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FORMULATION AND CHARACTERIZATION OF ANTIOXIDANT RELEASING FILMS ENRICHED WITH *AVICENNIA MARINA* BIOACTIVES

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Abstract:

Avicennia marina is a marine mangrove enriched with various bioactive compounds. Thin film mediated drug delivery has become one of the novel methods to deliver the drugs with enhanced bioavailability. In this study, antioxidant releasing films was developed using gelatin polymer fortified with A.marina bioactives. Gelatin based antioxidant films with glycerol (GAG) and without glycerol (GA), but both films enriched with A. marina bioactives was developed and characterized. AF was light green in colour and have mass of 370± 0.27 and 269 ± 0.54 mg for GAG and GA respectively. Folding endurance of GAG was found to be higher than GA. AF have shown greater solubility percentage for GAG than GA in both water and buffer, pH7.4. Tensile strength of AF -GA was higher compared to AF-GAG. However, percent of elongation at break was found to be higher for AF-GAG than AF-GA. Invitro assays revealed that AF had excellent antioxidant , protein denaturation inhibition, membrane stabilization and nitric oxide scavenging activity. Disintegration time of AF-GAG and GA was found to vary very slightly. However, dissolution time of AF-GAG and GA has shown moderate variation. GAG and GA have shown surface pH of 6.6 and 6.7 respectively. Hence, this antioxidant releasing films enriched with A. marina bioactives will be a better choice to improve the antioxidant status and immunity.

Key words: *Antioxidant films, Avicennia marina, bioavailability, bioactives, gelatin*

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INTRODUCTION:

Avicennia marina is mangrove trees belong to the family Acanthaceae. It is commonly found in the intertidal zone of estuarine areas. Like other plant species, mangroves also known to contain various bioactive metabolites like alkaloids, phenolics, steroids and terpenoids of medicinal importance. Phyto chemical characterization and antimicrobial efficiency of *A. marina* and *A. offinalis* has been clearly denoted [1]. *A.marina* has been found to harbor bioactive compounds like alkaloid, flavanoid, terpenoid, phenolics, tannin, saponin, steroids, glycosides and aminoacids. Methanolic extract of *A.marina* has been found to be effective against several bacterial species viz. *Staphylococcus aureus, Klebsiella pneumonia Pseudomonas aeroginosa, Bacillus subtilis, Escherichia coli, Enterobacter aeruginosa, Proteus sp, Salmonella paratyphi, Citrobacter sp.* and also very potent antioxidant property. Invitro antioxidant activity of pneumatophore of *A. marina* was also studied (Packia Lincy M, et al., 2013) [2].

Thin film drug delivery refers to incorporation of drugs in a polymeric substance, with or without plasticizer and considered as the novel system of drug delivery in the pharmaceutical formulation. The polymers should be non –toxic, biocompatible, biodegradable and also available at low cost. Thin films are more advantageous than conventional dosage forms like tablets and capsules, as it dissolve rapidly and no difficulty in swallowing [3-5]. It is immensely beneficial formulation for pediatric and geriatric population. An ideal thin film should possess high drug loading capacity, fast dissolution rate and formulation stability. It is also known to increase bioavailability of drugs and high patient compliance. Moreover, thin films are easy to administer via noninvasive routes, easy to handle, manufacture and transport and to develop cost effective formulation. Thin film incorporated with plant metabolites has become the recent trend in phyto drug delivery research. Phytochemical enriched ODF made up of Hypromellose and Hydroxycellulose using glycerol as crosslinker has been developed and characterized recently [6].

In this study, gelatin was used to develop antioxidant films enriched with *A.marina* bioactives. Gelatin is a proteinacious polymeric substance obtained from collagen protein. It possesses excellent film forming ability, biocompatibility, non-toxicity and biodegradability. Moreover, it can be easily obtained from bones and tendons of animals and more economic than other synthetic polymers. It also offers high solubility and employed in the manufacture of orodispersible films. Mammalian gelatin comprised better tensile strength and elongation at break percentage. Gelatin was used a matrix to prepare thin films loaded with essential oils and used as food preservative [7]. Comparitive study of mammalian and fish gelatin films was carried out to harness its suitability in edible film coating application [8]. Plasticizers are the crosslinking substances used to increase the flexibility and durability of the gelatin film. Certain substances like glycerol, sucrose, oleic acid, sorbitol, mannitol, citric acid, tartaric acid, malic acid, diethanolamine etc. can be used as plasticizers for thin films.

MATERIALS AND METHODS:

Sample collection and identification

Leaves of *A.marina* were collected from the shore area of Tuticorin, Tamil Nadu, India. The plant specimen was identified and authenticated by Dr. B. Mahesh kumar, Associate Professor, Department of Plant biology & Plant biotechnology, G Venkataswamy Naidu College, Kovilpatti, Tuticorin district, Tamil Nadu, India.

Preparation of *A. marina* extract

A. marina leaves were washed with tap water and shade dried. The dried leaves was then powdered and stored in airtight container. 10 g of dried *A.marina* leaf powder was then mixed with 300 ml of methanol and allowed to stand for a week with occasional shaking. After a week, the extract was filtered using whatmann no.1 filter paper and stored in a refrigerator until further use.

Antioxidant film preparation

2% gelatin solution was prepared and 25 ml of the gelatin solution was used to cast a single film. Different films viz. G- control gelatin film, GAG- gelatin film with 0.1 g *A.marina* extract and 0.2 % glycerol, GA- gelatin film contain 0.1 g *A.marina* extract without glycerol was casted by pouring 25 ml of appropriate solution mixture into circular petridish and allowed to dry at room temperature for overnight. After drying, the film was peeled off manually from the petridish and kept in a dessicator until further use.

Characterization of antioxidant film

All films was then evaluated for its colour, mass, solubility, surface pH, folding endurance, tensile strength, elongation at break, disintegration time, dissolution time. Pharmaceutical properties like total antioxidant activity, protein denaturation inhibition activity, membrane stabilization activity and nitric oxide scavenging activity were also evaluated.

***Invitro* antioxidant release profile by Phosphomolybdenum assay**

TAA was estimated by phosphomolybdenum assay [9]. *A.marina* film of size 2×2 cm^2 was added to beaker containing physiological buffer (pH 7.4). Samples were withdrawn at various time intervals viz. 0, 30, 60 , 90, 120, 150 s and made upto 1 ml using distilled water and 2 ml of Molybdate reagent solution (0.6 M sulfuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate) was added. The tubes were incubated at 95°C for 90min. After incubation, the tubes were cooled to room temperature for 20-30 min and the absorbance of the reaction mixture was measured at 695nm. Experiments were done in triplicates.

Protein denaturation inhibition assay (Tanford, 1968)[12]

A.marina film of size 2×2 cm^2 was added to beaker containing physiological buffer (pH 7.4). The reaction mixture (0.5ml) consisted of 0.45 ml bovine serum albumin (5% aqueous solution) and 0.1 ml of Samples were withdrawn at various time intervals viz. 0, 30, 60 , 90, 120, 150 s.. The samples were incubated at 37ºC for 30 min. After cooling the samples, 2.5 ml phosphate buffer saline (pH 6.3) was added. Turbidity was measured spectrophotometrically at 660nm. 0.5 ml distilled water was used as blank. The percentage inhibition of protein denaturation was calculated by the following formula. All experiments were performed in triplicates and the results were expressed as mean ± SD.

$$\text{Percent inhibition} = \frac{\text{Abs}_{\text{Control}} - \text{Abs}_{\text{sample}}}{\text{Abs}_{\text{Control}}} \times 100$$

Membrane Stabilization assay (Oyedepo and Femurewa, 1995) [12]

Preparation of Red Blood Cells (RBCs) Suspension

Fresh whole human blood (10 ml) was collected and transferred to the heparinized centrifuged tubes. The tubes were centrifuged at 3000rpm for 10 min and were washed three times with equal volume of normal saline. The volume of the blood was measured and reconstituted as 10% v/v suspension with normal saline.

Heat Induced Hemolysis

A.marina film of size 2×2 cm^2 was added to beaker containing physiological buffer (pH 7.4). The 2ml of reaction mixture is consisted of 1ml of *A.marina* film samples withdrawn at various time intervals viz. 0, 30, 60 , 90, 120, 150 s, 10% RBC suspension. Saline was used as control. Diclofenac sodium was taken as a standard drug. All the centrifuged tubes containing reaction mixture were incubated in a water bath at 56ºC for 30 min. At the end of the incubation, the tubes were cooled under running tap water. The reaction mixture was centrifuged at 2500rpm for 5 min and the absorbance of the supernatants was taken at 560nm. The experiment was performed in triplicates. % membrane stabilization activity was calculated by the formula

$$\text{Percent membrane stabilization} = \frac{\text{Abs}_{\text{Control}} - \text{Abs}_{\text{sample}}}{\text{Abs}_{\text{Control}}} \times 100$$

Nitric Oxide scavenging assay

Nitric oxide scavenging activity can be estimated by the use of Griess IlIosvoy reaction (Garrat, 1964).The compound sodium nitroprusside decomposes in aqueous solution at physiological pH (7.2) producing NO. Under aerobic conditions, NO reacts with oxygen to produce stable products (nitrate and nitrite). The quantities of nitrate and nitrite can be determined using Griess reagent (1% sulfanilamide, 2% H_3P0_4 and 0.1% N-(1-naphthyl) ethylenediamine dihydrochloride). Scavengers of nitric oxide compete with oxygen leading to reduced production of nitrite ions. For the experiment, sodium nitroprusside (10mM) in phosphate buffered saline was mixed with 0.1 ml of antioxidant film samples (2×2 cm^2) withdrawn at various time intervals viz. 0, 30, 60 , 90, 120, 150 s and incubated at 30ºC for 2 hours. The same reaction mixture without the film sample but the equivalent amount of distilled water served as the control. After the incubation period, 0.5 ml of Griess reagent was added. The absorbance of the chromophore that formed during diazotization of the nitrite with sulfanilamide and subsequent coupling with Naphthylethylenediamine dihydrochloride was immediately read at 540nm. Inhibition of nitrite formation by *A. marina* film and the standard antioxidant ascorbic acid were calculated relative to the control. All experiments were performed in triplcates and the results were expressed as mean ± SD.

$$\text{Percent inhibition} = \frac{\text{Abs}_{\text{Control}} - \text{Abs}_{\text{sample}}}{\text{Abs}_{\text{Control}}} \times 100$$

RESULTS:

Colour of *A.marina* film

Control gelatin film was transparent in nature with lustrous texture. However, GAG and GA was green in colour with lustrous texture (Fig.1 &2). The green colour of the film indicates the presence of *A.marina* bioactives in the film.

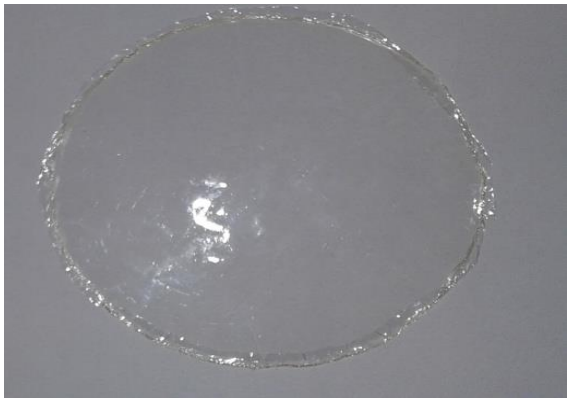

Fig.1. Gelatin film

Fig.2. Gelatin film with *A. marina* bioactives

Physicochemical properties of *A. marina* antioxidant film

Mass of control gelatin film was low, when compared with gelatin film with *A. marina* bioactives (GA, GAG). However, GAG have shown higher mass than GA (Table. 1). Control gelatin film has shown higher solubility in water than GA and GAG. Similar solubility pattern were observed for gelatin films in buffer (pH 7.4). A minor variation was observed among the surface pH of G, GA, and GAG. The folding endurance value of GAG has been observed to increase tremendously than control gelatin film (Table. 1). Tensile strength of GA and GAG was found to be lower than control film G. However, percent elongation at break of GAG was found to be higher than control film G. Disintegration and dissolution time of GA and GAG was observed to be lower than control gelatin film. However, Disintegration and dissolution time of GA was lower than GAG (Table. 1).

Table 1: Physico chemical parameters of *A. marina* antioxidant film

Parameters	Gelatin film, G	Gelatin film with A. marina bioactives w/o glycerol, GA	Gelatin film with A. marina bioactives with glycerol, GAG
Mass, mg	201 ± 1.27	269 ± 2.13	370 ± 1.20
Solubility in water , %	95 ± 2.55	85 ± 1.15	89 ± 1.23
Solubility in buffer, %	97 ± 1.57	88 ± 2.42	94 ± 1.18
Surface pH	6.6 ± 0.12	6.7 ± 0.17	6.6 ± 0.16
Folding endurance	30 ± 2.40	40 ± 1.20	220 ± 1.60
Tensile strength, MPa	34 ± 1.24	26 ± 2.20	23 ± 1.16
Elongation at break, %	9 ± 1.33	8 ± 2.40	21 ± 1.11
Disintegration time, s	122 ± 2.42	90 ± 1.10	94 ± 1.64
Dissolution time, min	4.55 ± 3.25	2.50 ± 1.10	4.40 ± 2.15

***Invitro* antioxidant release profile of film**

Antioxidant film GA, have shown increase in absorbance at 695 nm, whereas control gelatin film have shown no increase in absorbance (Fig.3). Increase in absorbance indicated the increase in antioxidant activity with time.

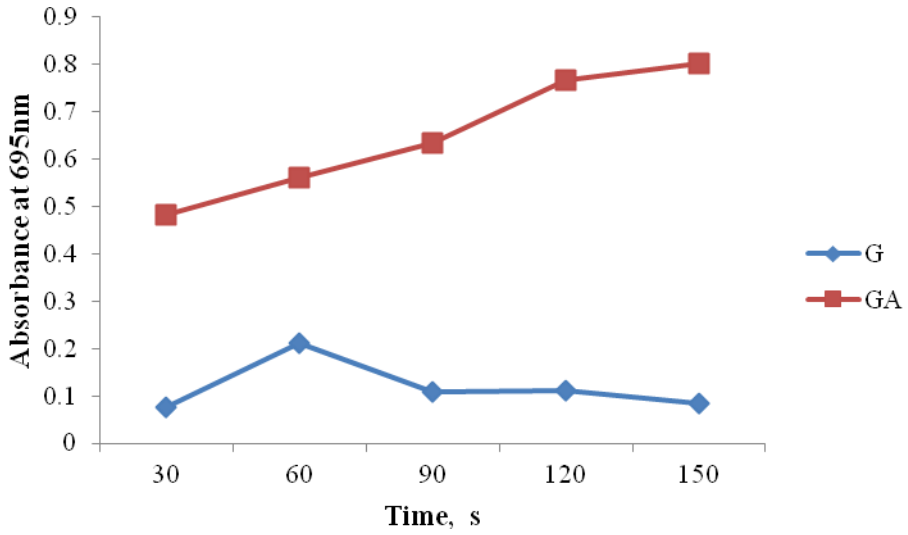

Fig.3: Total Antioxidant Activity of *A. marina* film

Protein denaturation inhibition activity of *A.marina* film

Gelatin film with *A.marina* extract has shown 22% and 54% Protein denaturation inhibition activity at 30 and 150 s respectively (Fig.4). This result indicated that *A.marina* film achieved effective concentration for protein denaturation inhibition within 150 s.

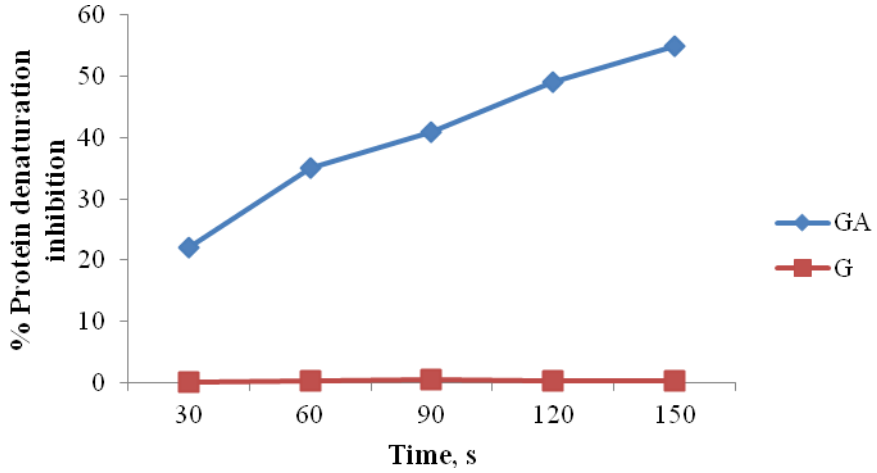

Fig.4: Protein denaturation activity of *A. marina* film

Membrane stabilization activity of *A.marina* film

Gelatin film with *A.marina* extract has shown 25% and 52% of membrane stabilization activity at 30 and 150 s respectively (Fig.5). This result indicated that *A.marina* film achieved effective concentration for membrane stabilization within 150 s.

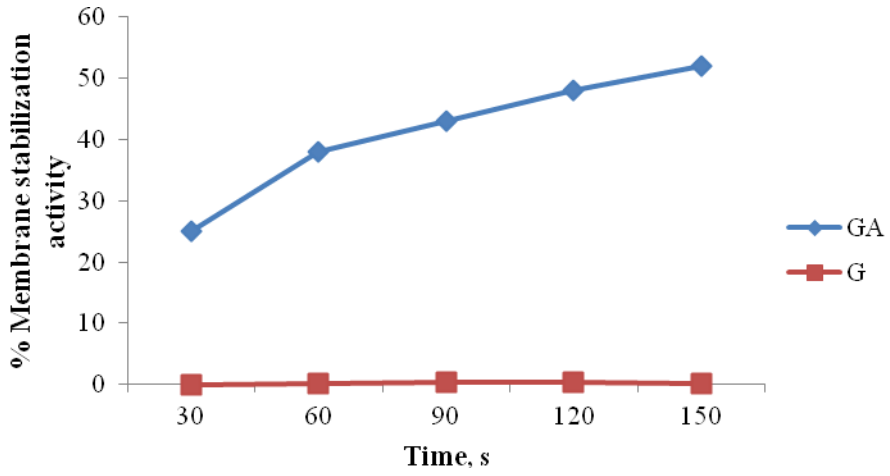

Fig.5: Membrane stabilization activity of *A. marina* film

Nitric oxide scavenging activity of *A.marina* film

Gelatin film with *A.marina* extract has shown 23% and 51% of nitric oxide scavenging activity at 30 and 150 s respectively (Fig.6). This result indicated that *A.marina* film achieved effective concentration for nitric oxide scavenging within 150 s.

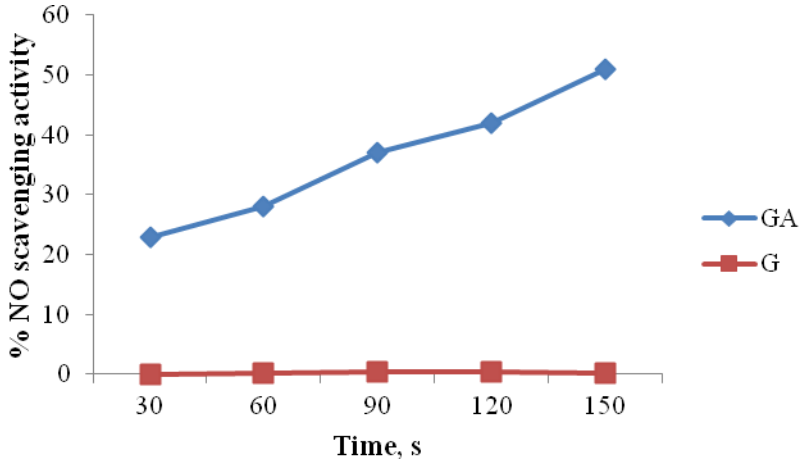

Fig.6: Nitric Oxide scavenging activity of *A. marina* film

DISCUSSION:

Antioxidant films enriched with plant bioactives is one of the novel method of drug delivery in phytomedicine. Gelatin has been served as a better polymer source for the preparation of antioxidant films [8]. Films enriched with metabolites obtained from selected Indonesian plants have been published recently [6]. *A.marina* a mangrove tree found abundantly in Tuticorin coast has been found to contain many bioactive compounds. In this study, a novel approach has been initiated to develop antioxidant rich films fortified with *A.marina* bioactives. This antioxidant rich film has pleasant appearance and colour. Moreover this *A.marina* film possessed excellent physicochemical properties like better solubility in both neutral and physiological pH. Dissolvation of *A.marina* film at physiological pH 7.4 has been considered to be very important for effective delivery of *A.marina* bioactives in to systemic circulation of human body. Surface pH of the film also affects the consumer compliance. Films with high acidic surface pH cause irritation in the buccal cavity. But, *A.marina* film developed in this study was found be less acidic.

Mechanical properties like folding endurance, tensile strength and percent elongation at break was drastically altered by the addition of glycerol.

Folding endurance value represented the flexibility of the film. *A.marina* film with glycerol has shown multifold increase in the folding endurance value than the film without glycerol. Multifold increase in folding endurance value may be due to the crosslinking nature glycerol in between the gelatin and *A.marina* bioactives. Tensile strength of film represented the maximum bearable stress of film. Elongation at break represented the elastic and flexible nature of film. However, addition of glycerol resulted in decrement of tensile strength and increase of percent elongation at break value of film. Disintegration time of *A.marina* film has revealed the fast disintegrating nature of the film. However, *A.marina* film dissolved completely within 5min. Several invitro assays confirmed the antioxidant, protein denaturation inhibition, membrane stabilization and nitric oxide scavenging potential of *A.marina* film. Moreover, *A.marina* film delivered the effective concentration dosage within 150 s. Hence, this antioxidant film could be used as a supplement to meet out the daily need of human beings to improve the antioxidant status and immunity.

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