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Presentation

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Chloroplast engineering: boon for third-world countries as therapeutic proteins

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Abstract: Chloroplasts are the site of photosynthesis in plants mostly seen in leaves and some eukaryotic algae that provides the primary sources of the world's food productivity. Plastids of higher plants are generally semiautonomous with a ~120–150 kb genome. Chloroplast transformation has become an attractive alternative to nuclear gene transformation due to its advantages, high protein levels, the feasibility of expressing multiple proteins from polycistronic mRNAs, and gene containment through the lack of pollen transmission. The review presents the recent trends and methods for plastid genome engineering and transgene expression and summarizes the potential of plastid transformation in various fields of biotechnology and also as a source of therapeutic proteins.

Keywords: *Chloroplast, Transformation, Therapeutic, Polyethylene glycol mediated method (PEG), Transplastomic genome*

I. Introduction

Chloroplast is an important primary cell organelle also known as plastids, mostly found in plants and in eukaryotic algae. Chloroplast helps to perform the primary synthetic process of photosynthesis. Other important activities that occur in plastids include evolution of oxygen, sequestration of carbon, production of starch, synthesis of amino acids, fatty acids, pigments, and is also a key aspects of sulphur and nitrogen metabolism[1,2].

The concept of chloroplast genetic engineering was developed in the 1980s by the work of Daniell and Mc Fadden (1987) [3], who show for the first time the uptake of genes by plant chloroplast. The plastid genome of higher plants is an attractive target for engineering because it provides readily obtainable high protein levels [4]. Chloroplast transformation generally results from homologous recombination, with a fragment of transforming DNA replacing the corresponding chloroplast DNA. Boynton et al., (1988) [5] reported successfully chloroplast transformation in *Chlamydomonas reinhardtii* through gene gun method. This method gain higher popularity due to simple operation and higher efficiency. Other method used is the polyethylene glycol (PEG) mediated transformation [6]. In 1989, stable chloroplast transformation in higher plants was achieved in Pal Maliga's Laboratory by the biolistic process, with which the *Escherichia coli* plasmids containing a marker gene and the gene of interest were introduced into chloroplasts or plastids. The foreign genes were inserted into plasmid DNA by homologous recombination *via* the flanking sequences at the insertion site [7]. This method of chloroplast recombination has proven to be more efficient than that

of nuclear gene transformation due to great potential, high protein levels, the feasibility of expressing multiple proteins from polycistronicm RNAs, gene containment through the lack of pollen transmission and pleiotropic effects due to sub-cellular compartmentalization of transgene products [4, 8, 9, 10]. Positive gene transformation in chloroplast has been carried out in plants like tobacco (*Nicotiana rustica*) and (*Nicotiana tabacum*) [11,7], Arabidopsis (*Arabidopsis thaliana*) [12], rice (*Oryza sativum*) [13] potato (*Solanum tuberosum*) [14], carrot (*Daucus carota*), and tomato(*Solanum lycopersicum*) [15, 16]. However, plastid transformation is mainly restricted to tobacco as its efficiency is much higher than in other plants [16].

Recently efficient plastid transformation using non-green tissues has been accomplished in carrot; in which the chloroplast transgenic lines were generated via somatic embryogenesis from tissues containing pro-plastids [17]. Keeping the importance of this technique an attempt is being made to highlight the recent trends in chloroplast engineering and its potential application as therapeutic proteins.

II. Transgene expression at chloroplast level

Chloroplast transformation was generally achieved by the biolistic process (bombardment) or the Polyethylene glycol mediated method (PEG). However, the bombardment method has become a favourable means for chloroplast or plastid transformation due to its higher transformation efficiency and simple operation [18] .Transformation is accomplished by integration of the transgene into a few genome copies, followed by 25 to 30 cell divisions under selection pressure to eliminate untransformed plastids, thereby achieving a homogeneous population of plastid genomes [2,19].

Chloroplast genes are basically transcribed by two RNA polymerases that recognize two different promoter regions, T7 like nuclear encoded polymerase and bacterium like plastid encoded polymerase. Both these polymerase promoters regions are found on plastid genes and encode for rRNA and mRNA. Transcription of transgenes inserted into the plastid genome is driven by plastid promoters usually the 16S rRNA promoter (P*rrn*16) or the *psbA* promoter [20, 21]. To date, the most commonly used site of integration is the transcriptionally active intergenic region between the *trn*I-*trn*A genes, within the *rrn* operon, located in the IR regions of the chloroplast genome. The foreign gene expression levels obtained from this site are among the highest ever reported [22]. It appears that this preferred site is unique and allows highly efficient transgene integration and expression. Many synthetic and hybrid promoter system have been developed that use GFP protein expression system or a specific sigma factor that bind to polymerase only at specific tissue. Still these systems are not completely adopted as it has been reported that these gene expression system affects nuclear genes as well and may cause impairment of plant growth [23].

The incorporation of suitable 5'-untranslated region (UTRs) of mRNA into chloroplast transgene along with stable 5' and 3' can also affect the protein expression level. Therefore, engineered UTR can be exploited more to enhance the expression of these transgenes in many of the higher plants to achieved desired products [24].

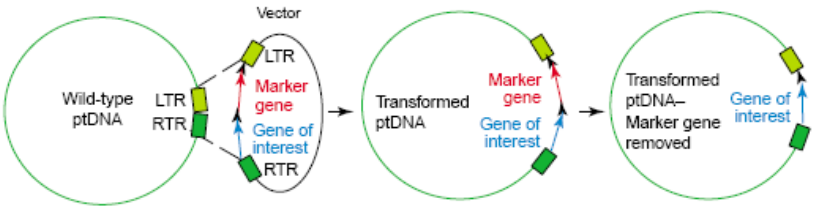

Fig1: Mechanism of transgene expression at chloroplast level (photo source: anonymous)

III. Application of Chloroplast engineering

Plastid transformation has been utilized in basic science, biotechnology and agronomy, pharmaceuticals and various other sectors of medicines and industrial production [25]. Some of the selected areas where chloroplast/ plasmid engineering has been applied are listed below.

III.1. Improvement of plant character

To improve plant traits and to produce plant capability to adapt stressful environment like biotic and abiotic stress factors, many genes have been engineered through chloroplast engineering. Characters like herbicide resistance, pest resistance and tolerance to drought and salinity stress has been improved using this technology. Transgenic chloroplasts have been reported to offer resistance to the fungal pathogen *Colletotrichum destructive* in tobacco [26]. *Cry* genes has also been expressed extremely well in the plastid genome and leaves of such plant prove to be toxic to insects feeding on such plant leaves [27]. Insect-resistant trans-plastomic soybean plants offers optimism for the transfer of the technology to important (food) crops [28] and thus is helpful for development of new stocks of resistance plants. New applications in these areas include the development of a plastid resistance gene against D-amino acids that potentially could be used as herbicides [29] and the successful expression of enzymes of the antioxidant system to provide increased tolerance to abiotic stresses [30] .Trans-plastomic carrot plants expressing BADH could be grown in the presence of high concentrations of NaCl (up to 400 m mol/L) [17].

III.2. Production of biopharmaceuticals

Chloroplast engineering is also suitable for high-level expression and economical production of therapeutic proteins in an environmentally friendly manner. Protein-based polymers derived from chloroplast transformation are affective with medical uses such as wound coverings, artificial pericardia, and programmed drug delivery [31]. This field is still at nascent stage but it emerges as an alternative for the production of medicines that is more chemical in nature. The production of therapeutic protein, human serum albumin (HSA) from transgenic chloroplasts of tobacco plant [32], high-level production of antigens for use as vaccines and their tests for immunological efficacy in animal studies, has been a revolutionary development in the pharmaceutical sector. Cholera toxin B sub-unit (CTB) of *Vibrio cholera* and virus type 1 (HIV-1) p24 antigen [33] against HIV virus has promising application in medicines development. Chloroplast-produced human IFN-g offered complete protection to human lung carcinomas against infection by the EMC virus. Chloroplast transformation can also serves as an effective expression system that can provide a clean, safe, and efficacious vaccine system.

III.3. In metabolic pathway engineering

Chloroplast represents the central organelle of a plant cell and many metabolic pathways involve the expression of genes present at chloroplast. An engineered chloroplast organelle can throw light on the way genes and their products express in plants. Particularly, chloroplast been the central house for photosynthesis and one of the key process in plant growth development that can be studied as metabolic pathway engineering [34].Further studies are carried out in different fruiting plants to increase the production of vitamins and minerals through plastid engineering. There is also growing interest in using trans-plastomic plants as factories for the production of so-called ‘green chemicals’: raw materials and building blocks for the chemical industry [35].

Many studies are carried out in the field of photosynthesis to understand the role of Rubisco protein through altered expression using chloroplast transformation. It is used for modifying the efficiency of Rubisco in favor of increasing catalytic activity or reducing the mechanism of photorespiration [36, 37].

III.4. In food

Developing protocols for important crops continues to pose a formidable challenge in plastid biotechnology and significant strides forward are likely to require conscientious efforts and long-term investments in both the academic and the industrial sectors. There is an urgent need to develop the concept of chloroplast transformation in economically important crop species such as carrot, cotton, rice, and soybean. Transformation of the plastid genome in commercial crops was achieved through somatic embryogenesis by bombarding embryogenic non-green cells or tissues [38].

Conversion of cellulosic biomass into fermentable sugars can be expressed from the plastid genome to very high levels. These include various cellulases, xylanases, glucosidases, pectatelyases and cutinases enzymes [38, 39].Stable integration of the ubiC gene into the tobacco chloroplast resulted in hyper-expression of the enzyme and accumulation of this polymer up to 25% of dry weight [38].

IV. **Conclusion**

The chloroplast engineering provide a good platform of foreign gene expression and holds great potential for the introduction of agronomic traits as well as the production of therapeutic proteins or vaccines in plants indigenous to developing countries such as India where people do not have access to these medicinal compounds. Also introducing the C_4 photosynthetic pathway and its proteins into the C_3 plant for better utilization of photosynthesis can be achieved by chloroplast engineering. Some of the major obstacles to extend this technology to major crop species include inadequate tissue culture and regeneration protocols, selectable markers and inability to express transgenes in developing plastids.

However, with recent advances in plastid engineering it has become a powerful biotechnological tool for the study of biogenesis and improvement of our understanding on crop development. Generation of trans-plastomic plants hold great promise for the commercialization of the technology and provides an efficient platform for the production of therapeutic proteins, vaccines, and biomaterials using an environmentally friendly approach.

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Phytochemical and Pharmacological Investigations of *Ricinus communis* Linn.

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Abstract: Medicinal plants have always played a vital role for the healthy human life. The family Euphorbiaceous is a family of flowering plants and contains nearly about 300 genera and 7,500 species. Amongst all, the species *Ricinus communis* or castor plant has high traditional and modern medicinal values. The individual parts of the plant like the seed, seed oil, leaves and the roots showed their importance in pharmacology. Traditionally, the plant has been used for the treatment of various diseases in traditional or folk remedies throughout the world. In modern pharmacology, this plant is reported to possess antioxidant, anti-inflammatory, anti-diabetic, central analgesic, antitumor, anti-nociceptive, antiasthmatic activity and other medicinal properties. These activities of the plant are due to the presence of important phytochemical constituents like flavonoids, glycosides, alkaloids, steroids, terpenoids etc. The aim of present article is to explore the chemical constituents, their structures and medicinal importance of *Ricinus communis*.

Keywords: *Ricinus communis;* Euphorbiaceae; Phytochemical Constituents; Pharmacology

I. Introduction

The species *Ricinus communis* Linn belongs to family Euphorbiaceae, monotypic genus, *Ricinus*, and subtribe, Ricininae.[1,2] This plant is popularly known as 'castor plant'. The other common names of this plant in different Indian languages are Jada, Gaba (Oriya); Bherenda (Bengali); Endi, Arand, Erand, Andi, Rend (Hindi); Erand (Marathi); Gandharvahasta, Vatari, Rubu, Urubu, Pancangula, Citra (Sanskrit); Haralu, Oudala, Gida (Kannada); Aran, Banangir (Kashmiri); Erandio, Erando (Guajrati); Arind (Punjabi).[2-4] It is found throughout the country and widely cultivated in the tropics and warm regions.[5,6] This is a fast-growing, perennial shrub or soft wooded small tree up to about 6 meters in height. This plant is cultivated for leaves, flowers or oil production and it grows wild in waste places. In the Indian system of medicine, different part of plants has been used for the treatment of different diseases.[1-7] It is also used as a lubricant, lamp fuel, a component of cosmetics, and in the manufacture of soaps, printer's ink, plastics, fibers, hydraulic fluid, brake fluid, varnishes, paints, embalming fluid, textile dyes, leather finishes, adhesives, waxes, and fungicides.[7]

In the Indian system of medicine, the leaf, root and seed oil of this plant have been used for the treatment of inflammation and liver disorders.[1] The plant has been found to be useful in hepatoprotective[8], antifilarial[9], antioxidant[10], antiasthmatic[11] and antimicrobial[12] activities. The root of this plant is also useful as an ingredient of various prescriptions for nervous diseases and rheumatic affections such as lumbago, pleurodynia and sciatica.[13] Roots of this plant showed anti-inflammatory and free radical scavenging[14], anti-fertility[15], anti-diabetic[16], and antimicrobial[17] properties.

In the following section, a comprehensive coverage of the literature covering the developments in the field of isolation of phytochemicals, the traditional and modern pharmacological applications and toxicological studies of ricin is presented.

II. Phytochemical Investigation

Phytochemicals are the compounds that are isolated from plant kingdom. The composition of these chemicals is dependent on their geographical locations and harvesting conditions. Hence, their quantitative and qualitative investigations are necessary to understand their applications. *R. communis* has wide range of applications. The phytochemists have worked tirelessly to find chemical constituents present in different parts of the plant. A homologous long chain 1,3-alkanediols and 3-hydroxyaldehydes have been obtained from the leaf cuticular waxes of this plant by Vermeer et al.[18] The cuticular waxes also comprised of alkanes, primary alcohols, aldehydes, fatty acids and triterpenoids.[18] Kang *et al* identified various alkaloids and flavonoids in the leaves of this plant.[19] The structures of these compounds were determined through spectroscopic analysis, chemical correlation and chemical degradation studies. A list of phytochemicals obtained from different parts is given below.

II.1 Leaves: Aldehydes (C_{26} and C_{28})[18], Alkanes (C_{26}-C_{29})[18], α-Amyrin (**1**)[18], β-Amyrin (**2**)[18], *N*-Butylmorpholine (**3**)[20], Chlorogenic acid (**4**)[21], Camphor (**5**)[22], 1,8-Cineole (**6**)[22], Citric acid[23], β-Caryophyllene (**7**)[22], Decanamine[20], *N*-Demethylricinine (**8**)[19,24], Di-butylphthalate[20], 2,5-Dihydroxybenzoic acid (Gentisic acid)[21], β-Eleosteric acid (**9**)[25], Ellagic acid (**10**)[10], (-)-Epicathechin (**11**)[21], Fumaric acid[23], Gallic acid (**12**)[10,21], Hexacosane-1,3-diol[18], 3-Hexen-1-ylacetate[20], Kaempferol (**13**)[19], Kaempferol 3-*O*-β-D-glucopyranoside (Astragalin, **14**)[19], Kaempferol 3-*O*-β-D-xylopyranoside (**15**)[19], Kaempferol 3-*O*-β-rutinoside (nicotoflorin, **16**)[19], Linoleic acid[25], Linolenic acid[25], Lupeol (**17**)[18], Myristic acid (**18**)[26], Malic acid[23], Methyl gallate[27], Neochlorogenic acid (an isomer of **4**)[21], 4-Octadecylmorpholine (**19**)[20], Oleic acid[26], Palmitic acid (**20**)[26], Palmitoleic acid[26], α-Pinene (**21**)[22], Primary alcohols (C_{22}-C_{38})[18], Quercetin (**22**)[19], Hyperoside (**23**)[21], Quercetin 3-*O*-β-Rutinoside (Rutin, **24**)[19], Quercetin-3-*O*-β-D-glucopyranoside (isoquercetin, **25**)[19,27,28], Quercetin 3-*O*-β-D-xylopyranoside (Reynoutrin, **26**)[19,27], Ricinine (**27**)[29-31], β-Sitosterol (**28**)[31], Stigmasterol (**29**)[31], Stearic acid (**30**)[26], Tartaric acid[23], Tannins[21].

II.2 Essential oils from leaves: Camphor (**5**)[32], Camphene (**31**)[32], 1,8-Cineole (**6**)[32], α-Pinene (**21**)[32], α-Thujone (**32**)[32].

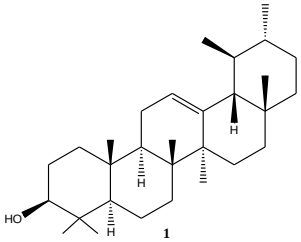
II.3 Roots: 3-*O*-Benzyol-stigmasta-5,22-dien-3β-21-diol (Ricinusterryl benzoate)[33], Dipiperenoyl methyl ester methylene (Ricipiperanyl ester)[33], Erandone (**33**)[34], 3-α-Hydroxypentatriacont-14-en-26-one (Ricipentatriacontanol)[33], Indole-3-acetic acid[35], Lupeol (**17**)[34], 1-Oleio-2-palmitoglyceryl phosphate[33], Quercetin-3-*O*-β-D-glucopyranoside (isoquercetin, **25**)[36], Quercetin 3-*O*-β-Rutinoside (Rutin, **24**)[36], Kaempferol 3-*O*-β-D-[6-O-acetylglucopyranosyl(1$\rightarrow$3)-β-D-galactopyranoside] (Ricinitin)[36].

II.4 Seed: Arachidic acid (**34**)[37], Eicosenoic acid[37], Ergost-5-en-3-ol (**35**)[38], Fucosterol[38], Glycine[39], Hydroproline[39], Leucine[39], Linoleic acid[37], Maltose[39], Oleic acid[37], Palmitinic acid[37], Phenylalanine[39], Probucol[38], Proline[39], Ricinoleic acid (9*Z*,12R)-12-Hydroxyoctadec-9-enoic acid, **38**)[27,40,41], Stearic acid (**30**)[37], Ricin[42], Ricinine (**27**)[39], γ-Sitosterol[38], Stigmasterol (**29**)[38], Tryptophan[39], Valine[39].

III. Pharmacological Applications

III.1 ***Traditional Pharmacological Uses:*** *R. communis* has very high traditional medicinal values. The oil extracted from the seeds of this plant has been used by local people since about 2000 BC.[2,4,43-45] The use of different parts of this plant for the treatment of various diseases in traditional or folk remedies throughout the world has been reviewed.[6] The oil is extracted after removing the hard protective cover of seeds. This oil is also known by other names like ricinus oil or castor oil.[46] This oil has been used in local medicines as a laxative, arthritic diseases and cathartic in Unani, Ayurvedic and other ethno medical systems.[43] The oil acts as an osmotic laxative in mild to moderate constipation. This holds water in the intestines and performs total cleansing of the large intestine.[44] This is one of the safest and most reliable purgatives we possess for the relief of obstinate constipation.[45] In addition, it is a traditional folk medicine used in the treatment of warts, cold tumors, and indurations of mammary glands, corns, and moles.[47-49] The oil is also externally applied and internally taken for sciatica; arthritis; gout and paralysis. The use of hot fomentation is beneficial for any cyst, inflammation, tumour or lump. The oil is beneficial in the treatment of styes, conjunctivitis

and foreign objects in the eyes. The oil is also useful to treat dry skin conditions such as psoriasis and eczema.[44] This is often given orally, alone or with quinine sulphate to induce labour in pregnancy. It is also included in the treatment of piles.[44] The decoction of leaves are heated and applied to a woman's breasts to improve secretion of milk. Decoction is a practice of obtaining dissolved chemicals from herbal or plant materials by first mashing and then boiling in water. The decoction has also been reputed to increase the secretion of milk when administered internally.[50] An infusion of leaves is used for stomache-ache, and as a lotion for the eye. Roots are administered in the form of a decoction for lumbago and allied complaints, in the form of a paste for toothache. In the decoction process, a herbal or plant material is mashed and then boiled in water to extract active compounds. Root bark is reported to be a powerful purgative.[50]



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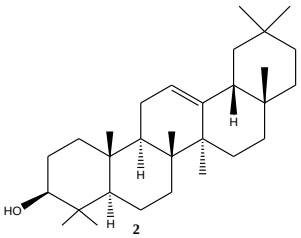

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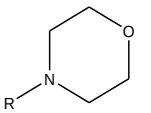
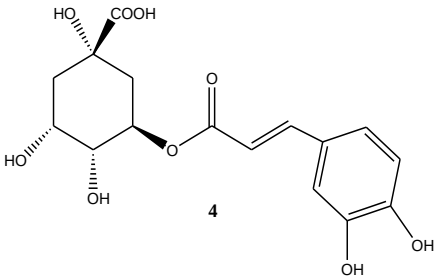


3 R = $(CH_2)_3CH_3$
19 R = $(CH_2)_{17}CH_3$



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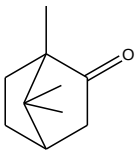

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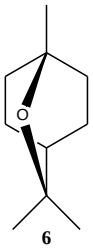

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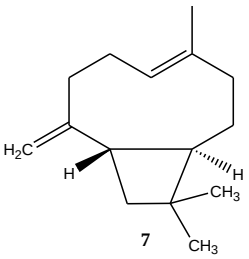

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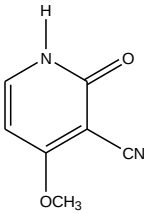
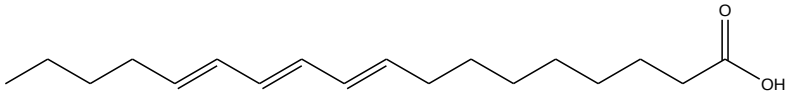


8 R = H
27 R = CH_3



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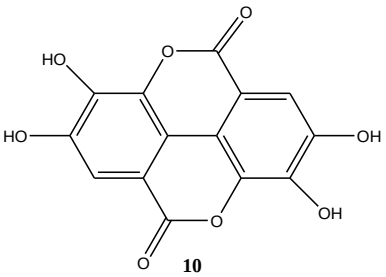

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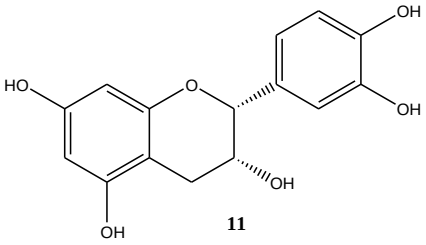

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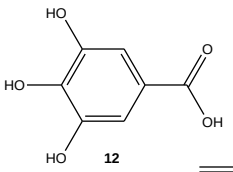

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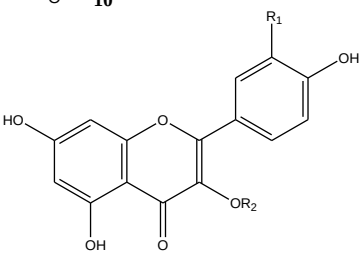
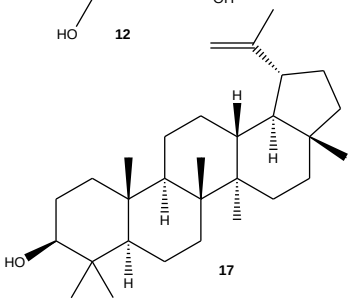


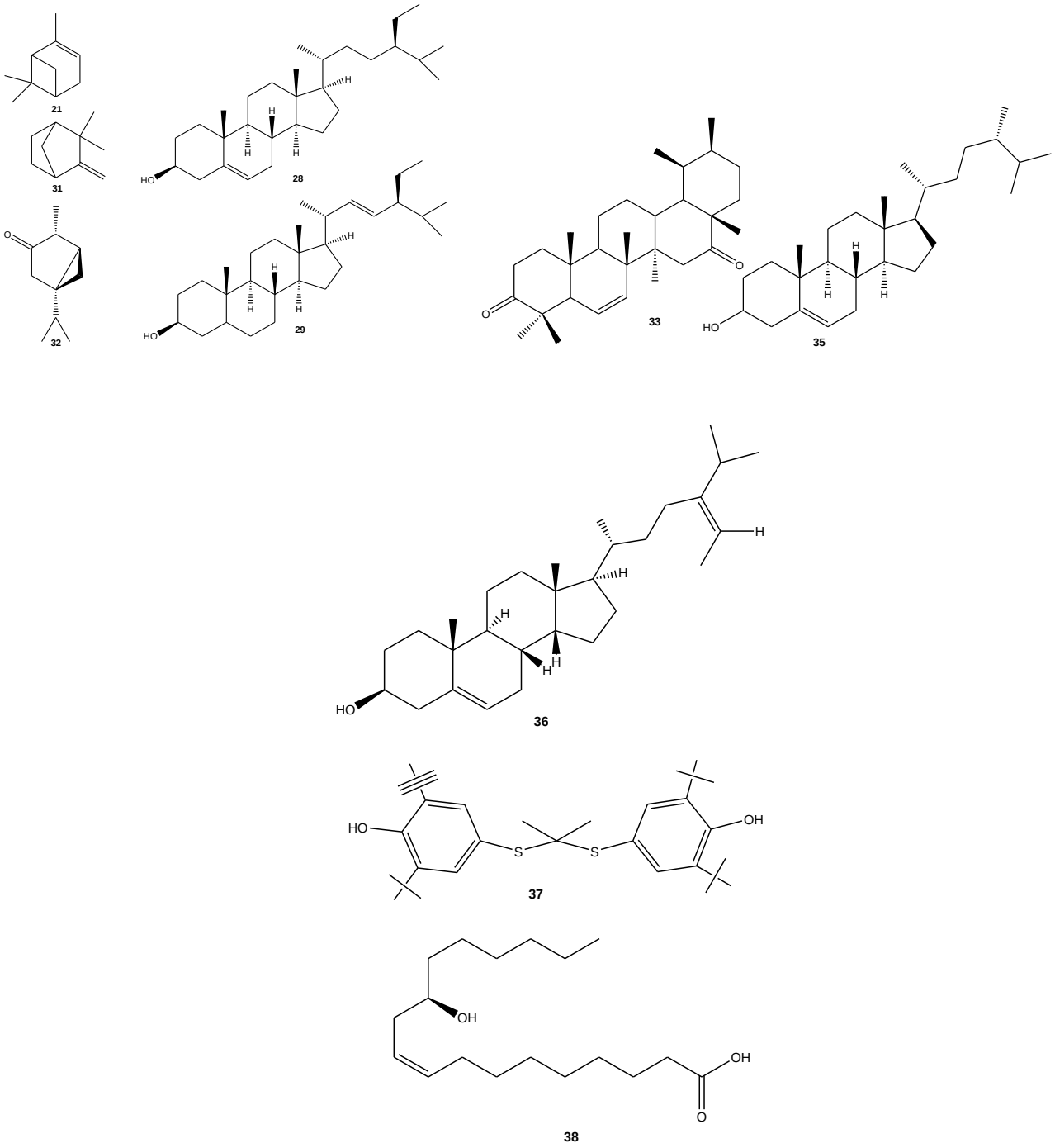
13 R_1 = H; R_2 = H
14 R_1 = H; R_2 = β-D-glucopyranosyl
15 R_1 = H; R_2 = β-D-xylopyranosyl
16 R_1 = H; R_2 = β-rutinosyl
22 R_1 = OH; R_2 = OH
23 R_1 = OH; R_2 = β-D-galactopyranosyl
24 R_1 = OH; R_2 = β-rutinosyl
25 R_1 = OH; R_2 = β-D-glucopyranosyl
26 R_1 = OH; R_2 = β-D-xylopyranosyl



17

$CH_3(CH_2)_nCOOH$

18 Myristic acid	n = 12
20 Palmitic acid	n = 14
30 Stearic acid	n = 16
34 Arachidic acid	n = 18



III.2 *Modern Pharmacological Uses*

III.2.1 Antioxidant activity: Antioxidants are compounds that prevent or delay the oxidation of oxidizable materials by scavenging free radicals. Free radicals are responsible for oxidative stress which promotes the development of chronic degenerative diseases including coronary heart disease, cancer and aging.[51,52] The plant *R. communis* has significant radicals scavenging abilities on 2,2-dipehyl-1-picrylhydrazyl (DPPH)[53], nitric oxide (NO)[54], and superoxide radicals[54]. The CH_3OH:H_2O (8:2) extract of leaves showed strong DPPH radical-scavenging activity.[10] The stem and leave extracts also produce antioxidant activity due to the presence of flavonoids in their extracts.[10]

III.2.2 Antidiabetic activity: Diabetes or diabetes mellitus is a group of metabolic diseases in which a person has high blood glucose (blood sugar). This may be due to inadequate insulin production, or because the body's cells do not respond properly to insulin, or both. An antidiabetic agent controls diabetes. World Health Organization (WHO) has recommended the evaluation of traditional plant treatments for diabetes as they are effective, non-toxic, with less or no side effects.[55] The

administration for 20 days of 50% ethanolic extract of roots has been found to be effective to the diabetic rats on fasting blood glucose, and also on total lipid profile and liver and kidney functions on 10^{th} and 20^{th} day.[16] The effective dose of 500 mg/kg body weight not only significantly lowered the fasting blood glucose (FBG) of the diabetic animals to almost normal level, but also increased the insulin levels and caused improvement in lipid profile and body weight of the diabetic animals. The extract also caused reversal of the damage of liver and kidneys seen in diabetic animals.[16]

III.2.3 Antimicrobial activity: An antimicrobial is an agent that kills microorganisms or inhibits their growth. Antimicrobial substances are grouped according to the microorganisms against which they act. The antimicrobial activity of the oil isolated from leaves was investigated in order to evaluate its efficacy against twelve bacteria and four fungi species, using disc diffusion and minimum inhibitory concentration methods.[32,56] The results are comparable to the antibiotic ampicillin, used as a positive control. The isolated leaf oil showed strong antimicrobial activity against all microorganisms tested with higher sensitivity for *Bacillus subtilis*, *Staphylococcus aureus* and *Enterobacter cloacae*.[32] These findings showed that the variation in quantities of the main components such as camphor (**5**) and 1,8-cineole (**6**), might be responsible for the different antimicrobial activity.[32] The MIC and IC_{50} values of essential oil on bacteria ranged from 120 µg/ml to 300 µg/ml, and from 210 µg/ml to 870 µg/ml, respectively, whereas on fungi the value ranged from 140 µg/ml to 250 µg/ml and from 350 µg/ml to 590 µg/ml.[32] Naz et. al. studied the *in vitro* antimicrobial properties of methanol (CH_3OH), ethanol (C_2H_5OH) and water (H_2O) extract of leaves against gram positive and gram negative bacterial strains and *Aspergillus flavus* and *A. fumigatus* fungal strains.[57] Methanol extract was found to be more efficient than the other two extracts.[57] The methanol and ethyl acetate ($CH_3COOC_2H_5$) extracts of leaves showed good activity against *P. aeruginosa*, *S. aureus*, *K. Pneumonia* and *Proteus vulgaris.* The antimicrobial assay revealed that these extracts possess good zone of inhibition.[58] The secondary infections in the immune compromised oral cancer cases were due to bacterial and fungal species. The co-administration of *R. communis* with the immune suppressant drugs for the prevention of infection against oral cancer treatment patient showed significant result.[59]

III.2.4 Anti-Inflammatory activity: Inflammation is a localized response that produces redness, warmth, swelling and pain due to infection or injury. In the Indian system of medicine, the leaves, roots and seeds have been used for the treatment of inflammation.[14,60,61] Methanolic extract of the root was studied for anti-inflammatory activity in carrageenan induced hind paw edema model in Wistar albino rats.[62] Ricinine (**27**), Quercetin (**22**) and *n*-butanol soluble fraction of methanol extract gave promising result for anti-inflammatory activity.[62] Root crude methanolic, enriched *n*-hexane fraction isolates at doses 100 mg/kg p.o. exhibited significant ($P < 0.001$) anti-inflammatory activity in carrageenan-induced hind paw oedema model.[34] The compound ricinoleic acid (**38**), the main component of castor oil also showed remarkable analgesic and anti-inflammatory effects.[41] The results showed that **38** may be seen as a new capsaicin-like, non-pungent anti-inflammatory agent suitable for peripheral application.[41]

III.2.5 Antinociceptive activity: Nociception is a physiological term to describe neural process of encoding and processing noxious stimuli.[63] The methanol extract of the leaves possesses antinociceptive activity in acetic acid induced writhing test, formalin induced paw licking and tail immersion test in mice.[64] The extract showed analgesic effect at a dose of 150 mg/kg on formaline induced pain corresponding to neurogenic and inflammatory pains.[64] The extract also increases latent time after 90 minutes of drug treatment in tail immersion model.[64]

III.2.6 Anti-fertility activity: The seed extract have been found to possess anti-fertility activity. The ether soluble portion of the methanol extract of seeds when administered subcutaneously to adult female rats and rabbits showed anti-implantation and anti-conceptive activity.[65] The extract protected the animals from getting pregnant for over three gestation periods.[65] Further, the extract did not show any long term effect on the pups that were born after the extract effect.[65] The seed extract was found to possess anti-implantation and abortificient effects. It was also observed that the seed extract prolonged the oestrus cycle of guinea pigs. The dioestrus phase was significantly prolonged as well. After stopping the administration of the extract, the normal dioestrus phase and oestrus cycle started to resume. The seed extract also reduced the weight of the uterus without affecting that of the ovaries significantly. The anti-fertility effect of *R. communis* in female guinea pigs might be extrapolated to human beings.[66] The 50% alcohol extract of the roots possess significant reversible anti-fertility effect.[67] There was a drastic reduction in the epididymal sperm counts in male rats. The extract also

caused changes in the motility, mode of movement and morphology of the sperms. The reductions in the fructose and testosterone levels further suggested the reduced reproductive performance.[67]

III.2.7 Anti-hepatotoxicity: The hepatoprotective activity is the ability to protect liver damage.[68] Liver cirrhosis and drug induced liver injury are major health problem in western and developing countries.[69] Herbal and Plant products are popular and potential hepatoprotective agents.[70,71] An ethanol extract of the leaves showed significant protection against galactosamine-induced hepatic damage.[72] The maximum activity was obtained in the butanol fraction of the ethanol extract. Further purification led to the isolation of two active compounds ricinine (**27**) and N-demethylricinine (**8**). The compound **8** was found to be more active than **27**.[72] In another study by Padmapriya et al [8], ethanolic leaf extract was evaluated at a dose of 100 mg/kg body weight against Ketoconazole (Phytoral) induced liver damage in mice. The result showed relative significant reduction in hepatic enzymes of treated mice and confirmed the traditional uses of this plant as a potential hepatoprotective agent.[8] Pingale *et al* used the powder of leaves against hepatosuppression induced by carbon tetrachloride (CCl_4). It was found that the powder had high potential in healing liver parenchyma and regeneration of liver cells. It showed best ability to protect liver and may act in humans even as potent liver tonic due to the presence of large number of antioxidants.[73] Natu *et al* also studied the protective effect of leaves in experimental liver injury caused by carbon tetrachloride in albino rats.[74] They reported the pharmacological effects of the whole leaves, cold aqueous extract and a glycoside extracted from the leaves. The whole leaves provided protection against liver necrosis while the cold aqueous extract provided protection only against fatty changes.[74] The glycoside protected the liver from cell necrosis. The leaves had significant parasympathetic activity and parasympathetic predominance can be expected to cause an increase in blood supply to the liver and protection against hepatotoxic agents.[74]

III.2.8 Cytotoxic Activity: Cytotoxicity is the ability of any substances to be toxic to cells. Ricin is a heterodimeric protein isolated from the seeds. It possesses cytotoxic activity by virtue of its ability to fatally disrupt protein synthesis.[75] Therapeutically, it can be used to specifically target and destroy cancer cells.[75] The leaves on the other hand, have another range of cytotoxic phytochemicals which induces apoptosis *via* translocation of phosphatidyl serine to the external surface of cell membrane and loss of mitochondrial potential. These compounds included three monoterpenoids: camphor (**5**), 1,8-cineole (**6**) and α-pinene (**21**) and a sesquiterpenoid: β-caryophyllene (**7**).[76] The *R. communis* agglutinin I (RCA I), was found to preferentially binds to and is internalized by tumour endothelial cells leading to VEGFR-2 down-regulation, endothelial cells apoptosis and tumour vessel regression. It has no effect on normal blood vessels.[77] A volatile extract from the leaves was cytotoxic to several human tumor cell lines in a dose-dependent fashion.[76] Apoptosis was induced in SK-MEL-28 human melanoma cells at a concentration of 20 μg mL^{-1}. Translocation of phosphatidyl serine to the cell membrane's external surface and loss of mitochondrial membrane potential have also been detected with this extract.[76] The effect of different concentrations of essential oil from leaves on HeLa cell survival was also studied. The cytotoxicity of the oil was quite strong with IC_{50} values less than 2.63 mg/mL for both cell lines.[32] Ricin A[78], a lectin isolated from *R. communis* possess antitumor activity, it was more toxic to tumor cells than to non-transformed cells, judged from the ED_{50} of the lectin towards tumor cells and non-transformed cells.[79]

IV. Toxicological Analysis

The seed from *R. communis* contains two toxins that are poisonous to humans, animals and insects.[80-84] One of the main toxic proteins is ricin which is a potent cytotoxin but a weak hemagglutinin, whereas the other one RCA (*Ricinus communis* agglutinin) is a weak cytotoxin and a powerful hemagglutinin.[86] Ricin works by inactivating the ribosomes present in cells. Due to this the ribosomes do not produce proteins. Cells need these proteins to survive and reproduce, so when ribosomes are inactivated, cells die. The seed poisoning by ingestion is due to ricin, not RCA, because RCA does not penetrate the intestinal wall. The RCA does not affect red blood cells (RBCs) unless given intravenously. If RCA is injected into the blood, it will cause the red blood cells to agglutinate and burst by hemolysis.[83] The effect of ingestion of castor bean in a puppy was studied by Mouser et al.[80] Ingestion of masticated seeds resulted in high morbidity, with vomiting and watery to hemorrhagic diarrhea. The prognosis varied with the number of seeds ingested, the degree of mastication, individual susceptibility and the delay in treatment. Despite supportive therapy, the puppy

died several hours after presentation for acute vomiting, diarrhea and lethargy. Histopathologic findings included superficial necrotizing enteritis of jejunum and occasional, random foci of coagulative necrosis in the liver. Ricin toxicological effect was confirmed by liquid chromatography/mass spectrometry using Ricinine as a marker.[80] Coopman *et al* studied the suicidal death of a 49 year-old man who committed suicide by intravenous and subcutaneous injection of a castor bean extract.[81] The patient developed nausea, vomiting, diarrhea, dyspnoea, vertigo and muscular pain after taking injection. Despite symptomatic and supportive intensive care, he died due to multiorgan failure.[81] It is the first time that ricin had been identified in a case of castor bean poisoning. Based on the clinical symptoms and the results of the toxicological analysis, it was concluded that the death was caused by intoxication with plant toxins originated from the plant.[81]

V. Summary and Future Prospects

Traditional medicines are always at the centre of attention to cure various ailments. *R. communis* is native plant of India and all the parts of this plant have been medicated. Various crude fractions and purified components have shown potential medicinal and pharmacological activities. The antioxidant and free radical scavenging activities of phyto-components isolated from this plant give us an impression that the plant might be the future drug for diversified panel of tumors and cancers. The plant is also reported to possess anti-diabetic, antimicrobial, anti-inflammatory, anti-nociceptive, anti-fertility, anti-hepatotoxicity and other medicinal properties. These activities of the plant are due to the presence of important phytochemical constituents like flavonoids, glycosides, alkaloids, steroids, terpenoids etc.

A systematic scientific approach from phytochemicals either in pure or crude form to modern drug development can provide valuable drugs from traditional medicinal plants. Development of such medicines with international safety and efficacy can give better and satisfactory treatment of various diseases. To ensure ample production of phyto-constituents with in limited space and time, new approaches must be adopted. This is because the prospecting of bioresources for economic development is emerging as a new economic venture.

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Ameliorated Effects of Green Tea Extract on Lead Induced Kidney Toxicity in Rats

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Abstract:

In the present study, the protective effect of an aqueous extract of green tea (GTE) against renal oxidative damage induced by lead was undertaken. Adult males rats were divided into 4 groups: Control group receives distilled water as sole drinking source. GTE group received green tea extract (6.6% w/v).Pb group received Pb at dose of 0.4 % w/v in distilled water. Pb + GTE group received mixture of Pb and GTE as sole drinking source. Renal oxidative damage was observed in Pb-treated rats as evidenced via augmentation in kidney lipid peroxidation (LPO) as well as depletion in kidney antioxidant enzymes; catalase (CAT), superoxide dismutase (SOD) and glutathione peroxidase (GPx). Histopathological analysis revealed degeneration in the endothelium of glomerular tuft and the epithelium of lining tubules. In conclusion, GTE appeared to be beneficial to rats, to a great extent by attenuating and restoring the damage sustained by lead exposure.

Key words: Green tea; Lead; Histopathology; Nephrotoxicity; Oxidative stress.

I. Introduction

In recent years, the level of heavy metals, particularly lead has increased in air, water and soil in both urban and periurban areas [1]. It is well known that heavy metals induce toxic effects on different systems and apparatuses. Furthermore, because of their long half-life, heavy metals also induce accumulation phenomena, which in turn produce an experimental increase of their concentration in blood and tissues. Among heavy metals, lead represents the main environmental poison. Lead is a non-essential toxic heavy metal widely distributed in the environment and chronic exposure to low levels of lead has been a matter of public health concern in many countries [2]. Lead may be absorbed through the skin, gastrointestinal tract or lungs and distributed to three major compartments-blood, soft tissue and bone. Blood lead is in equilibrium with lead in soft tissue. The soft tissues that take up lead are liver, kidneys, brain and muscle. Lead is not metabolized in the body but it may be conjugated with glutathione and excreted primarily in the urine. Lead is a multi-targeted toxicant affecting gastro intestinal tract, hematopoietic system, cardiovascular system, central and peripheral nervous system, kidneys, immune system and reproductive system [3]. Lead can damage all tissues, particularly the kidneys and the immune system. Recent evidence suggested that the

kidney might also be one of the major organs for chronic lead toxicity. Lead may exert toxic effect on several organ system but those in the kidney are the most insidious. Nephrotoxicity results because kidney is the main route of elimination of lead [4]. Effect of lead on renal system is characterized by dysfunction of proximal renal tubules manifested by glycosuria, generalized amino aciduria, hyperphosphaturia, hyperphosphataemia and rickets are noted in acute lead poisoning. Long-term exposure to lead is known to cause irreversible functional and morphological changes, which include interstitial, tubular atrophy, and ultra-structural changes in renal tubule mitochondria [5]. Chronic lead toxicity is caused by the change of renal function parameters. Lead induced oxidative stress contributes to the pathogenesis of lead toxicity for disturbing the delicate prooxidant/antioxidant balance that exists within mammalian cells. Lead exposure cause generation of ROS (Reactive Oxygen Species)and alteration of antioxidant defense in animals and occupationally exposed workers [6]. Nephroprotective agents are the substances which possess protective activity against Nephrotoxicity. Medicinal plants have curative properties due to the presence of various complex chemical substances. Early literatures have prescribed various herbs for the cure of renal disorders [7]. The term renal failure primarily denotes failure of the excretory function of kidney, leading to retention of nitrogenous waste products of metabolism in the blood [8]. In addition to this, there is a failure of regulation of fluid and electrolyte balance along with endocrine dysfunction. The renal failure is fundamentally categorized into acute and chronic renal failure [9]. Tea in the form of green tea (GT) or black tea is one of the most widely consumed beverages in the world today second only to water [10]. Since ancient times GT consumption has been known to maintain and improve health. Polyphenols are plant metabolites occurring widely in plant food and exhibit outstanding antioxidant and free radical scavenging properties [11]. GT is an excellent source of polyphenols such as catechins[12], orgallotannins, flavonols, flavandiols, and phenolic acids [13]. In particular GT catechins and their derivatives are known to contribute beneficial health effects ascribed to tea by their antioxidant [8], antimutagenic, and anticarcinogenic properties [14]. GT consumption has been linked to lowering of various forms of cancers [15]. GT constituents also have been shown to have cardioprotective, neuroprotective, antidiabetic, and antimicrobial properties [16]. In addition, GT has been found to be useful in the treatment of arthritis, high cholesterol levels, infection, and impaired immune function [17]. GT consumption also has resulted in improved kidney functions in animal models of renal failure [18]. Hence, the goal of the present study has been to investigate the efficacy of green tea, as a source of water soluble antioxidants on renal function abnormalities of mature rats exposed to an oxidative stress induced by lead.

II. Materials and Methods

II.1. Animal treatment

40 male wistar rats (age: 14-16 weeks and about 170-200g body weight) were purchased from Animal House, department of Biology, Algeria University. All animals were conditioned at room temperature (22-25°C) at a natural photoperiod for one week before experiment execution. A commercial balanced diet and tap water *ad libitum* were provided. The duration of experiment was 4 weeks. All the procedure performed on animals were approved and conducted in accordance with the National Institute of health Guide (Reg. No. 488/160/1999/CPCSEA). They were randomly divided into 4 groups (10 rats each) as the following: Group I (Control group) receives distilled water as sole drinking source. Group II (GTE group) received green tea extract (6.6% w/v) of beginning of experiment. Group III (Pb group) received lead acetate at dose of 0.4 % w/v in distilled water. Group IV (Pb + GTE group) received mixture of lead acetate and GTE as sole drinking source.
The GTE was made by soaking 30 g of instant green tea powder in 500 ml of boiling distilled water for 15 minutes. The solution was filtered to make 6.6% GTE. This solution was provided to rats as their sole source of drinking water. During the experimental duration, body weights were recorded avery week . After 4 weeks, the animals of different groups were sacrificed under light anesthesia 1 day after the end of the treatment. Both kidneys were removed, cleaned and weighed for histological and biochemical evaluation.

II.2. Oxidative stress evaluation

The kidneys were quickly removed, perfused immediately with ice cold hypertonic saline solution and homogenate 10% prepared in 1.15% w/v of potassium chloride for measurement of antioxidant activity.

II. 2.1. Renal lipid peroxidation

Malondialdehyde (MDA) content as indicator of lipid peroxidation was determined in the serum and tissues, by a colorimetric method.
Malondialdehyde(MDA) occurs in lipid peroxidation and was measured in kidney tissues after incubation at 95 °C with thiobarbituric acid in aerobic conditions (pH 3.4). The pink colour produced by these reactions was measured spectrophotometrically at 532 nm to measure MDA levels [18].

Renal antioxidant enzymes:
II. 2. 2. Assay of catalase (CAT)

Catalase (CAT) activity was measured using the method of Aebi[19]. Twenty µl of the supernatant were added to a cuvette containing 780 µl of 50 mM potassium phosphate buffer (pH 7.4) and then the reaction at 25 °C was initiated by adding 200 µl of 500 mM H_2O_2 to make a final volume of 1 ml. The decomposition rate of H_2O_2 was measured at 240 nm for 1 min on a spectrophotometer. A molar extinction coefficient of 0.0041 $mM^{-}1$ was used to determine the CAT activity. The activity was defined as an nmoles H_2O_2 /min/mg protein.

II. 2.3. Assay of superoxide dismutase (SOD)

Superoxide dismutase (SOD) activity was estimated according to Beauchamp and Fridovich, [20]. The reaction mixture contained 50 mM of kidney homogenates in 0.1 M of potassium phosphate buffer (pH7.4), 0.1 mM EDTA, 13 mM L-methionine, 2 µM riboflavin and 75 µM Nitro Blue Tetrazolium (NBT). The developed blue color in the reaction was measured at 560 nm. Units of SOD activity were expressed as the amount of enzyme required to inhibit the reduction of NBT by 50% and the activity was expressed per mg of protein.

II. 2. 4. Assays of glutathione peroxidase (GPx)

Glutathione peroxidase (GPx) activity was measured according to Flohe and Gunzler [21]. GPx catalyzes the oxidation of reduced glutathione by cumene hydroperoxide. In the presence of reduced glutathione reductase and nicotinamide adenine dinucleotide phosphate reduced form (NADPH), the oxidized reduced glutathione is immediately converted to the reduced form with a concomitant oxidation of NADPH to NADP+. The decrease in absorbance at 340 nm was measured. The enzyme activity was expressed as nmol of GSH oxidized/min/ mg protein. Protein concentration was determined according the method described by Lowry et al. [22] using bovine serum albumin (BSA) as a standard.

II.3. Histological studies

At the end of the experiment, kidney from each sacrificed rat was dissected out; and trimmed of excess fat. Then, it was fixed in 10% buffered formalin and was processed for paraffin sectioning by dehydration in different concentrations of alcohol, cleared with xylol and embedded in paraffin blocks. Sections of about 5 µm thickness were stained with Harris haematoxylin and eosin (H&E) for histological study [23].

II. 4. Statistical analysis

Results were analyzed using a one-way analysis of variance (ANOVA) followed by Duncan's test. Comparison within groups was considered statistically significant at $p<0.05$. All the data were expressed as mean ±SD of number of experiments (n=10).

III. Results

III. 1. Body and Organ Weights

At the end of the experimental course, there was no significant difference in body and relative kidney weights between GTE and untreated rats. However a significant loss of weekly body weight accompanied by a significant increase in the relative kidney weights were recorded in rats treated with lead acetate compared to the control. The administration of GTE to Pb treated groups has an ameliorated effect either in the loss of body weight or in the increase of relative Kidney weights (Table1).

Table1. Effect of green tea consumption on body weight (g), kidney weight (g), and relative kidney weight (%) of rats treated with lead acetate.

Groups	Body weight (g)	Absolute kidney weight (g)	Relative kidney weight (g/100g body weight)
Control group	218.90±3.75	1.27±0.05	0.58±0.01
GTE group	221.90±4.19^{b}	1.26±0.06^{b}	0.57±0.09^{b}
Pb group	173.90±6.44^{a}	1.22±0.02^{a}	0.71±0.07^{a}
Pb+GTE group	192.18±7.40^{ab}	1.21±0.04^{ab}	0.63±0.08^{ab}

Values are means ±S.E.; N (number of animals) = 10; LSD (least significant difference) at the 5% level = 0.01650.
[a]Superscript in the same row differ significantly at *P <0.05* withcontrol (C). [b] Superscript in the same row differ significantly at *P <0.05* with lead group (Pb).

III. 2. Renal lipid peroxidation (LPO)

Administration of Pb led to a significant increase ($p < 0.05$) in lipid peroxidation as evidenced by the increase in kidney tissue MDA levels by 44%, when compared to the control group. However, co-administration of GTE to treated rats reduced the augmentation in MDA levels to 19% for Pb-treated rats (Table 2). Results in Table 2 show the influence of lead acetate on the activities of CAT, SOD and GPx. Subacute levels of the tested metal resulted in a state of kidney injury and extensive oxidative damage in rats as manifested by the significant alteration in these enzymes. In fact, in treated rats, a significant depletion was noted in the activities of CAT, SOD and GPx. However, the co-administration of GT mitigated the change in the activities of SOD, GPx and CAT (Table 2).

Table 2. Effect of lead acetate on renal lipid peroxidation and antioxidant enzymes of male rats and the ameliorative role of green tea.

Treatments	LPO (nmoles/mg protein)	CAT (nmoles H_2O_2 /min/mg protein)	SOD (mg protein)	GPx (nmoles of GSH oxidized/min/mg protein)
Control group	3.28±0.19^{a}	312.2±14.3ab	18.8±0.80^{a}	4.26±0.25^{a}
GTE group	3.22±0.18^{a}	324.4±9.4^{a}	324.4±9.4^{a}	4.19±0.24^{a}
Pb group	5.87±0.21^{d}	198.4±11.8^{f}	10.3±0.84^{d}	2.58±.09^{b}
Pb+GTE group	4.05±0.14^{b}	271.4±11.7^{cd}	13.5±0.70^{bc}	3.78±0.12^{a}

Each value is a mean of 10 rats ± S.E.M; $^{a, b, c, d}$ values are not sharing superscripts letters (a, b, c, d) differ significantly at $p < 0.05$.

III.3. Histopathological examination

The representative pictures of histopathological examination in the kidney tissue are shown in Figure 1 (A-C). Kidney sections from the control group rats and green tea-treated rats showed intact histological structure of glomeruli and renal tubules. However, abnormalities in kidney of treated rats were detected in glomeruli and in convoluted tubules (Figure 1-B) compared to those of controls (Fig. 1A). The main characteristic findings were the appearance of vacuolization and swelling in the

endothelium of glomerular tuft**,** swelling in the lining epithelium of tubules and inflammatory cells infiltration in between the degenerated tubules with fibrosis and hyalinosis between the tubules in focal manner. However, the co-administration of the GTE with Pb (Fig. 1C) showed marked improvement in their histological structure in comparison to the treated groups (Pb) alone.

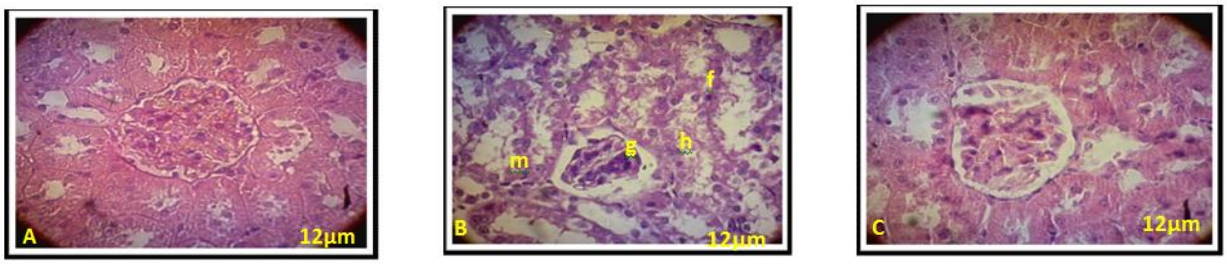

*Fig.1: Kidney paraffin sections stained by haematoxylin and eosin (H&E) for histopathological changes. Control and GTE group (**A**) showing intact histological structure. Pb-treated group (**B**) showing swelling and vacuolization in the endothelial cells lining the tuft of the glomeruli (g) with fibrosis (f) and hyalinosis (h) between the tubules in focal manner and inflammatory cells infiltration (m) and few fibroblastic cells proliferation (arrow) in between the degenerated tubules (d) in focal manner at the corticomedullary portion Kidney (**C**) GTE+Pb shows the architecture nearto normal (x 200).*

IV. Discussion

Lead is a ubiquitously found environmental and industrial pollutant that has been detected in nearly all phases of environment and biological system. Its persistence in human and animal tissues has quite often been associated with considerable health risks [24]. Several strategies, mechanisms and agents were utilized to prevent Pb nephropathy in animal model [25- 26]. Tea, most widely consumed beverage worldwide since ancient times, is known for its beneficial health effects. In particular, green tea polyphenols, chiefly catechins and their derivatives have been shown to retard various forms of cancers due to its antimutagenic, anticarcinogenic and antioxidant properties [2728]. It was also found to be cardioprotective, neuroprotective, antidiabetic and antibacterial besides other health benefits [28; 16]. In toxicological studies, body, organ and relative organ weights are important criteria for evaluation of organ toxicity [29]. In the present study, oral administration of Pb resulted in a significant reduction in the body weight gain, and an increase in the relative kidney weight. The reduction in body weight gains may be due to the combined action of cholinergic and oxidative stress [30]and/or due to the increased degradation of lipids and proteins as direct effects of organophosphate compound exposure [29]. The reduction in body weight may be due to the combined action of cholinergic and oxidative stress and/or due to the overall increased degradation of lipids and proteins as a result of the direct effects of Pb [30]. Moreover, the increase in kidney weight could be attributed to the relationship between kidney weight increase and various toxicological effects or to the reduction in body weight gain of experimental animals [30-31]. These results are consistent with many previous investigators with Pb and other metals [31] Co-administration of GTE improved body and kidney weights of intoxicated rats. In the present study, the oral administration of Pb to adult male rats provoked an increase in serum creatinine urea, and uric acid levels of rats. It is well known that the kidney is the main site of elimination of xenobiotics[32]. These findings reflect the diagnosis of renal failure [33]. The potentially reactive derivatives of oxygen, ascribed as ROS such as O_2, H_2O_2 and OH are continuously generated inside the human body as a consequences of exposure to a lot of exogenous chemicals in our ambient environment and/or a number of endogenous metabolic processes involving redox enzymes [34]. Under normal circumstances, the ROS generated are detoxified by the antioxidants present in the body and there is equilibrium between the ROS generated and the antioxidants present [35]. Harmful effects caused by ROS occur as a consequence of an imbalance between the formation and inactivation of these species. However, owing to ROS overproduction and/or inadequate antioxidant defense, this equilibrium is hampered favoring the ROS upsurge that culminates in oxidative stress [36]. The ROS readily attack and induce oxidative damage to various biomolecules including proteins, lipids, mitochondria, lipoproteins and DNA [36]. Oxidative stress affects many cellular functions by various mechanisms such as alteration in gene expression through activation of transcription factor NF-kB or induction of permeability transition in mitochondria with lethal consequences [36]. Our results revealed that the effect of exposure to Pb for 4 weeks, in

rats induced nephrotoxicity evidenced by histopathological observation and biochemical parameters perturbations in kidney of rats. This treatment has a negative effect on renal lipid peroxidation as well as the renal antioxidant defense system.A significant increase in the LPO level following administration of the Pb observed in the present study. Since ROS are highly reactive and can oxidize cellular macromolecules (e.g. lipids, DNA, nucleic acid and proteins) which may lead to genetic alterations. Lipid peroxidation is linked with excessive generation of ROS, which may be contributed by exogenous or endogenous sources and is the most destructive process in the living cells has been implicated in causing a wide range of biological effects such as increase membrane rigidity, osmotic fragility, decreased cellular deformation, reduced erythrocyte survival, and membrane fluidity [37- 38]. Lipid peroxidation products, such as malondialdehyde and 4-hydroxy-2-nonenal (the most cytotoxic) cross link the membrane, damage the DNA and are mutagenic leading to functional changes [39]. Therefore, we used lipid peroxidation as a marker of oxidative stress and studied the effect of Pb administration on renal lipid peroxidation. Treatments of animals with Pb lead to the induction of lipid peroxidation, as monitored by measuring the rate of production of Thiobarbituric acid reactive substances (TBARS), expressed as malondialdehyde equivalents, reflecting the formation of activated species in rat kidney. Accumulation of lipid peroxide is believed to be a major contributor to the loss of cell function under oxidative stress conditions [40]. This further indicate that renal injury induced by Pb in present study is the result of oxidative stress that arise as a result of excessive generation of ROS, which have been reported to attack various biological molecules including lipids and causing lipid peroxidation.

Results of the current study revealed that green tea extract (GT) reversed the elevation of lipid peroxidation. Hence, it is possible that the mechanism of green tea extract may be attributed to epicatechins (antioxidant present in green tea) that scavenge a wide range of free radicals including the most active hydroxyl radical, which may initiate lipid peroxidation. Therefore, it may decrease the concentration of lipid free radicals [41]. Moreover, it was reported previously that it chelates metal ions, especially iron and copper, which, in turn inhibit generation of hydroxyl radicals and degradation of lipid hydroperoxides [42].

Nephrotoxicity could also be explained by the impaired antioxidant enzyme activities in the kidney of the rats. Indeed, the antioxidant enzymes SOD, GPx and CAT limit the effects of oxidant molecules in tissues and act in the defense against oxidative cell injury by means of their being free radical scavengers [43]. These enzymes work together to eliminate active oxygen species. In this respect, SOD accelerates the dismutation of H_2O_2, also termed as a primary defense, as it prevents further generation of free radicals whereas, CAT helps in the removal of H_2O_2 formed during the reaction catalyzed by SOD [43]. In the current study, our results indicated that Pb exposure inhibited SOD, CAT and GPx activities in kidney of rat. This depletion may be due to the decreased synthesis of enzymes or oxidative inactivation of enzyme protein. Our histopathological data substantiate kidney dysfunction. Indeed, the renal histoarchitecture of the Pb treated rats showed swelling in the endothelium of glomerular tuft**,** swelling in the lining epithelium of tubules and inflammatory cells infiltration in between the degenerated tubules.Most of the biochemical alterations accompanied by histopathological changes were alleviated following GTE administration. This could be attributed to the antioxidant capacity of GTE that reduce the lipid peroxidation which in turn restore the integrity of the cell membrane and improve the disturbance in permeability. Since the oxidative damage as the central mechanism of metals toxicity occurs primarily through production of reactive oxygen species (ROS), including hydroxyl radicals and hydrogen peroxide that are generated during the reaction and react with biological molecules, eventually damaging membranes and other tissues [44; 45]. The use of antioxidants to counteract the formed ROS is the corner stone in alleviation of such hazards. So, the major nutraceutical compounds in green teas are tea catechins that have the most effective antioxidant activity. Tea catechins are an efficient free radical scavenger due to their one electron reduction potential [45-46]. In addition, tea contains minerals that function as co-factors in antioxidant enzymes: zinc, selenium and manganese. Polyphenols have additional mechanisms in which they reduce oxidation level besides direct role as antioxidants: (1) Binding of metal ions such as iron and copper and preventing their participation in oxidation reactions (leading to the formation of hydroxyl radical). (2) Prevention of redox sensitive transcription factors activation that amongst others things serve as mediators of inflammatory reactions. (3) Suppression of oxidation stimulants such as induced nitric oxide synthase (iNOS), cyclooxygenase 2 (COX- 2), lipoxygenase 2 (LOX-2) and xanthine oxidase. (4) Induction of antioxidant enzymes such as glutathione S -transferase and super oxide dismutase [35].

V. Conclusion

The results of present study show that lead acetate treatment caused oxidative damage and histopathological alterations in the kidney of male rats. In contrast GT reduces oxidative damage by virtue of its antioxidant properties thus improving the structural integrity of cell membrane and eventually alleviates the histopathological changes. Based on our present observations, we propose that GT may provide a cushion for prolonged therapeutic option against toxins-induced nephrotoxicity without harmful side effects.

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Antioxidant activity of *Aplysia depilans* ink colleted from Bizerte Channel (NE Tunisia)

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Abstract: The ink secretion of molluscan species was identified as one of the novel sources of bioactive compounds. The present study aims to evaluate the *in vitro* antioxidant activity of *Aplysia depilans* ink extract. The antioxidant activity of ink extract were evaluated using 2,2- diphenyl-1-picrylhydrazyl (DPPH) scavenging activity, Hydroxyl radical scavenging activity, Ferric ion reducing power (FRP) and Ferrous ion chelating (FIC) activity. The results from the present work revealed the strongest antioxidant activity of *Aplysia depilans* ink. The electrophoretic profile showed band with molecular weight of 60 kDa. The highest antioxidant activity in ink extract probably may be due to the presence of this protein with lower moleculair weight.
Keywords: *antioxidant activity; cephalopods; ink extract; Aplysia depilans; sea hare*

I. Introduction

Lipid oxidation causes some health hazards in human beings such as cardiovascular disease, cancer, and neurological disorders as well as aging process [1-2]. To prevent or slow down lipid oxidation, several antioxidants including synthetic and natural antioxidants have been widely used. However, synthetic antioxidants are suspected of being toxic upon long-term exposure [3]. As a consequence, natural antioxidants have gained increasing attention. In the past few decades, mining of bioactive compounds from marine sources are considered promising because of its rich species diversity [4]. Earlier studies by various researchers reported that molluscs have good antioxidant properties [5-6]. Several natural bioactive compounds like peptides, sterols, terpenes, polypropionates, nitrogenous compounds, prostaglandins, fatty acid derivatives, miscellaneous compounds and alkaloids were reported from molluscs which were identified as essential with specific types of activities [7]. Cephalopod ink has been proved to be an alternative medicine and has a wide range of therapeutic applications [8].
On the basis of its traditional use and literature reference, the present study focuses the antioxydant activity of *Aplysia depilans* ink extracts by different methods.

II. Materials and methods

II.1. Collection of ink samples

30 adult specimens of the sea hare, (*Aplysia depilans,* Gmelin, 1791), ranging in length from 20-25 cm, were collected during low tides from the Bizerte Channel, Tunisia (37°8’ and 37°14’ N, 9°46’ and 9°56’ E). The Channel represents a transition area between Bizerte Bay and Bizerte Lagoon (fig 1).

The animals were transported to the laboratory in a container with sea water. The ink fluid was obtained by disturbing the animals and extracted with water. All aqueous ink samples were centrifuged at 15,000 g for 15 min as described by [9] and the supernatant was taken and lyophilized to a black residue using a lyophilizer and stored for further use.

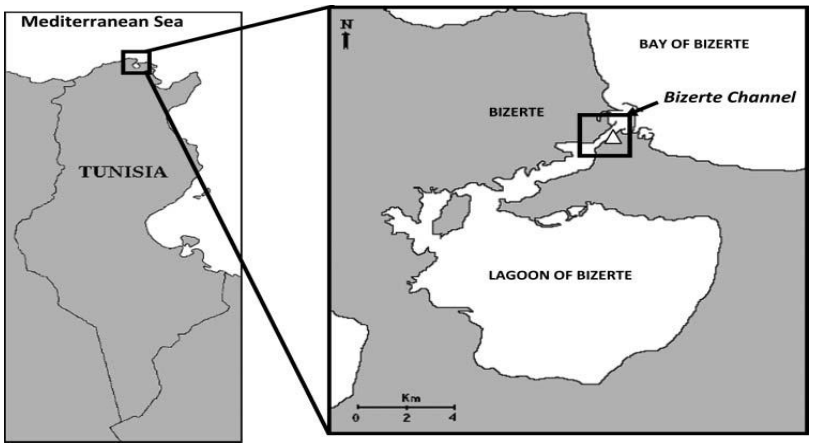

Figure 1: *Map of the Bizerte Lagoon showing the sampling site (Channel) from where sea hare (Aplysia depilans, Gmelin, 1791) were collected (Neffati et al.; 2012).*

II.2. Estimation of protein concentration

Protein concentration was determined by the method of Bradford [10] using bovine serum albumin (BSA) as a standard.

II.3. Chemicals

All chemicals and solvents were purchased from Sigma-Aldrich, and they were of highest purity and analytical grade.

II.4. Determination of *in vitro* antioxidative activities

The extract of *Aplysia depilans* ink was tested for antioxidative activities by 2,2,-diphenyl-1-picrylhydrazyl (DPPH) scavenging activity, Hydroxyl Radical Scavenging Activity, Ferric Reducing Antioxydant Power (FRAP) and Ferrous Ion Chelating Activity (FIC).

II.5. DPPH radical scavenging activity.

The DPPH radical scavenging assay employed is as described by [11]. Various dilutions of the methanolic solution and standard (ascorbic acid) (0.003-0.3 mg/mL, in triplicate) were added to DPPH solution (0.035 mg/mL). The mixture was left in the dark for 30 min before reading the absorbance at 517 nm with methanol as blank. The control consisted of methanol in place of sample. Radical scavenging activity was expressed as a percentage and calculated using the formula:
% Scavenging = ((Abs. control - Abs. sample) / Abs. control) * 100.
Where Abs control is the absorbance of the control reaction and Abs sample is the absorbance in the presence of the sample.
Result was presented as IC_{50} the concentration of the substrate which causes an inhibition of 50% of the activity of DPPH.

II.6. Hydroxyl radical-scavenging activity

The hydroxyl radical scavenging activity was measured by the desoxyribose method [12] and compared with ascorbic acid. It was carried out by measuring the competition between desoxyribose and the compounds that generate hydroxyl radicals from the Fe^{3+}/ascorbate/EDTA/H_2O_2 system.

Attack of the hydroxyl radicals on desoxyribose led to formation of thiobarbituric acid-reactive substances (TBARS) which were measured by the method of Ohkawa *et al,* [13]. The hydroxyl radical is the most reactive oxygen species (ROS) that attacks almost every molecule in the body and also leads to DNA damage in a cell. It initiates the peroxidation of cell membrane lipids increases MDA levels which is cytotoxic, mutagenic and carcinogenic. Results was expressed as a percentage and calculated using the formula:
(%) OH scavenging = ((Abs control - Abs sample) / Abs control) * 100
Where Abs control is the absorbance of the control reaction and Abs sample is the absorbance in the presence of the sample.

II.7. Ferric ion reducing antioxidant power (FRAP).

The reducing power of methanolic solution was determined according to the method of Oyaizu [14] and compared with ascorbic acid. Substances, which have reduction potential, react with potassium ferricyanide (Fe^{3+}) to form potassium ferrocyanide (Fe^{2+}), which then reacts with ferric chloride to form ferric ferrous complex that has an absorption maximum at 700 nm. One mL of test sample solution or standart at various concentrations (0.003-0.3 mg/mL) was mixed with phosphate buffer (0.2 M) and potassium ferricyanide (1 %). The mixture was incubated at 50°C for 20 min. Aliquots of trichloroacetic acid (10 %) were added to the mixture, which was then centrifuged at 3000 rpm for 10 min. The upper layer of solution (2.5 mL) was mixed with distilled water and a freshly prepared ferric chloride solution (0.1 %). The absorbance was measured at 700 nm. Ascorbic acid was used as standard. A blank was prepared without adding standard or test compound. Increased absorbance of the reaction mixture indicates increase in reducing power. The percent increase in reducing power was calculated using the following equation:

((Abs test – Abs blank) / Abs blank) * 100

Where Abs test is absorbance of test solution; Abs blank is absorbance of the blank.
IC_{50} corresponds to the concentration necessary to reduce 50 % of ferric ferrous complex. The lower value indicates a higher reducing power. IC_{50} is expressed in mg/mL for the pure compounds.

II.8. Ferrous ion chelating activity (FIC)

The chelating of ferrous ion by the methanol sample solution was estimated by the method of Singh and Rajini [15]. Various dilutions of the metanolic solution and standard (in triplicate) were added to $FeSO_4$ (0.1 mM) and ferrozine (0.25 mM). The tubes were shaken well and left to stand for 10 min. The absorbance was measured at 562 nm, against blank containing water in place of ferrozine. The control consisted of water in place of the sample. The ability of a sample to chelate ferrous ion was calculated as follows:

chelating effect (%) = ((Abs. control – Abs. sample) / Abs control) * 100

IC_{50} corresponds to the concentration necessary to prevent 50 % from initial Fe^{2+}-ferrozine complex formation. The lower value indicates that the compound is more chelator. IC_{50} is expressed in mg/mL for the pure compounds.

II.9. SDS-Polyacrylamide gel electrophoresis

Electrophoresis of the crude ink was carried out by the method of Laemmli [16] on a 1-mm vertical gel consisted of 5% stacking gel mix, and main running gel mix of 12.0% acrylamide. Ink samples containing 2% SDS and 1% 2-mercaptoethanol were incubated at 100°C for 10 min. A few sucrose crystals were dissolved in the samples before being applied (30 µL) to the gel. Electrophoresis was carried out at 20-mA constant current for 60 min and protein bands were visualized by staining in Coomasie brilliant blue. Standard molecular weight markers sizes ranging from 35 to 225 kDa were used to determine the molecular weight of individual proteins.

II.10. Statistical analysis

IC_{50} values were calculated by linear regression. Means ± SD were calculated. The data were analyzed for statistical significance using one way ANOVA followed by Tukey post test. P values less than 0.05 were considered significant. Statistical analysis was performed using the Statistical Package for Social Science (SPSS 17.0 for Windows, SPSS Inc., Chicago, IL, USA).

III. Results

III. 1. DPPH radical scavenging activity

DPPH is usually used as a reagent to evaluate free radical scavenging activity of antioxidants [14]. DPPH is a stable free radical and accepts an electron or hydrogen radical to become a stable diamagnetic molecule [17]. The reduction capability of DPPH radical is determined by the decrease in absorbance at 517 nm induced by antioxidants. Ascorbic acid is the reagents used as standards. The sample is able to reduce the stable radical DPPH to the yellow-colored diphenylpicrylhydrazine. *Aplysia depilans* ink is likely to have the effect of scavenging free radical with an IC_{50} = 0.94 mg/mL when compared with ascorbic acid IC_{50} = 0.0396 mg/mL (Fig 2). The involvement of free radicals, especially their increased production, appears to be a feature of most, if not all human diseases [18]. It has been found that ascorbic acid reduce and decolorize DPPH by his hydrogen donating ability [19], aplysia extract is probably involved in their antiradical activity.

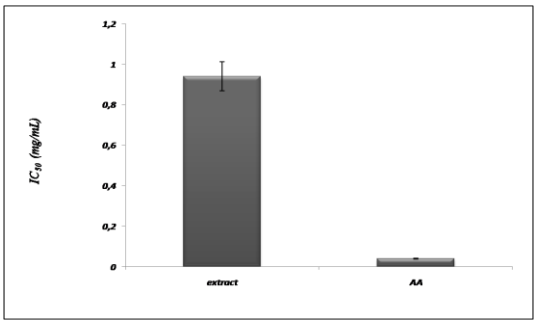

Figure 2. *DPPH radical scavenging activity, (Extract: Aplysia ink extract, AA : ascorbic acid)*

III.2. Hydroxyl radical-scavenging activity

The hydroxyl radical is the most reactive oxygen species (ROS) that attacks almost every molecule in the body and also leads to DNA damage in a cell. It initiates the peroxidation of cell membrane lipids [20-21], increases MDA levels which are cytotoxic, mutagenic and carcinogenic [22]. Aplysia ink extract showed significant hydroxyl radical scavenging activity with an IC_{50} of 7.43 µg/ml when compared with ascorbic acid IC_{50}: 6.24 µg/mL and gallic acid IC_{50}: 2.17 µg/mL

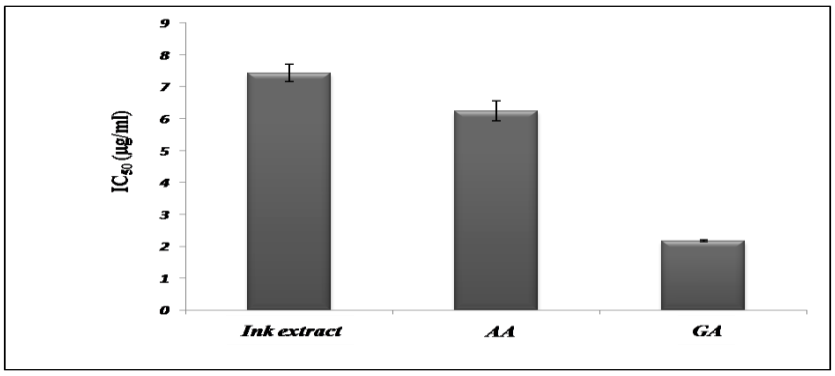

Figure 3: *Hydroxyl radical scavenging activity in (%) (Extract: Aplysia ink extract, AA : ascorbic acid; GA gallic acid).*

III.3. Reducing power determination

It has been observed a direct correlation between antioxidant activity and reducing power of certain compound. Figure 4 shows the reductive capability of aplysia ink compared to ascorbic acid. For the measurements of the reductive ability, it has been investigated from the Fe^{3+}–Fe^{2+} transformation in the presence of compounds using the method followed by Oyaizu [14]. The extract exhibited reducing power activity with an IC_{50} at 39.4 µg/mL wich is lower than that of ascorbic acid IC_{50} : 3.56 µg/mL

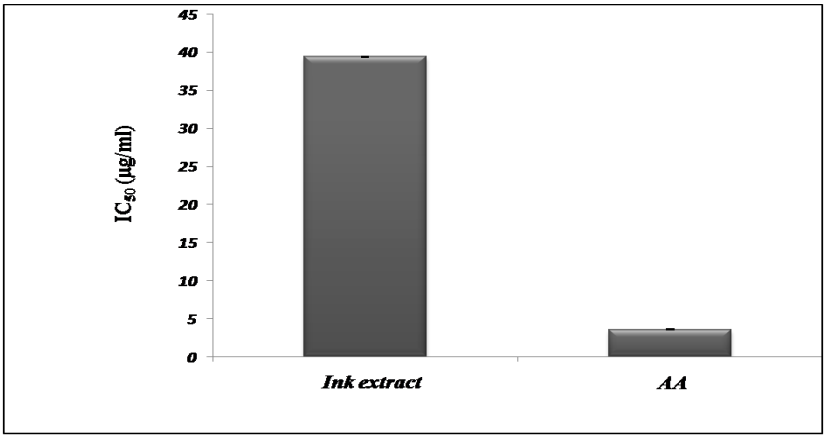

Figure 4: *Reducing power activity (Ink extract: Aplysia ink extract, AA : ascorbic acid)*

III.4. Ferrous ion chelating (FIC) activity.

The method of ferrous ion chelating activity is based on chelating of Fe^{2+} ions by the reagent ferrozine, which is a quantitative formation of a complex with Fe^{2+} ions [23]. The formation of a complex is probably disturbed by the other chelating reagents, which would result in the reduction of the formation of violet-colored complex. Measurement of the rate of reduction of the color, therefore, allows estimation of the chelating activity of the coexisting chelators. In this assay both compound and standard compound interfered with the formation of ferrous complex with the reagent ferrozine, suggesting that it has chelating activity and captures the ferrous ion before ferrozine. The ferrous ion chelating effect of the aplysia ink showed an IC_{50} at 2.31 µg/ml compared with ascorbic acid (IC_{50}: 5.77 µg/mL). Metal chelating capacity was significant as they reduced the concentration of the catalyzing transition metal in lipid peroxidation [24]. It was reported that chelating agents are effective as secondary antioxidants because they reduce the redox potential, thereby, stabilizing the oxidised form of the metal ion [25]. The data obtained from Figure 5 reveal aplysia ink demonstrate a high capacity for iron binding more than standard, suggesting that its action as antioxidant may be related to its iron-binding capacity.

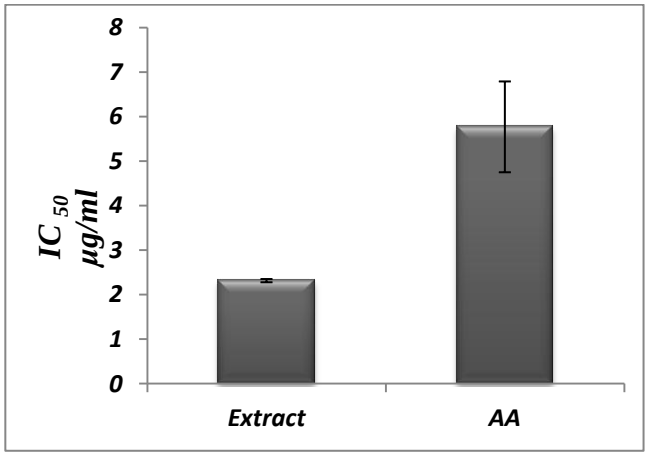

Figure 5: *Iron chelating activity (Extract: Aplysia ink extract, AA: ascorbic acid)*

III.5. SDS-PAGE analysis

SDS PAGE showed a major protein band with a molecular weight of 60 KDa (fig 6). The 60 Kda band was the target protein of the study.

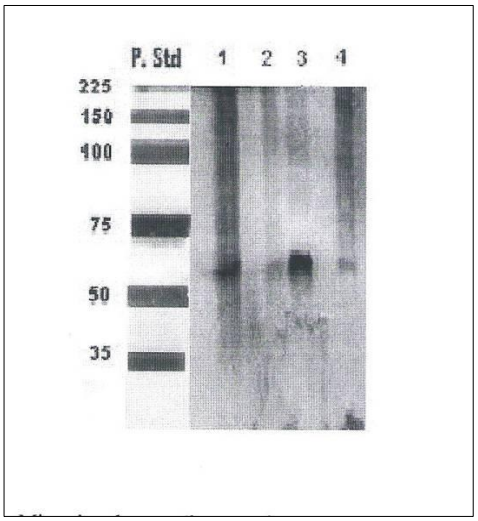

Figure 6: *Electrophoretic profile of ink samples showing a prominent band (60 KDa). Number on the left indicate the molecular weight markers.*

IV. Discussion

Marine mollusks are protecting themselves from predators through their unique way; one of them is releasing ink when disturbed. This ink secretion contains a rich array of chemical secretions to escape from predators. The chemical composition of the ink of all inking mollusk will not be the same. In this study we have accessed antioxidant activity of *Aplysia depilans* ink samples using several assays as shown in fig 2, 3 and 4.

Ink extract was effective in scavenging DPPH radicals. DPPH radical scavenging activity was based on the ability of antioxidants to donate a hydrogen atom or an electron to stabilize radicals by converting them to the non-radical species [26- 27]. DPPH is a radical having an odd electron and reacts with hydrogen donated from antioxidant.

The ink extract exhibited reducing power activity with an IC_{50} = 39.4 µg/mL wich is lower than that of ascorbic acid IC_{50} = 3.56 µg/mL. The ferrous ion chelating effect showed an IC_{50} = 2.31 µg/ml compared with ascorbic acid (IC_{50} = 5.77 µg/mL). Ferric reducing power activity is generally used to measure the capacity of a substance in reducing TPTZ-Fe (III) complex to TPTZ-Fe(II) complex [28-26]. The result indicated that ink extract was able to act as reducing agent which provided electron for stabilization. Additionally, some compounds in the ink could chelate prooxidative metals, thereby lowering or retarding the initiation of lipid oxidation process. Ink from *Sepia officinalis* and *Loligo formosana* was reported to function as antioxidant [29- 30].

The results from the present work revealed the strongest antioxidant activity of sea hare ink. The highest antioxidant activity in ink extract probably may be due to the presence of peptides or proteins with lower moleculair weight [31]. SDS polyacrylamide gel electrophoresis of aqueous extracts of ink samples showed the presence of proteins with the molecular weight of 60 kDa. This protein may be responsible for the antioxydative activitie of the ink samples. This result supports the previous work done by Xin Guo *et al.* [32] hwo reported that proteins isolated from squid ink with molecular weight of 10-50 kDa and more, showed higher antioxidant activity than other fractions. SDS polyacrylamide gel electrophoresis of aqueous extracts of ink samples of some molluscan showed the presence of proteins with the molecular weight ranging from 62 to 249 kDa. These proteins may be responsible for various biological activities of the ink samples [9]. Also, Rajaganapathi *et al*, [33] has purified from the

purple ink of the sea hare *Bursatella leachii* a protein with 60-kDa moleculair weight, that showing anti-HIV activity.

V. Conclusion

In recent years many bioactive compounds have been extracted from various marine animals like tunicates, sponges, soft corals, cephalopods. Sea hare come under the class Cephalopoda (phylum - Mollusca), which eject ink from their ink sac to escape from their predators. From this study, it is clear that *Aplysia depilans* ink contains a large number of compounds with antioxidant properties. These compounds might be one of the factors which are playing a crucial role in the defensive mechanism of the inking mollusk. These results give additional support that ink secretions are source of biologically important compounds for biomedical research. Further studies are under way to explore the compound present in the ink extracts that may lead for a source of drug from this marine animal.

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V. References

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