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ANTIMICROBIAL ACTIVITY OF SOYBEAN AND SUNFLOWER OIL POST FRYING VEGETABLES FROM *ALLIUM* AND *BRASSICA* FAMILY

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ABSTRACT

Antimicrobial properties of left-over, oxidized, soybean and sunflower oil, after frying organosulphur rich vegetables fritters (one-pod garlic, garlic, one-pod onion, onion - *Allium* family and cabbage, cauliflower, broccoli, kohlrabi - *Brassica* family) for 3, 9 and 15 minutes, were studied. Oxidative degradation was evaluated from thiobarbituric acid and acid value. Sulphur content of oil was determined and antimicrobial effect was studied against *Escherichia coli* and *Bacillus subtilis*. Kohlrabi transferred remarkably high sulphur on frying to oil. Malonaldehyde declined remarkably on frying one-pod garlic in soybean oil. Only cabbage and cauliflower could decrease acid value in soybean oil. Maximum concentration of TBARS substances, sulphur, free fatty acid and microbial growth had been found to occur in soybean oil. All four vegetables from *Allium* family showed antimicrobial activity in sunflower oil whereas cabbage, cauliflower and the *Allium* vegetables exhibited antimicrobial properties against *Bacillus subtilis* and *Escherichia coli* selectively in both oils.

KEY WORDS

Antimicrobial activity, Sulphur content, *Allium*, *Brassica*, Acid value and TBA value.

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INTRODUCTION

Prevention of food contamination from food borne pathogenic bacteria presently uses mostly broad spectrum antimicrobial chemicals[1,2]. Safety of chemical additives has become a major concern of consumers because of their toxicity[3]. Thus, to minimize overuse of antibiotics in the treatment of infectious diseases and to inhibit appearance of multi-drug resistant bacterial strains (resistant to two or more antibiotics), the exploration of natural and safe antimicrobial substances to replace synthetic chemicals is gaining importance[4-6]. *Allium* and *Brassica* vegetables have long been known for their antimicrobial activity[7,8] against various microorganisms, including Gram-positive and Gram-negative bacteria and fungi. The principal antimicrobial compounds of *Allium*[9,10] and *Brassica*[11] are volatile sulphur compounds derived from S-alk(en)yl-L-cysteine sulfoxide, as well as glucosinolates in case of *Brassica*[12-14] named as allicin (S-allyl-L-propene thiosulfinate) and methyl methanethiosulfinate respectively. Relative instability of the antimicrobial compounds and strong odour of their mother plants seem to limit the use of them as a practical food preservative.

Edible oils extracted from plant sources are important in foods and in various other industries[15]. Oil extract of these sulphur-rich vegetables at boiling temperature has potential antimicrobial activity and could be used in food preparation to get the synergistic effect of the oil and the vegetables[16]. Thus to explore ethnomedicinal properties, this research work investigated the antimicrobial properties of the left over oxidized oil after frying fritters of vegetables namely onion (*Allium cepa*), one-pod onion (*Allium aggregatum*), garlic (*Allium sativum*) and one-pod garlic (*Allium oleraceum*) from *Allium* family and cabbage (*B. oleracea,* var. *capitata*), cauliflower (*Brassica oleracea,* var. *botrytis*), broccoli (*B. oleracea,* var. *gongylodes*) and kohlrabi (*B. oleracea,* var. *gongylodes*) from *Brassicaceae* family. It also tried to correlate the activity with certain parameters like accumulation of sulphur and oxidation products.

MATERIALS AND METHODOLOGY

Chemicals

2-Thiobarbituric acid was purchased from Loba chemie (India). All other solvents and reagents were procured from Merck (India). The culture medium used was procured from (Himedia Laboratories Ltd. India). All chemicals used were of analytical grade.

Oil

Among the cooking oils, soybean oil and sunflower oil were selected and were purchased from local market of south Kolkata.

Vegetables used in Frying

Amaryllidaceae family - The 4 varieties used were onion (*Allium cepa*), one-pod onion (*Allium aggregatum*), garlic (*Allium sativum*) and one-pod garlic (*Allium oleraceum*).

Brassicaceae family - cabbage (*B. oleracea,* var. *capitata*), cauliflower (*Brassica oleracea,* var. *botrytis*), broccoli (*B. oleracea*, var. *gongylodes*) and kohlrabi (*B. oleracea,* var. *gongylodes*) were the selected members of this family.

All the vegetables mentioned were bought from local market of south Kolkata.

Sample Preparation and Frying Operation

Recipe for 10 fritters: Bengal gram flour- 10 grams, vegetable (finely chopped) - 10 grams, water - 10 ml, salt - 1gm.

The batter was prepared and 10 small round fritters were fried for 3 different time limits, 3, 9 and 15 minutes. A volume of 50 ml of each type of oil was heated to respective smoking point in Teflon coated frying pan and fritters were fried. Each set was repeated thrice. After frying the samples were poured in dark coloured glass containers till the brim leaving no head space to ensure no oxidation and refrigerated. Same amount of oil of each type was heated to same temperature and exposed for same time durations as above. This was treated as control.

The entire operation was done on a triplicate basis.

These oils were used to estimate the accumulation of sulphurous compounds and oxidation products as well as their antimicrobial property.

To determine antimicrobial activity of the oil, gram positive and gram negative bacterial species used were *Bacillus subtilis* and *Escherichia coli* respectively. The isolated strains of *Escherichia coli* and *Bacillus subtilis* were obtained from microbiology laboratory of J D Birla Institute, Kolkata.

Determination of Sulphur Content

A volume of 25 ml of the sample was pipetted out, diluted to 50 ml with acetone. The pH was adjusted to 1 with 2 M HCl. The solution was heated to boil. To it was added 15 ml of boiling 0.05 M $BaCl_2$ solution. Subsequently 35 ml of 0.5 M standard EDTA solution and 5 ml of ammonia solution was added and boiled for 15-20 minutes and cooled. About 10 ml of buffer solution of pH 10 and a few drops of Eriochrome black T (EBT) indicator was added and titrated with 0.05 M $MgCl_2$ solution to a clear red colour. Weight percent of sulphur was calculated using the formula:

Sulphur = (32 x 0.0116 x volume of $BaCl_2$ and EDTA complex x 100) / (233 x weight of sulphur in grams).

Determination of Thiobarbituric Acid Number

In a test tube, 200 mg of oil sample was taken and 5 ml of thiobarbituric acid reagent was added. The mixture was stoppered and warmed in water bath at 95 °C for 120 minutes. After that the mixture was cooled and the absorbance was measured (As) at 530 nm in a 10 mm cell against water. A reagent blank absorbance (Ab) was also carried out.

Thiobarbituric acid number =

(50 * (As – Ab)) / Weight of the sample

Determination of Acid Value

In a conical flask, 25 ml of diethyl ether was mixed with 25 ml alcohol and 1 ml of phenolphthalein solution. The mixture was carefully neutralized with 0.1 N sodium hydroxide. This is called Neutral Solvent. 2 grams of oil were taken and the sample was dissolved in the neutral solvent and was titrated with aqueous 0.1 N sodium hydroxide, shaking vigorously until a pink colour was formed that persisted for 15 seconds. Acid value was calculated as:

Acid value = (Volume of sodium hydroxide solution (ml) x 5.61) / Weight of the oil sample.

Determination of antimicrobial activity

Preparation of test organisms

The cultures of test organisms (*Escherichia coli* and *Bacillus subtilis*) were maintained in agar slants at +4 °C. Bacterial inoculums obtained from these reference stock cultures were inoculated in nutrient agar medium, which was incubated at 37 °C for 18-24 hours. From the fresh grown cultures decimal dilutions were made in sterile lactose broth to the concentration of 10^6 CFU/ml and used for testing the oil samples.

***In vitro* antimicrobial activity testing**

Sterile petri plates using nutrient agar medium were prepared and 100 µl of oil samples were poured in each plate before the agar had solidified. Then 10 µl of inoculums prepared in lactose broth of both the bacterial strains were added to the plate. They were kept at 37 °C for 24 hours and the colony count (CFU/ml) was calculated.

$$\textbf{Number of cells per ml} = \frac{\textbf{Number of colonies}}{\textbf{Amount plated * dilution}}$$

Statistical Analysis

The data was reported as mean (n=3) ± SD (n=3). Students' correlation tool was used to find the relationship between two parameters. Statistical analyses of the data were performed by split-plot design Analysis of Variance (ANOVA). Probability value of $P \leq 0.05$ was considered to denote the statistically significant differences at 5% level of significance.

RESULTS AND DISCUSSION

Sulphur Content

Both *Brassica* and *Allium* have in common S-alk(en)yl-L-cysteine sulfoxides as major non-protein amino acids[17]. S-Alk(en)yl-L-cysteine sulfoxides acts as a soluble pool for organic sulphur[18]. Among five S-alk(en)yl-L-cysteine sulfoxides differing in R-side group, four are in *Allium* and one in *Brassica.* S-Methyl-L-cysteine sulfoxide (SMCSO) is present both in *Allium* and *B*rassica family, propenyl derivative is present in both onion and garlic, whereas allyl substitution (viz. alliin) occurs in garlic and propyl only in onion. SMCSO and alliin generate methyl methanethiosulfinate (MMTSO) and allicin. MMTSO, the primary breakdown product of SMCSO, has been found to degrade into volatile sulphur compounds, including methyl methanethiosulfonate ($MMTSO_2$) and dimethyl disulfide (DMDS)[19,20]. Due to the presence of these sulphurous compounds in the vegetables, the sulphur content of the frying oil was found to increase significantly (P>0.05) with increasing time (Figure No.1). Oil in which kohlrabi was fried showed maximum sulphur content. In kohlrabi besides the compounds discussed many volatile products have also been isolated namely 3-methylthiopropyl, 4-methylthiobutyl and allyl isothiocyanate. The two corresponding organic cyanides 3-methylthiopropyl and 4-methylthiobutyl cyanides and dimethyl, trimethyl and tetramethyl disulphides are also present in higher amounts. *n*-Nonenal, (*E*)-2-hexenal, (*E*,*E*)-2,4-heptadienal and some isothiocyanates of unknown structure occur in the volatile fraction as minor constituents[21].

Thiobarbituric Acid Number

Thiobarbituric acid number indicates the amount of non-volatile aldehydes formed during primary oxidation including malonaldehyde which is a carcinogen[22]. This increases with increase in frying time and then may decompose into secondary oxidative substances[23].

From Figure No.2, it can be said, soybean oil recorded higher values in thiobarbituric acid number compared to sunflower oil. This indicates more volatile aldehyde formation in soybean oil. In soybean oil, big garlic, one-pod onion and big onion showed a significant increase ($P>0.05$) in the TBA values with increasing time. It is interesting to note that one-pod garlic illustrated a reverse trend of decreasing TBA value with increasing time. Thus it can be inferred that one-pod garlic in soybean oil could reduce the malonaldehyde accumulation markedly. In sunflower oil, one-pod onion steadily increased ($P>0.05$) the TBA value in subsequent fryings, whereas big onion and one-pod garlic though initially recorded an increase in TBA value has suffered a decrease with increasing frying time. Big garlic again followed an opposite trend of decrease followed by an increase. This indicates that though initially big garlic in sunflower oil was able to reduce the TBARS formation, failed to do so with increased thermal exposure.

All the vegetables of the *Brassica* family had shown either a steady increase or an increase followed by decrease with subsequent increase in frying span. In this family none of the vegetables could reduce the malonaldehyde accumulation in either of the oil. This suggests that sulphur present in these vegetables was not able to positively affect the TBARS formation or control malonaldehyde accumulation.

Acid Value

Acid value is an indicator of the free fatty acid content formed as a result of primary oxidation of oil[24]. As it is being observed from Figure No.3, acid value can be arranged as soybean oil > sunflower oil. This indicates that the amount of free fatty acids generated during primary oxidation is much more in soybean oil than in sunflower oil. From Figure No.3, it can be stated that, one-pod garlic, one-pod onion and big onion in soybean oil had undergone an rise in the values with initial increase in time which had reduced significantly ($P>0.05$) during the third frying, whereas big garlic had shown a steady decrease in the values in successive fryings. In sunflower oil, all vegetables of *Allium* family except one-pod onion has shown diminishing values, where one-pod onion exceptionally has shown an increase followed by a decrease in the value. This can be explained by the fact that free fatty acids are unstable compounds which fast transform into secondary products to become stable[25].

In sunflower oil, acid value in case of cabbage and cauliflower though rised in first two fryings finally declined with third frying. Broccoli and kohlrabi recorded a gradual steady ($P>0.05$) increase in the values in the same oil. On the contrary soybean oil in which broccoli and kohlrabi fritters were fried showed an increasing acid value followed by a decreasing pattern. Cabbage and cauliflower in soybean oil however with increase in frying time, had undergone an unusual decline in the value to as low as 13.61 and 14.92 respectively from an initial value of 82.77 and 230.66 respectively. This reduction in value in three successive fryings implies decrease in free fatty acid formation in the frying oil due to subsequent reaction[25]. Thus it can be inferred that only cabbage and cauliflower in soybean oil were successful in reducing free fatty acid formation with increasing frying time.

Antimicrobial Effect

Oil and its components are able to inhibit growth of certain microorganisms[26]. Again vegetables contain some compounds that have inhibitory or growth promoting effect on microorganisms[27,28].

Figure No.4 depicts the growth of *Escherichia coli* in the left-over oils post frying of fritters of *Allium* and *Brassica* family. It clearly demonstrates that a gradual decrease in the CFU had occurred in both the oils exposed to degradation. Microbial count in general is higher in soybean oil. Though the relationship between fatty acid structure and antimicrobial activity is not clear, it can be suggested that higher amount of polyunsaturation in sunflower oil might have contributed to the inhibitory profile of the fatty acid[29].

In soybean oil, all the vegetables from *Allium* family except one-pod garlic have exhibited an antimicrobial pattern on longer frying. Among the cruciferous vegetables, cabbage and cauliflower in sunflower oil, cauliflower, broccoli and cauliflower in soybean oil have recorded gradually decreasing CFU with increasing frying duration. The antimicrobial property of the oils might be due to the principal antimicrobial compounds of *Allium*[9,10] and *Brassica*[12,13,14] namely allicin (S-allyl-L-propenethiosulfinate) and methyl methane thiosulfinate, respectively which belong to thiosulfinate group.

Antimicrobial activity of left-over oils post frying of fritters of *Allium* and *Brassica* family tested with strains of *Bacillus subtilis* is depicted in Figure No.5. The graph illustrated that sunflower oil exposed to heating and all the *Allium* vegetables fried in sunflower oil had undergone a decrease in values with increasing frying time thereby indicating an antimicrobial property. One-pod garlic fried soybean oil, has shown a reduction in the colony forming units with increase in frying duration. From *Brassica* family cabbage had undergone a decrease in the CFU in both the oils. In cauliflower fritters fried soybean oil also exhibited an antimicrobial property with increasing frying time. One interesting observation was that though kohlrabi accumulated maximum sulphur it did not exhibit antimicrobial activity. The increase in the microbial counts with increasing frying time in certain cases might be due to compounds formed during the oxidative degradation which may induce the growth of the bacteria by serving as medium for nutrition. However some antioxidants like quercetin present in the vegetables might have inhibitory effect on the growth of microbes[30,31]. This might cause decrease in the bacterial growth.

Statistical Analysis

The overall view of the results of the ANOVA indicted that the variation in the vegetable had significant impact on the oxidative parameters. Thus it can be said that the vegetables had a control on the oxidation of oil. The bacterial strains responded to the variation in frying time as well as the assorted vegetables.

Correlation Study

Correlation between various parameters of lipid oxidation, sulphur accumulation and antimicrobial activity was determined. Table No.2 represents the correlation values of TBA value - microbial count, sulphur content - microbial count and acid value - microbial count of both the strains. The samples exhibiting antimicrobial activity has shown high to moderately high negative correlation with TBA value, sulphur content and high positive correlation with acid value. The samples following this trend are one-pod garlic, big garlic, one-pod onion, big onion cabbage in sunflower oil, cauliflower in soybean oil against *B.subtilis* and big garlic, one-pod onion, big onion, cabbage in sunflower and one-pod onion, big onion in soybean oil against *E.coli*. The exceptions to this trend were shown by big garlic (small positive correlation + 0.3542 with sulphur content) and cauliflower in soybean oil, one-pod garlic (small positive correlation + 0.1223, + 0.3522 with TBA value respectively) in sunflower oil against *E.coli*. One-pod garlic, cabbage in soybean oil positively correlated with TBA value (+ 0.9925 and + 0.5736) against *B.subtilis*. Cauliflower in sunflower oil was found to negatively correlate with acid value (-0.4163) against *E.coli*. It is recognized that although an increment of fatty acids as linoleic acid improve the growth of bacteria, but an excessive concentration can inhibit the bacteria growth[32].

Since none of the oils selected contain sulphur therefore the antimicrobial property of the control oil could not be correlated with sulphur and both the oils were found to show antimicrobial activity against *E.coli* whereas contrary to sunflower oil, soybean oil did not exhibit antimicrobial activity against *B.subtilis*. Respective sunflower and soybean oil having antimicrobial activity have highly correlated with (negatively) TBA and (positively) acid value.

These correlation values emphasize that in general increase in concentration of sulphur and oxidised TBARS substance along with decrease in fatty acid content enhanced the antimicrobial activity of the oils.

Table No.1: Antimicrobial activity of the oils

S.No	Oils	Organism	Big onion	One-pod onion	Big garlic	One-pod garlic	Cabbage	Cauliflower	Broccoli	Kohlrabi	Oil
1	Soybean oil	*E.Coli*	+	+	+	-	-	+	-	-	+
		B.subtilis	-	-	-	+	+	+	-	-	+
2	Sunflower oil	*E.Coli*	+	+	+	+	+	+	-	-	+
		B.subtilis	+	+	+	+	+	-	-	-	+

Table No.2: Correlation of different parameters with microbial counts of *E.coli* and *B.subtilis*

	Oil	One-pod garlic	Big garlic	One-pod onion	Big onion	Cabbage	Cauliflower	Broccoli	Kohlrabi
TBA: *E.coli*									
soybean	0.4027	-0.7792	-0.9993	-0.9836	-0.6481	-0.5376	0.1223	0.9963	0.9738
sunflower	-0.9805	0.3522	-0.1611	-0.9942	-0.0068	-0.9735	-0.9446	0.9685	0.9933
TBA: *B.subtilis*									
soybean	-0.6906	0.9925	0.5798	0.9863	0.8919	0.5736	-0.4001	0.7315	0.7917
sunflower	-0.9999	-0.1556	-0.7516	-0.9928	-0.2902	-0.9976	0.9999	0.9148	0.9697
Sulphur:*E.coli*									
soybean	---	0.7421	0.3542	-0.9527	-0.5253	0.8279	-0.8071	0.9035	0.9893
sunflower	---	-0.9044	-0.1982	-0.9941	-0.7666	-0.8671	-0.7415	0.9959	0.9848
Sulphur:*B.subtilis*									
soybean	---	-0.0368	0.5255	0.9574	0.8132	-0.8515	-0.394	0.4465	0.8396
sunflower	---	-0.5759	-0.7760	-0.9443	-0.9173	-0.9753	0.9189	0.9972	0.9990
Acid value : *E.coli*									
soybean	0.9919	-0.9685	0.7046	0.3658	0.3379	-0.9842	0.9614	-0.3718	-0.7540
sunflower	0.9309	0.8741	0.8643	0.4202	0.9744	0.2551	-0.4163	0.9778	0.9691
Acid value : *B.subtilis*									
soybean	-0.8893	0.4966	-0.9919	-0.3804	-0.6752	0.9909	0.6874	0.2802	-0.4132
sunflower	0.8459	0.9999	0.9869	0.2056	0.8707	0.5284	0.0993	0.9305	0.9285

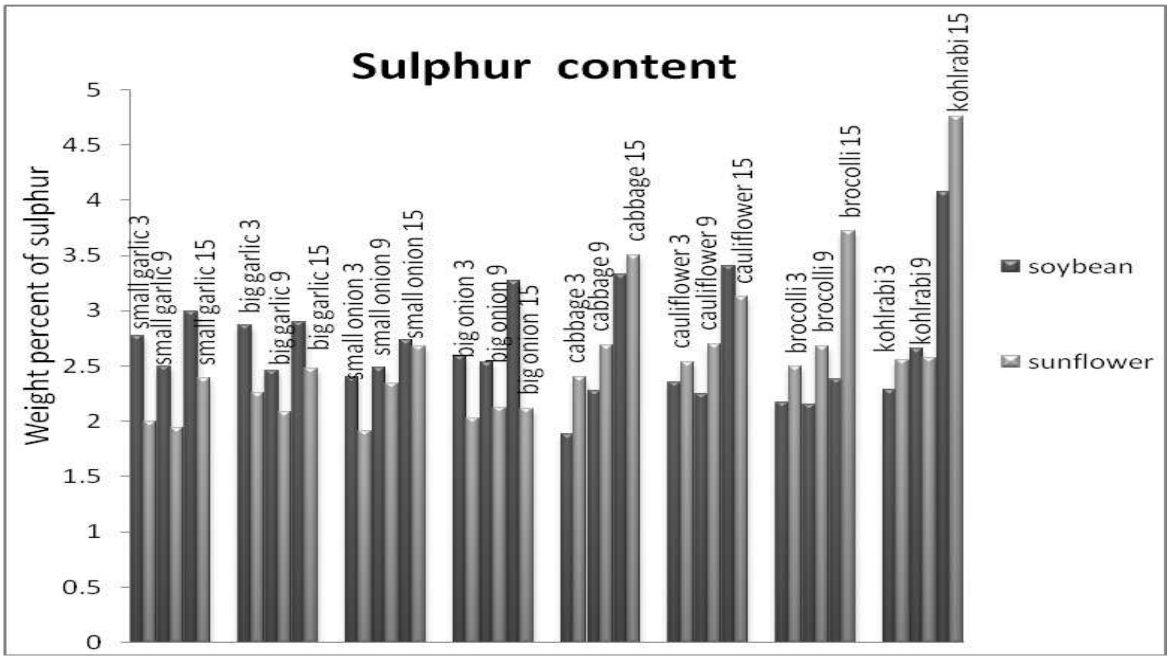

Figure No.1: Graph showing the sulphur content(in mgs) in both *Allium* and *Brassica* family in 2 types of oil with increasing frying time

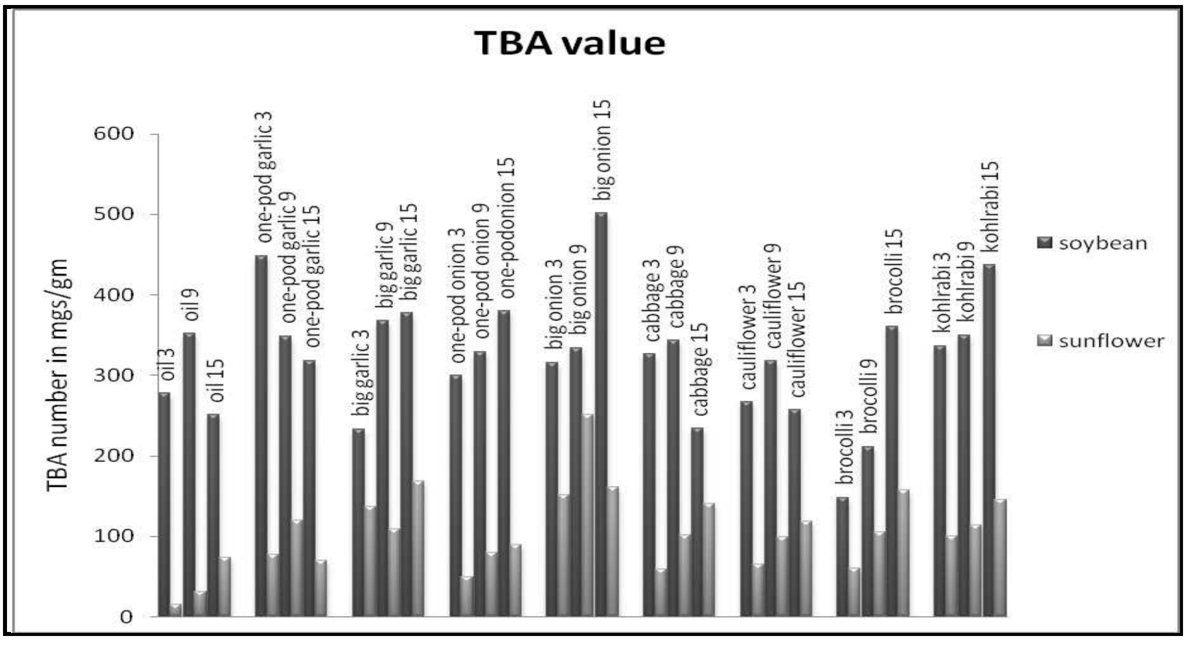

Figure No.2: Graph showing the TBA number in *Allium* and cruciferous family in 2 types of oil with increasing frying time

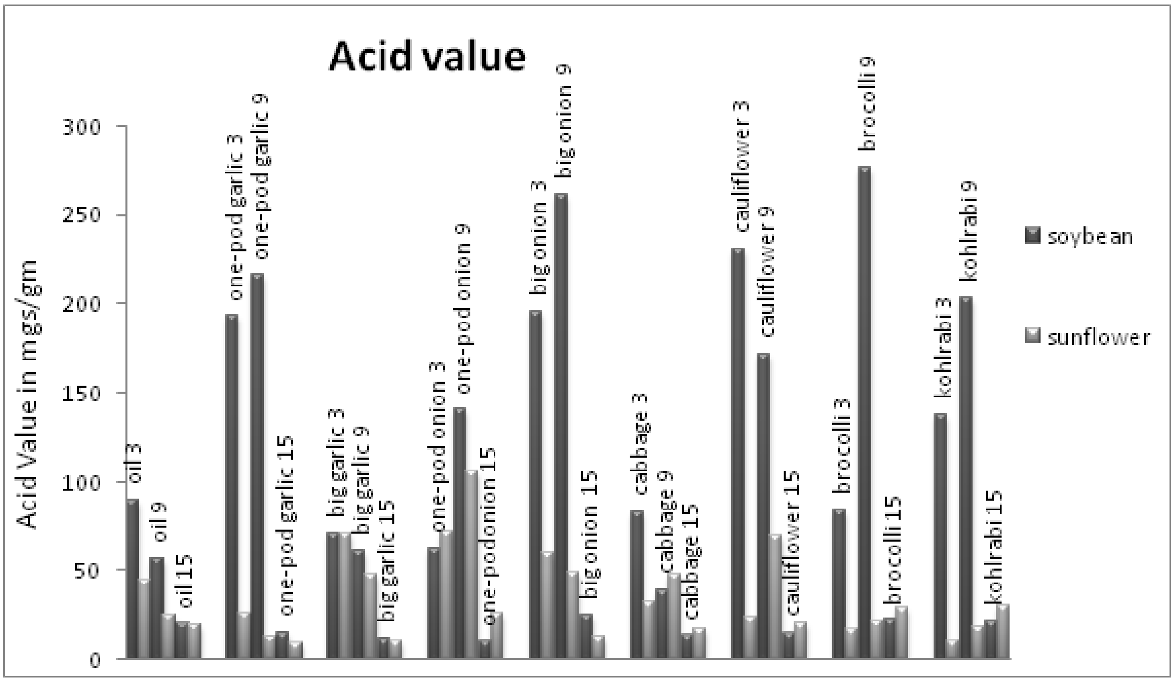

Figure No.3: Graph showing the acid value in *Allium* and *Brassica* family in 2 types of oil with increasing frying time

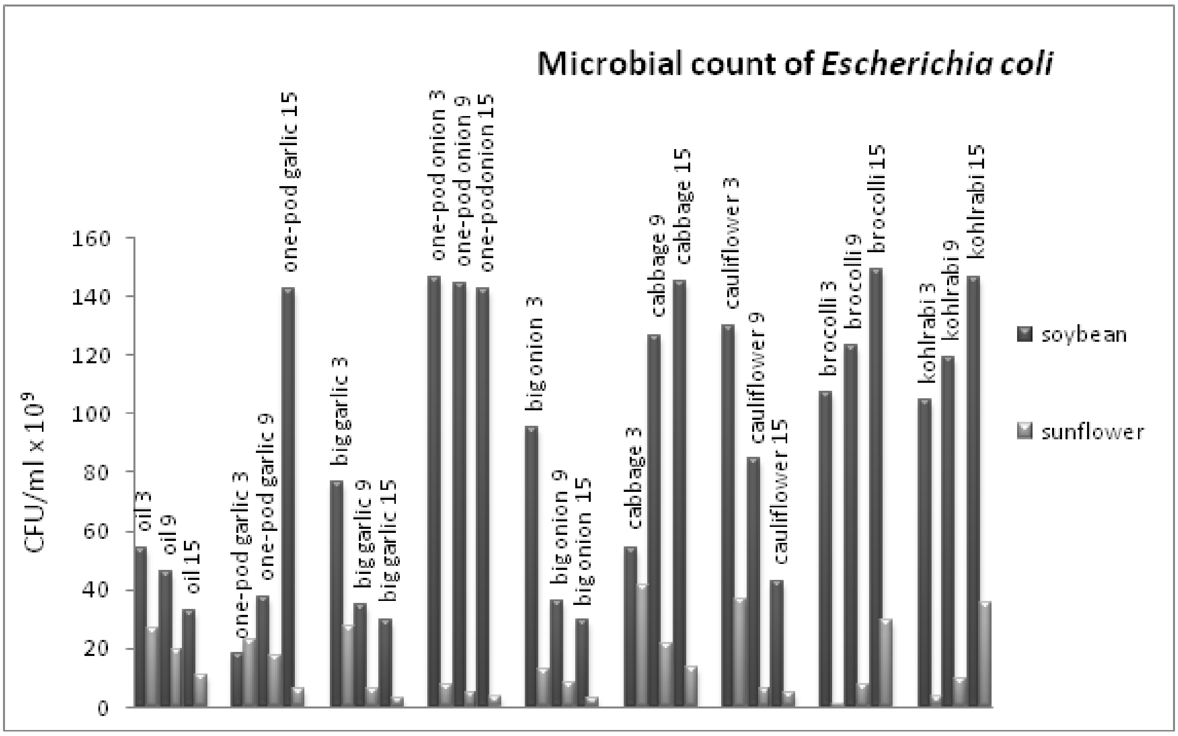

Figure No.4: Graph showing effect of the *Allium* and *Brassica* family in 2 different types of oil on the growth pattern of *E. coli*

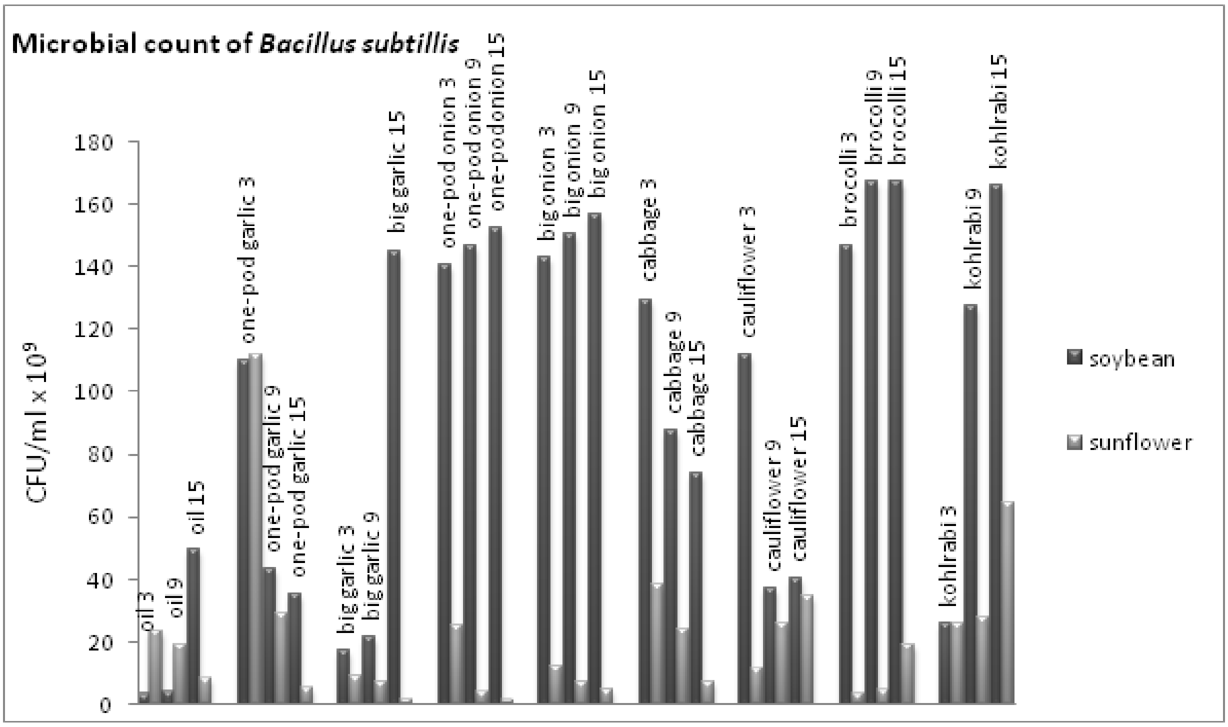

Figure No.5: Graph showing effect of the *Allium* and *Brassica* family in 2 different types of oil on the growth pattern of *Bacillus subtilis*

CONCLUSION

This study revealed that maximum concentration of TBARS substances, sulphur, free fatty acid, and higher microbial growth of both strains had occurred in soybean oil. One-pod garlic was able to reduce malonaldehyde formation in soybean oil. All four vegetables from *Allium* family showed antimicrobial activity in sunflower oil against both the microorganisms. In soybean oil all members expect small garlic depicted an inhibitory effect on *E.coli* and small garlic against *B.subtilis* (Table No.1). Cabbage (except in soybean oil against *B.subtilis*) and cauliflower (except in sunflower oil against *E.coli*) from the *Brassica* family exhibited maximum antimicrobial properties against both the strains. Thus, it can be concluded that organosulphurs present in vegetables had influenced the oxidative degradation of oil, as well as the antibacterial activity of the oil. The future scope of the study is in further analyzing the microbial activity of left-over oil under varying conditions and medium against different strains.

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