement; <sup>7</sup>United Kingdom; <sup>8</sup>amount of Brazil nut ingested; <sup>9</sup>male & female.

forms of 2S albumin were administered by daily gavage dosing of 0.1 and 1 mg to rats for 42 days. Intraperitoneal administration was used as a positive control.

The enzyme-linked immunosorbent assay (ELISA) and passive cutaneous anaphylaxis methods were used to analyze Sera. Oral exposure to native or reduced 2S albumin resulted in specific IgG1 and IgG2 responses. In conclusion, the study demonstrated that disruption of the protein structure of Brazil nut 2S albumin reduced the sensitization potential of the rat food allergy model, whereas the immunogenicity of 2S albumin was preserved. This observation can be useful for the development of immunotherapy for Brazil nut allergy. On the other hand, de Melo et al. (1994) investigated the oral administration of various

doses Brazil nuts to rats as a possible route of immunization, but they found that instead of immunization, it induced systemic tolerance. Multiple feeding, than by a single dose feeding more effectively induced immune tolerance.

### Brazil nut possible allergies symptoms

In general, the symptoms of anaphylactic reactions associated with Brazil nut can vary from urticaria to anaphylactic shock, and the diagnosis can be made based on medical history or using the skin prick test (SPT) to measure the presence of specific IgE. Table 5 shows some patients with symptoms from oral ingestion or contact with body fluids (semen) of an individual who had consumed Brazil nuts (Bansal et al.,

2007). The proteomic approach was used to characterize the main isoforms of Brazil nut 2S albumin. Although most isoforms have molecular weight of approximately 12 kDa with a high amino acid sequence homology, significant heterogeneity was found (Moreno et al., 2014). Alcocer et al. (2012) stated that due to its extremely high content of sulfur amino acids, the allergen fraction has attracted much scientific attention as a target protein in transgenic biotechnology research, studies on processing of plant storage proteins and as an experimental protein in nutritional supplementation experiments. Some studies investigated the addition of Brazil nut protein to other foods such as soy. However, these experiments showed the allergen presence. For example, the nutritional quality of soy can be compromised by a relative

deficiency of methionine in the protein fraction of the seeds. In order to improve nutritional quality, the 2S albumin fraction from Brazil nut has been introduced into transgenic soybeans. An allergen can be transferred from one food to another through genetic engineering (Nordlee et al., 1996). Generally, immunological methods for detection of Brazil nut allergens in foods are based on polyclonal antibodies tested in animals. Phage display technology allows obtaining high affinity antibodies, avoiding animal immunization, and it can therefore apply the principle of replacement supported by animal welfare guidelines.

De La Cruz et al. (2013b) studied specific binders against Brazil nut employing a Brazil nut protein extract and a purified 2S globulin extract. A fragment (phage) that specifically recognizes Brazil nut proteins was isolated. The selected phage was also used as affinity probe to develop an indirect phage-ELISA for the detection of Brazil nut in experimental binary mixtures and processed foods. This study described for the first time the isolation of specific recombinant antibody fragments and showed the way for the development of immunoassays for the analysis of food that can be produced *in vitro* and do not rely on animal immunization.

Despite several previous reports on the allergenic potential of the 2S albumin protein fraction, Rundqvist et al. (2012) reported that the protein alone does not cause an allergic response in rats, but the addition of the components of a Brazil nut lipid fraction would be important. Structural details of *Ber e 1* suggest that it may contribute to the understanding of the protein allergenic properties and its potential interaction partners. The overall fold of 2S albumin is similar to that of other albumins, but the hydrophobic cavities resemble that of a homologous non-specific lipid transfer protein. The allergen proved to interact with  $\text{Cu}^{2+}$  ions. This  $\text{Cu}^{2+}$  binding has a minimal effect on the electrostatic potential on the protein surface, but the charge distribution within the hydrophobic cavity is significantly changed. Since the hydrophobic cavity is likely to be surrounded by a lipid,  $\text{Cu}^{2+}$  interaction, it can in turn affect the interaction to trigger an allergic response. On the other hand, the labeling of foods containing Brazil nut is of fundamental importance for the safety of consumers susceptible to food allergy. In order to protect sensitized individuals, reliable methods to detect trace amounts of Brazil nut should be available for the food industry and for health and food safety authorities. A TaqMan real time polymerase chain reaction (PCR) method was developed for specific detection of proteins in foodstuffs. The method uses specific Brazil nut primers, targeting the 2S albumin fraction, and a positive amplification control based on the 18S rRNA gene. The applicability of this specific system for Brazil nuts was evaluated on 66 samples of different commercial foods. The real time PCR assay proved to be a useful tool for detection of Brazil nut DNA, and it can be used as a routine analysis to assert accuracy on food labeling (De la Cruz et al., 2013a).

## Other issues

Despite its beneficial nutritional properties, the mycobiota prevailing in Brazil nuts may include mycotoxins-producing fungi. Since these substances can also be associated with environmental allergies and have deleterious effects, especially on the liver, the associated risks should be considered. Brazil nut production involves several producing countries and a global supply chain. Due to the presence of aflatoxins (AFLs) produced by toxigenic fungi during storage and production of Brazil nuts, there is strong need for contamination control and monitoring (Baquião et al. 2013; Pacheco et al. 2013; Pacheco and Scussel, 2009). In samples collected in the early stages of the production chain (communities/forest), the frequency of aflatoxin positive samples was lower than that in samples that were processed or obtained from retail centers (Pacheco and Scussel, 2007). Mycotoxins have been extensively studied in terms of their mechanism of action, mutagenicity and carcinogenic activity (Andrade et al., 2012; Freitas-Silva and Venâncio, 2011). The knowledge of these mechanisms has led to the development of biomarkers such as biotransformation products and macromolecular adducts (Bando et al., 2007).

AFLs ingested through contaminated foodstuffs are absorbed in the gastrointestinal tract and are biotransformed in the liver being activated and can therefore exert carcinogenic effects. The AFB1 activated form (AFB1 epoxide) can covalently bond to DNA, RNA and proteins. These bonds form adducts, which are primary biochemical lesions produced by AFB1 epoxide. The formation of adducts is a characteristic of liver carcinomas (Redzwan et al., 2012; Pereira and Santos, 2011). Therefore, DNA adducts reflect the variation in the aflatoxin intake daily diet, whereas albumin adducts integrate the exposure over several weeks. Quantification of exposure to AFB1 using precise and accurate laboratory methods is a relatively recent development. As an indicator of weeks or months of exposure, AFB1 Lysine (AFB1-lys) can be hydrolyzed with the patient serum. It can be quantified using isotope dilution liquid chromatography-tandem mass spectrometry (ID-LC-MS/MS) and quantified as a biomarker.

In a study conducted in the USA, for example, about 1% of the USA population had detectable levels ( $\geq 0.02$  mg/L) of AFB1-lysine. Of those with detectable levels, the geometric mean was 0.038 (0.024 to 0.060 g/L), equivalent to 0.842 (0.530 to 1.34 pg/mg) albumin (Schleicher et al., 2013). In addition to the high protein level of some foods, such as Brazil nuts, several studies strongly indicate that there is need for guidance during post-harvesting and food handling steps and interventions in the preparation of food intended to reduce exposure to AFLs in different populations and ethnic groups (Leong et al., 2012; Jolly et al., 2006). The identification and measurement of biomarkers to assess human exposure to mycotoxins by simple, rapid,

precise and accurate analytical methods can help prevent or minimize the health harms that arise from human exposure to these substances. In conclusion, the risk assessment of Brazil nut consumption, concerning allergy, is necessary in order to provide actual information to consumers and improve the food safety and public health.

Concerning the therapeutic potential from Brazil nuts, their benefits are well known. According to Yang (2009), epidemiologic studies have shown inverse association between the intake of nuts and chronic diseases such as cardiovascular diseases and cancer. The content of lipids, minerals and phytochemicals and their health benefits associated with the consumption of Brazil nuts have been continuously reviewed. Their nutritional composition seems to have beneficial effects due to their antioxidant properties that are associated with a reduced risk of developing atherosclerosis and cancer. Still regarding their influence on human metabolism, Cominetti et al. (2012) demonstrated that obese people who consume Brazil nut daily could improve Se status and lipid profile, especially high-density lipoprotein and cholesterol levels, thereby reducing cardiovascular risks. The consumption of 3 to 5 nuts a day for 16 weeks, improved the lipid profile and microvascular function in obese adolescents, possibly due to the high content of unsaturated fatty acids and bioactive substances (Maranhão et al., 2011). In addition, it was reported a long-term decrease in inflammatory markers after a single intake of Brazil nuts in healthy individuals (Colpo et al., 2014). Nevertheless, the long-term effect of their consumption on the inflammatory markers should be further investigated.

## CONCLUSION

The Brazil nut allergy is a clinical status dependable of prevention. Some strategies for consumers such as observe the ingredients content of food products and complete information for clinical treatments are necessary. More effort by the medical assistance is important to report clinic cases related to Brazil nut allergy to government agencies in order to apply the risk assessment concepts and to communicate the population about the risks of Brazil nut consumption by sensible individuals.

## Conflict of Interest

The authors have not declared any conflict of interest.

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The background of the entire page is a blue gradient with rounded corners. On the left side, there is a vertical image of a hand holding a clear glass filled with water. On the right side, there is a vertical image of a hand holding several colorful pills (red, yellow, green, white, and black).

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