# Natural Food Preservation System as Allyl Isothiocyanate and Edible Brown Seaweed Laminaria japonica Against Aspergillus flavus and Rhizopus stolonifer

Amira A. EL-Fallal[a], Mohamed I. Abou-Dobara[a], Ahmed K.A. EL-Sayed[a], Phillip Pendleton[b*] and Reham A. El Fayoumy[a]

[a] Botany and Microbiology Department, Faculty of Science, Damietta University, New Damietta City, Egypt.

[b] School of Chemistry Engineering, The University of Adelaide, SA 5005, Australia.

**Abstract**— Allyl isothiocyanate (AITC) is a major compound in mustard essential oil and found in some species of *Brassicaceae*. It is well-recognized antimicrobial agent against many foodborne pathogens. Its using as a natural preservative is limited by its high volatility, strong pungency, and poor water solubility. We demonstrate AITC antifungal activity against *R. stolonifer* and *A. flavus* which cause food spoilage. In agar diffusion disk assay, the inhibition zone diameter for *A. flavus* and *R. stolonifer* were 30.00 mm and 33.00 mm respectively at 20.26 mg/ml of AITC. The minimum inhibitory dosage (MID) for both fungi were 5 µL in disk volatilization assay. A new natural food preservative system by vapor and solution deposition methods into raw and de-oiled *Laminaria japonica* powder, an edible seaweed, we proved AITC vapor-phase activity against both fungi. Up to 97% colony deactivation occurred within 72 h for both *R. stolonifer* and *A. flavus*, both vapor- and solution-deposited were parallel in their decay behavior. The de-oiled *L. japonica* powder was adsorbed AITC twice that of the raw powder at 72 h approaching 99% theoretical maximum amount. The activity of AITC after contact proves that the *L. japonica* + AITC system would represent a viable natural system for food preservation.

**keywords**— Allyl isothiocyanate; *Laminaria japonica;* antimicrobial activity; *Aspergillus flavus*; *Rhizopus stolonifer.*



## 1 INTRODUCTION

Food safety is a scientific discipline describing handling, preparation, and storage of food in ways that prevent food borne illness. This includes anumber of routines that should be followed to avoid potentially severe health hazards. The microorganisms which contaminate food and cause foodborne illness and in many cases lead to death and cause public health problem; bacteria, viruses, and parasites, fungi and yeasts can cause food to spoil but do not cause foodborne illness unless not producing aflatoxins, if the fungi are aflatoxins producers can cause serious health problems in the long term, in same time food spoilage by fungi can cause big economic problem in food industry [7].

Traditional food preservation strategies including physical preservation, and synthetic antimicrobial chemical addition may cause adverse changes in organoleptic properties of foods and/or the loss of nutrients in addition to some of these chemicals have side effects on human health. These factors reduce consumer acceptance [23], requiring improved methods for food preservation, including novel, natural preservatives.

Middle Eastern peoples rely on bread, vegetables and fresh products as their primary source of carbohydrates and vitamins, processed food, fermented sausages, chicken, beef and fish as protein. Each of these is susceptible to spoilage by fungi just like *R.stolonifer* [9] and *A.flavus* [17]. *A. flavus* produce enzymes that break down the food resulting to spoilage. In addition to enzymes, *A. flavus* also produce mycotoxins onto the food. Ingestion of mycotoxin-contaminated food is fatal. Hundreds of people in developing countries die every year after consuming grains contaminated with mycotoxins [17]. Food spoilage due to *R. stolonifer* includes off-flavours, mycotoxins contamination, discoloration, and rotting, its spoilage can occur either in the field or in storage which can cause disaster in industrial economy [13].

The increasing negative consumer perception of the synthetic preservatives has required a return to natural preservatives [10]. In past natural extracts from herbs and spices were used to extend food shelf life but in the same time they have organoleptic effect which they could not avoid [6]. Allyl isothiocyanate (AITC) is the main effective compound in mustard essential oil which has antifungal activities against wide range of fungi. Although this antimicrobial is currently used as an additive for food preservation in Japan, AITC is permitted as a food preservative due to it has been shown to contain residual chloride compounds in addition to its organoleptic effect in food [11]. AITC has received US FDA approval as a safe flavoring agent, with human exposure set generously at a maximum of 25 mg/kg/day [11], however it has not yet been approved as

a food preservative.

Because of high volatility of AITC's liquid phase, there is challenges of using it as antimicrobial preservative although it has been recognized as generally safe. Several delivery systems used to overcoming this problem by adsorption and desorption from porous solids such as mustard powder seeds [5], calcium alginate beads [12] maize [18], and mesoporous silica [19]. Each of these represents food to increase its shelf life and protect it from contamination or spoilage. Siahaan *et al.* [21] demonstrated antimicrobial activity towards *Escherichia coli, Salmonella Typhimurium, Bacillus cereus* and *Staphylococcus aureus*, after AITC desorption from powdered, brown seaweed *Saccharina japonica*. In this research, we applied new natural food preservative system consist of allyl isothiocyanate (AITC) as natural antimicrobial compound found in mustard essential oil and another edible carrier, brown seaweed, *Laminaria japonica* which found in the seawaters of the Middle East, japan and other countries around world. This natural food preservative system can overcoming the annoyable physical properties of AITC which limit its using as food preservative. The de-oiled powder of *L. japonica* was examined as a suitable carrier for AITC, with subsequent antimicrobial tests of vapor released activity after liquid and vapor deposition methods against tested food borne fungi *A.flavus* and *R.stolonifer*. Siahaan *et al.* [21] had demonstrated that the larger the mean particle diameter of the carrier for AITC (900 and 500 μm), the greater amount of AITC adsorbed. Since the current work aimed at demonstrating a more economic process and test its antimicrobial activity in minimized amount, the current work focused on the smaller particle size as a delivery medium prepared via vapor and via solution phase AITC deposition (500 μm).

## 2 Materials and Methods

### 2.1 Materials

Brown seaweed *L. japonica* was collected from Guemil-eup, Wando-gun, Jeonnam, South Korea. Both AITC and dimethyl sulfoxide (DMSO) were 99.9 % grade. The media used for the fungal growth DOX broth and DOX solid. (Sigma Aldrich, Australia).

### 2.2 Fungal cultures preparation

**Corresponding Author:** Reham A. El Fayoumy, Botany and Microbiology Department, Faculty of Science, Damietta University, Egypt
Tel: + 201064838628,
e-mail: reham_ahmad64@yahoo.com

The tow strains fungi *A. flavus* and *R.stolonifer* were prepared by streaking frozen culture stocks on to DOX solid media and left to grow until visible colonies appeared (5 days, 30 °C) to make future assays.

### 2.3 Antifungal activity of AITC against tested fungi

#### 2.3.1 Agar diffusion disk assay

A disk diffusion assay is typically used to determine the relative antimicrobial efficiency of the test compound (AITC) [3]. In this study, mold stock inoculum suspensions were prepared from 7-day cultures grown on Dox agar, the inoculum were prepared by removing the sporulated fungi from the agar plate with a loop and suspending them in 10 ml of sterile water. The fungal suspensions were filtered once through a sterile gauze to remove hyphae, and adjusted spectrophotometrically at a 530-nm wavelength to optical densities that ranged from 0.09 to 0.3 [4].

(DOX) agar plates (90 mm) were inoculated with 0.1ml of the molds stock inoculum and simultaneously in three directions with a sterilized cotton swab to get uniform growth. The inoculated agar was allowed to dry for 15 to 30 min.

Series of dilutions of AITC ranging from 0.5 to 20.26 mg/ml, were prepared with 20% DMSO, sterile filter discs (6mm diameter) were saturated with 20 μl of each respective each dilution of the AITC at six serially diluted concentrations (0.5, 1.01, 5.06, 10.13, 15.19 and 20.26 mg/ml) and placed onto inoculated agar with sterilized Forceps, the 20% DMSO without AITC served as control. Once the moistened filter discs were placed onto the seeded agar, they were left at room temperature for 30 min to allow for the diffusion of AITC, and then incubated at 30 °C for 5 days. After incubation, the zones of inhibition were measured. Studies were performed in biological and technical triplicates at the least, and the diameters of inhibition zones were averaged.

#### 2.3.2 Disk volatilization

This method evaluates the antimicrobial activity of volatile substances without any solvent on the same tested bacteria and fungi. The protocol is technically similar to the above disk diffusion method [16]. In this study, the mold stock inoculum suspensions were prepared just like previous assay agar diffusion disk, four doses of AITC 2μl, 5μl, 7μl and 10μl was added to 6mm sterile filter paper disks and leave it for 30 sec until the disk saturated with the AITC then put it on the lid of the perti dish and incubated at 30 °C for 5 days. After incubation, the zones of inhibition were measured. Studies were performed in biological and technical triplicates at the least, and the diameters of inhibition zones were averaged. The minimum inhibitory dose (MID) of AITC for each microorganism was defined as the minimum dose to completely inhibit microorganism growth on all plates [11].

### 2.4 Natural food preservative system by controlled release of AITC using powdered *L. japonica* as edible carrier

### 2.4.1 Preparation of *L. japonica* powder

Fresh *L. japonica* samples were washed with water, cut into small pieces and freeze dried (72 h). The dried samples were ground using a mechanical blender, then sieved by mesh (500 μm) and stored at -20 °C. These samples were classified as raw material samples. The lipid content of selected samples was extracted using supercritical carbon dioxide (SC-$CO_2$), with the resulting powders classified as de-oiled materials.

For the extraction procedure, *L. japonica* sample (250 g) was loaded onto a thin layer of previously SC-$CO_2$-extracted and cleaned cotton, placed at the base of the stainless steel extraction vessel (500 mL). The powder was then covered with a second layer of cotton and the unit was sealed. Supercritical $CO_2$ was pumped at constant pressure into the extraction vessel by ahigh pressure pump, up to the desired pressure controlled by a back-pressure regulator. Extraction conditions were $T$ = 50 ºC, $P$ = 25 MPa for 2 h. The $CO_2$ flow rate was constant for all extractions (44.7 x $10^{-5}$ kg $s^{-1}$). Both raw and de-oiled samples were used as carriers for AITC.

### 2.4.2 AITC loading of *L. japonica* powder

The loading of *L. japonica* with AITC was achieved via two ways vapor and solution adsorption. The vapor adsorption method involved placing a glass test tube (length 50 mm; ID 12 mm) containing liquid AITC (500 μL) inside a sealed, larger glass vial containing a small petri-dish (dia. 5 cm) in which a sample of de-oiled *L. japonica* (0.5 g) was placed. These larger vials were supported in sealed containers at 65 ± 0.05 ºC. The same method was used for loading raw *L. japonica* samples.

The solution adsorption of AITC by *L. japonica* samples (de-oiled and raw) was achieved by mixing *L. japonica* powder (0.5 g) directly with an AITC solution (500 mg/ml in 20% DMSO) at constant temperature (25 ± 0.05 ºC). For each method, the amount of AITC adsorbed was determined by monitoring sample weight increase with time (4, 8, 12, 24, 48, and 72 h).

### 2.4.3 Antifungal testing sample preparations

#### a- Preparation of culture samples and cell suspensions for initial growth determination

The tow fungal strains which cause food spoilage: *Aspergillus flavus* – *Rhizopus stolonifer* were grown for 7-day on DOX agar at 30 °C. The inoculum was prepared by removing the a sporulated fungi from the agar plate with a loop and suspending them in 15 ml of DOX media broth and incubated for 48 h at 25 $^0$C in shaker which shacking at 200 rpm, then the overnight fungal suspensions were filtered once through a sterile gauze to remove any hyphae. These overnight cultures served as inoculum. The microbial growth in each broth after the overnight pre-cultivation was examined by measuring optical density of the broth at 540 nm ($OD_{540}$). Absorbance at $OD_{540}$ was proportional to the culture concentration. These absorbances were used to monitor microorganism growth to a desired concentration. The total number of microorganisms was evaluated using a 0.5 McFarland standard to make a uniform population, culture suspensions. Each of the overnight growth cultures was appropriately diluted to provide a uniform initial microorganism concentration, for each culture sample, set to approximately $10^6$ CFU/mL. Serial dilutions ($10^{-1}$ – $10^{-8}$) of each culture (1 mL) were prepared by suspending them in a sterile saline solution to give 10% v/v for each dilution factor. Samples (1 mL) were then spread DOX agar in triplicate, according to their dilution factors. These plates were then incubated (72 h, 25 °C). Initial cell counts were made for each colony.

#### b- Antifungal test of released AITC

Each fungi was tested in one of three arrangements contained in vacuum desiccators (10 L at 25 °C). The first contained AITC vapor-loaded *L. japonica*, the second AITC solution-loaded *L. japonica*, and the third, as a control system, contained neither AITC nor *L. japonica*. Each desiccator also held glass tubes (5 mL) containing overnight-grown cultures of a selected fungi (2 mL). Samples tubes of the cultures were removed periodically after 4, 8, 12, 24, 48, and 72 h. On removal, the samples were serially diluted and an aliquot (1 mL) was spread on DOX plates to determine the effect of exposure to AITC vapor (released from the seaweed carrier) on further microorganism growth. These plates were held at constant temperature at 25 °C for 72 h. Any further growth, determined as a colony viable count on the spread plate, was adjusted for its corresponding dilution factor.

### 2.5 Statistical analyses

All measurements were made in triplicate and the data evaluated as mean ± standard deviation including statistical analysis using the one-way analysis of variance (ANOVA) statistical analysis package in MS Excel.

## 3 Results and Discussion

The discussion below presents the preliminary measures taken to prove AITC antifungal activity against foodborne fungi, *A. flavus* and *R. stolonifer*. These fungi have been found as spoilage pathogens in some food consumed in Middle Eastern countries. Although the antimicrobial compound AITC suppresses, and in some cases destroys many bacteria, fungi and yeast [19], since an edible carrier was being considered in this work, it was necessary to establish if this carrier has any reducing effects on AITC antifungal activity towards the above fungus.

### 3.1 Antifungal activity of AITC against tested fungi

#### 3.1.1 Agar diffusion disk assay

The inhibition effects observed were proportional to the concentration applied. A higher concentration led to higher antifungal activity. Low concentration of DMSO 20% to make different concentration of AITC to overcome the problem of poor solubility of AITC in water which make obstruction for diffusion AITC through the agar and show its antifungal effect on the tested fungi [1]. Fig.1 and table.1 showed that *R. stolonifer* was more sensitive to AITC than *A. flavus*, at 1.01 mg/ml concentration of AITC while there is no effect for *A. flavus* there is a good effect against *R. stolonifer* which the inhibition zone diameter was 11.33mm, and at the highest concentration 20.26 mg/ml, *R. stolonifer* gave biggest inhibition zone 33 mm but *A. flavus* gave 30.00 mm only, the acidity nature of AITC may explained these results [8], which in addition to the antifungal activity of AITC against *R.stolonifer* the acidic nature of AITC restrict the growth of *R.stolonifer* than *A. flavus*, which *R.stolonifer* fail to grow on acidic pH [2]. At the lowest concentration applied 0.5 mg/ml there is no effect for the two tested fungi, The minor effect detected for AITC at lower doses in this assay, could be explained either by its lipophilic nature and slower diffusion through the agar, or by its higher volatility when applied, as previously noted. Thus, to be effective, it must be applied at higher doses as illustrated by the results [22]. the control performed with the solvent (DMSO) used in preparation of the compound AITC was ineffective in the suppression of the fungal growth of the both fungus which gave us no inhibition zone.

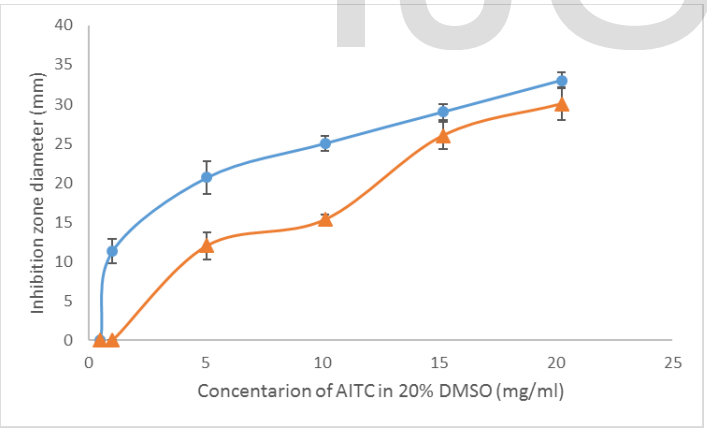



Fig.1. Agar diffusion disk assay showing the antimicrobial activity of AITC at different concentration against the two tested fungi.

For each figure: - ● - for *Rhizopus stolonifer*, - ▲ - for *Aspergillus flavus*

Table .1: Agar diffusion disk assay observations for fungi:
Different concentrations of AITC in solution phase (20% DMSO as solvent)
1. Lowest solution concentration: 0.5 mg/mL – no effect on fungi
2. Highest solution concentration: 20.26 mg/mL

| Fungi | inhibition zone diameter, mm |
|---|---|
| *Rhizopus stolonifer* | |
| *Aspergillus flavus* | |

### 3.1.2 Disk volatilization

The results of this assay help to demonstrate the results of the previous assay. The results expressed as minimum inhibitory doses (MID); the minimum dose completely inhibit the microorganism in all the plate [11] and in our assay cause 90mm inhibition zone where the plates we used were 90mm its width. The effect of AITC by disk volatilization assay was stronger than agar disk diffusion assay where AITC was in solution form, this is can be explained as in some previous work that indicates that AITC can be more effective as a vapor treatment, which AITC vapor was discovered to be 500 to 1,000 times more effective as an antimicrobial agent than the same amount of AITC liquid agar [20]. There are some theories as to why AITC vapor causes greater antimicrobial activity than the liquid form. For example, the liquid has low solubility in broth, there is the potential for possible degradation of AITC in aqueous solution, and the liquid would have limited contact with microbial cells compared to the vapor [14]. Fig .2 showed that *R. stolonifer* was more sensitive than *A. flavus* just like the previous assay, which at lowest dose 2 µL *R. stolonifer* gave 76.00 mm diameter inhibition zone but *A. flavus* gave 65.00 mm only, but in same time the both fungi have same MID at 5µL. Table 2. *Isshiki et al did this assay in his research and showed similar results for A. flavus* [11].

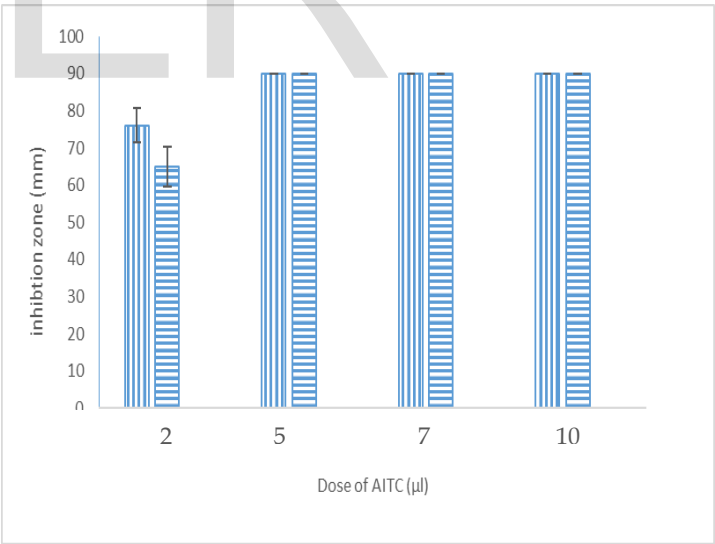



Fig .2. Disk volatilization assay showing the antimicrobial activity of AITC in vapor phases at different doses against the two tested fungi

Each figure: - ▥ - for *Rhizopus stolonifer*, - ▤ - for *Aspergillus flavus*

Table .2: Inhibition activity represented by minimum inhibitory dosage (MID) for fungi.

| Fungi | MID per dish, µL |
|---|---|

| | |
|---|---|
| *Rhizopus stolonifer* | |
| *Aspergillus flavus* | |

### 3.2 Natural food preservative system by controlled release of AITC using powdered *L. japonica* as edible carrier

Desorption of molecules from aporous materials for the inhibition action of the molecules being desorbed which make controlled release of this antimicrobial agent is achieved via two consecutive processes: (i) an initial burst of the contained molecules to produce a relatively high concentration in a relatively short time period, and (ii) a continuous, but slower rate of release to maintain a suitblconcentration to keep the antimicrobial activity of released materials [19].

In this current research we used *L. japonica* as a natural carrier for this system because it is edible, so no possible side effects on contact with food. Its preparation for use is relatively simple which it is widely found on the beaches of marine waters around many countries around world such as middle east countries, so it can be harvested easily, cheaply and legally. In other work, *Saccharina japonica* which belong to the same family of *L. japonica* was tested as a carrier for AITC against other species of bacteria. Which fourier transform infrared red spectroscopy (FTIR) measurements proved that AITC antimicrobial activity was unaffected or reduced due to the seaweed surface-AITC interactions being via physical adsorption only with no chemical interactions inducing any changes in the AITC chemical composition and thus no effect on antimicrobial activity [21].

#### 3.2.1 Adsorption of AITC by *L. japonica* powder via vapor and solution deposition

The time-dependent loading, or adsorption, profiles for AITC onto raw and de-oiled *L. japonica* particles via vapor and solution depositions are shown in Fig. 3. The absolute amounts adsorbed with time data were curve-fitted to the general expression $y(t) = y_\infty\ at/(1+at)$ to enumerate the uptake at saturation, $y_\infty$, equivalent to infinite time. Thus, the ordinate in Fig. 3 is dimensionless as a fraction of theoretical maximum mass AITC adsorbed, allowing comparison between systems. For the solution deposition of AITC, no effort was made to optimize the initial solution concentration; the initial value was that used by Siahaan *et al.* [21]. Overall, the loading profiles were similar in shape to those of Park and Pendleton [19] and of Siahaan *et al.* [21], showing a relatively large fraction adsorbed in the first 24 h: 31.2% via solution and 25.6% via vapor depositions onto raw particles, but 67.9% and 63.1% for the same onto de-oiled particles. In contrast with the previous reports, the current powders continued to adsorb relatively rapidly up to 48 h. The overall fraction uptake difference between methods of AITC deposition onto each set of powders was relatively constant for $t > 8$ h, with solution deposition exceeding the vapor method: 3% difference for de-oiled powders; 9% difference for raw powders. Since the solution deposition was made via direct immersion of the powder, the slightly lower uptakes with vapor deposition were attributed to vapor losses during handling. As expected, de-oiled samples adsorbed considerably more than the raw powder. The rate of change of relative uptake at 72 h implied an approach to saturation had been almost achieved. This observation was consistent with previous reports, however, the relative uptake for the current systems up to 48 h exceeded greatly those for *S. japonica* [21].

The data in Fig. 3 indicated that the maximum amount adsorbed at 72 h by the de-oiled powder was approximately twice that of the raw powder, approaching 99% theoretical maximum. This difference was attributed to the hydrophobic nature of the lipid content, providing poor water-lipid interfacial transfer, leading to restricted diffusion of AITC into those cells containing lipids. Clearly, by removing the residual lipids by SC-$CO_2$ extraction and, assuming no chemical interaction with the surface, as shown with *S. japonica* [21], *L. japonica* would be a strong candidate porous, natural, edible carrier for AITC as an antimicrobial system.

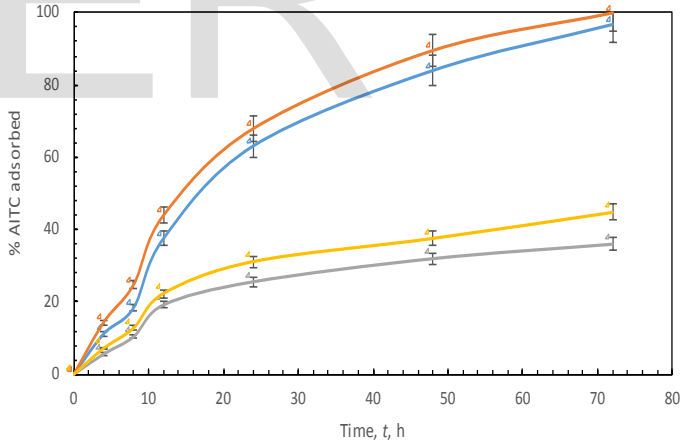


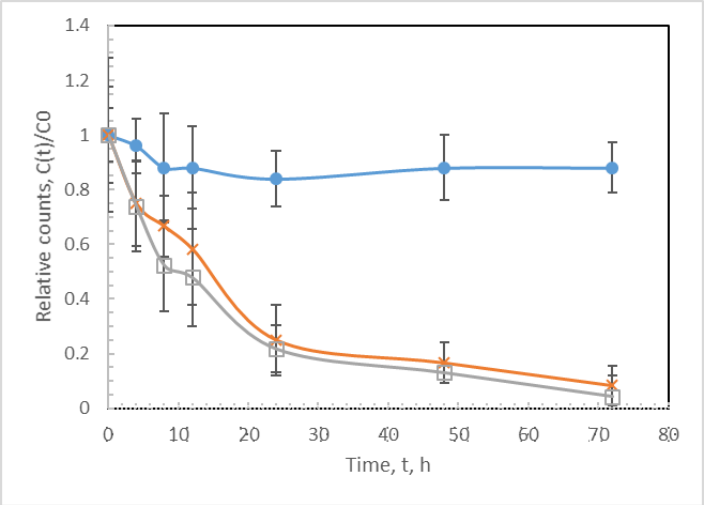

Fig. 3. AITC adsorbed by de-oiled (solution, ●; vapor, ○) and raw (solution, ▲; vapor, △) *L. japonica* (500 μm maximum particle size, 25 °C).

#### 3.2.2 Antimicrobial effect of vapor AITC released from de-oiled *L. japonica* powder

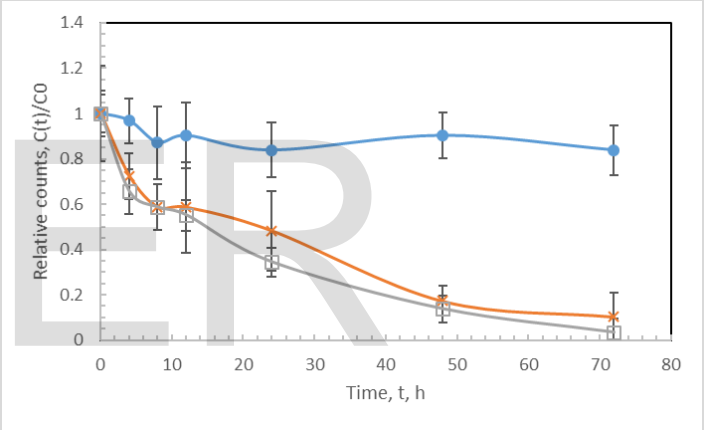
When AITC adsorbed via silica [19] or *S. japonica* [21] surfaces, it was by dispersion forces, i.e. no chemical changes occurred to the adsorbed phase. So its desorption produced an atmosphere with retained antimicrobial activity. The antifun-

gal activity of AITC released from de-oiled *L. japonica* powder was tested against fungi *A. flavus* and *R. stolonifer*. Data of these experiments are spread in Fig. 4. For each system, antifungal activity was tested as vapor and solution loaded powders and a control one lacking AITC. Previous researches considered absolute count of fungus reduction with time; count reduction, $c(t)$, normalized to initial count, $c_0$, with time, $t$, allowed direct comparison of the depletion curves. The data in Fig. 4 are average values and their standard deviation.

The overall shape of each curve in Fig. 4 approximates exponential decay, with vapor deposited systems paralleling the solution deposited systems. The AITC-free systems maintained a constant relative count. The expected initial burst in AITC desorbed over the first 12 h resulted in 42-45% removal of *A. flavus* in both systems and same also for *R. stolonifer* with vapor deposited system but showed more removal reached to 63% in case of solution deposited system, and that is mean when there is little increase in AITC concentration via solution deposition than vapor way this little increase in concentration has great inhibition effect on *R. stolonifer* and that is proved its sensitivity from AITC more than *A. flavus* just like we mentioned before in previous assays, which beside the antifungal effect of AITC against *R. stolonifer* the acidity of AITC affect on the growth of this fungi in contrast of *A.flavus* which inhibit by its antifungal effect only [2]. More indications of the sensitivity of *R. stolonifer* more than *A. flavus*, after 24 h exposure *A. flavus* showed more resistant which its removal was only 52-66%, in contrast *R. stolanifir* was more sensitive which its removal was almost 80%. But the relative count reduced to approximately total removal by 48 h which reached to almost 90% removal for both. And exposure up to 72 h resulted in near total removal for each fungi (97%). Fig.4. Ma [15] also demonstrated in his research a system similar to our system by using oriental mustard meal to release AITC vapor to inhibit molds in bread and he found that Fifty mg mustard meal showed fungistatic activity, and ≥100 mg were fungicidal and that is indicating that the increasing in concentration of AITC by the time lead to more lethal effect for the exposure fungus. From these results we concluded that Since AITC vapor deactivation of microbial growth for both vapor- and solution-deposited AITC were parallel in their decay behavior and the different in the inhibition of all tested bacteria couldn't be noticeable and very small, in addition to the application of vapor would be a relatively cheaper, so vapor deposition is a food safety perspective a safer option and more economic than solution deposition. (Fig. 4).




*Rhizopus stolanifir*




*Aspergillus flavus*

Fig. 4. Antimicrobial effect of AITC vapor on the two tested fungi released from de-oiled *L. japonica* powder, loaded via vapor (×) and solution (□) adsorption, and control (●)

## Conclusions

In this work we proved that both the raw and the de-oiled powders of seaweed *L. japonica* adsorbs AITC from vapor- or solution-phase contact, with the latter producing larger quantities adsorbed; de-oiled powder adsorbs more than its raw. These carrier powder provide an initial burst of vapor followed by continuous release, producing a suitable antifungal atmosphere controlling growth of selected foodborne fungi (*A.flavus* and *R.stolonifer*). Since AITC vapor deactivation of microbial growth for both vapor- and solution-deposited AITC were parallel in their decay behavior and the different in the inhibition of all tested fungi couldn't be noticeable and very small, in addition to the application of vapor would be a relatively cheaper, so vapor deposition is a food safety perspective a safer option than solution deposition. Powdered, de-oiled *L.*

*japonica* would be a suitable, natural edible food-compatible carrier of antimicrobial agents for control over the growth of spoilage fungi, so it can be natural food preservation system enhancing food security and safety during storage and transportation.

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