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PHYTOCHEMICAL ATTRIBUTES AND ANTIMICROBIAL ACTIVITY OF GREEN TEA AND ROSEMARY LEAVES: A COMPARATIVE STUDY

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ABSTRACT

Green tea and rosemary are known as highly potent antioxidant drugs. Little idea is known about their leaf fatty acids, steroids and terpenoids. Thus, the aim of this work was to investigate the lipoidal matter composition of their leaves and to test for antimicrobial activities of both leaf extracts as a comparative study as well as their 1:1 extract mixture. GC/MS analysis of the unsaponifiable part of the light petroleum fraction of 70% ethanolic extract resulted in the identification of phytol as the major constituent (36.24%) in green tea and α- amyrin as the major constituent (38.8%) in rosemary leaves. Palmitic acid was detected as the main saturated fatty acid in both plants with much higher concentration in green tea (35.70%) than in rosemary (12.3 %). Analysis resulted in identification of new constituents that were not detected previously in both plants. Also give confirmation of some synthetic pathways as in rosemary. Screening the antimicrobial activities of both leaf extracts ensured their antimicrobial effect. Gentamycin and fluconazole were used as the positive antibacterial and antifungal control respectively. Both aqueous extracts were more potent against all the tested microbes than the alcoholic ones. Green tea was found to be more potent than rosemary. No valuable synergism was observed on mixing both plant extracts. MIC of both extracts was 25 mg/ml. The susceptibility of *Salmonella typhi* isolates and *Candida albicans* lab isolates in this study may provide a new plant source for safe and efficient anti bacterial drugs.

KEYWORDS: green tea, rosemary, lipoidal matter, antimicrobial.

INTRODUCTION

Green tea (Camellia sinesis (L.)Kuntze F. Theaceae and Rosemary (*Rosamarinus officinalis* L.) F. Lamiaceaeare are natural medicinal plants known many years ago as highly potent antioxidant drugs. Both are rich in well- studied flavonoids and polyphenolic compounds but the lipoidal matter contents are still not fully clarified.

Camellia sinensis is the species of plant whose leaves and leaf buds are used to produce Chinese tea. Green tea is made from leaves that have undergone minimal oxidation during processing.[1] Green tea contain a variety of enzymes, amino acids, carbohydrates, lipids, sterols, related compounds, dietary minerals, and phytochemicals such as polyphenols, flavanols, and caffeine. Polyphenols found in green tea include but are not limited to epigallocatechin gallate, epigallocatechin, epicatechingallate, and epicatechin; flavanols such as kaempferol, quercetin, and myricitin also have been detected in green tea.[1] Numerous claims have been made for the health benefits of green tea based on its chemical composition. Preliminary research on many of these claims is promising, but many also require further study to evaluate.[1, 2]

The steeping of green tea leaves in near boiling water releases a rich variety of catechins as well as caffeine, theanine. The most abundant polyphenol in green tea is epigallocatechin gallate.[3]

The antiviral effect of green tea catechins was investigated against influenza virus.[4] The oral administration of 1.5% green tea extract to Tamoxifin -intoxicated rats, produced significant increments in the antioxidant enzymes and reduced glutathione concomitant with significant decrements in liver transaminases levels.[5] Green tea has been shown to have antimicrobial effects against a variety of gram positive and gram negative bacteria and a variety of viruses (e.g., HIV, herpes simplex, influenza).[6,7]

Studies concluded that catechins work synergistically with tetracycline against *Staphylococcus species.*[8]

Rosemary is a woody, perennial herb with flagrant, evergreen, needle-like leaves and white, pink, purple or blue flowers, native to Mediterranean and Asia regions. The plant is cultivated since long ago for its strong antioxidant and antimicrobial activities.[9] This plant species also has many other beneficial activities such as antiviral[10] anti-inflammatory[11] and

anticarcinogenic activity.[12] It is considered to be one of the most important sources of both volatile and non-volatile bioactive compounds.[13-14]

Rosemary contains a number of phytochemical, including rosmarinic acid, camphor, hispidulin, naringin, caffeic acid, ursolic acid, betulinic acid, and the antioxidants carnosic acid (the most abundant) and carnosol.[10, 11, 12]

A quantitative evaluation of the antibacterial activity of the two rosemary essential oils α-pinene and myrcene was determined against some Gram-positive and Gram-negative bacteria. Both exerted a moderate antibacterial activity, although the dose response curves showed that the α-pinene-rich oil had a higher antibacterial activity than the myrcene-rich.[15, 16]

From another point of view, the antibacterial effect of the ethanol extract of rosemary leaves was found to be increased by increasing the concentration.[17]

A previous comparison between rosemary and green tea leaves concluded that the ethanolic extract shows high content of phenolic compounds and in turn highly antioxidative activity for both plants as compared with aqueous extract. Although, the phenolic compounds of green tea leave was almost about 3-fold, their extracts show extremely the same reducing power and chelating ability.[18]

MATERIALS AND METHODS

MATERIALS

Rosemary (*Rosmarinus officinalis* L.), Chinese green tea (*Camellia sinensis* L.) Kuntze that were collected in spring 2014 were purchased from a local herb-shop, identified by Prof. Samir El-Kady, Faculty of Agriculture, Kafr El –Shikh University and voucher specimens were kept in herbarium of Faculty of Pharmacy, Damanhour University as 118G and 96R for green tea and rosemary respectively.

All solvents and Chemicals were purchased from Sigma Chemical Co. (St., Louis, USA). α-amyrin (purity 99.3%) was obtained from Sigma-Aldrich.All other reagents were from Sigma (St. Louis, MO, USA) and were of analytical grade.

Micro-organisms were obtained from American type culture collection (ATCC, Rockville, Maryland) or from Northern regional research laboratories (NRRL, Peoria,

Illinois).*Salmonella typhimurium* was isolated from Egyptian Hospital (Tanta University Hospital).

APPARATUS

Melting points were determined using Fisher- Johns melting point apparatus. Solvent evaporation and concentration was performed on Rotavapor Heidolphvv 2000. Concentration of aqueous extracts was performed using Lyophilyser (Hosokawa, England)

GLC analysis was Perkin- Elmer GC/MS model: Clarus 580/560S. Oven initial temperature 50 ^{0}C for 0 min, ramp 3^{0}C/ min to 270^{o}C, Inj= 250 ^{0}C, Carrier gas= He, Solvent Delay= 5.00 min, Transfer Temp= 260^{0}C, Source Temp= 200 ^{0}C, PEGA 10% Column 30.0 cm x250µm. Spectrophotometer (Optima SP-300, Japan) was used for determination of MIC values of the extracts.

TLC analysis was done on precoated silica gel 60 F254 plates (Germany).
UV detection of the TLC plates was done using Camag, Switzerland UV Lamp. Column chromatographic separations were performed on silica gel (70-230 mesh) (E- Merk, Germany).

General experimental procedure

Isolation of Lipids[19]

The fine powdered leaves (100 g) of both plants were separately defatted with petroleum ether (40-60^{O}C) till exhaustion. The solvent was stripped off under reduced pressure so as to yield the lipoidal matter dark greenish residue (0.8 g) and yellowish brown residue (0.6 g) for *Camellia sinesis* and *Rosumarium officinale* respectively. Both lipoidal matter residues were subjected to saponification for subsequent investigation of both unsaponifiable and saponifiable fractions for each.

Preparation of unsaponifiable matter and fatty acids

The light petroleum extracts (0.8 g &0.6g) were separately refluxed with alcoholic potassium hydroxide (10%) for 2 h, after stripping off ethanol and dilution with water, the unsaponifiable matter in each was extracted with ether. Both residues left after evaporation of ether were weighed and found to be 0.45 g in case of *Camellia sinesis* and 0.34g in case of *Rosumarium officinale* and kept for further investigation. Samples of the unsaponifiable fraction were subjected to GLC analysis.

Preparation of fatty acids methyl ester

The two soapy aqueous layers of the two plants were separately acidified with 10% hydrochloric acid and the liberated fatty acids were extracted with ether. The brownish fatty residue in case of *Camelia sinesis* left after evaporation of ether was weighed (0.20 g) and kept for further study. The yellowish residue 0.15 g in case of *Rosumarium officinale* was also kept for methylation. These total fatty acids were subjected to methylation.[20] Methylated fatty acid residues (0.15 and 0.09 g) for *Camelia sinesis* and *Rosumarium officinale* respectively were kept for investigation by gas chromatography.

ISOLATION OF COMPOUND R1 FROM *ROSUMARIUM OFFICINALE*

On cooling the unsaponifiable fraction of the extract, a whitish powder was separated on the beaker wall that was collected by a clean, dry spatula and dissolved in chloroform then repeatedly crystallized from acetone to give 2mg of white crystals **R1.** Compound **R1** has melting point of 183-185^{0}C. Two TLC silica gel F 254 precoated plate chromatograms of the isolated crystals with an authentic sample of α- amyrin on petroleum ether : ethyl acetate : acetonitrile (8.2 : 1.2 : 0.1 v/v/v)and on ethylacetate: n- hexane (7:3 v/v). The plates were then sprayed with anisaldehyde-sulphuric acid reagent, air-dried for complete removal of the spray reagent and then was heated at 110°C for 10 minutes. R_f of isolated and authentic samples was identical (R_f = 0.68& 0.58 in the two systems respectively). Also, a mixed spot gave the same R_f value in both solvent systems. Compound **R1** was identified by IR spectrum as KBr disc showed ƛ max (KBr) cm^{-1}: 3450, 2895 and 2895.The IR spectrum of the isolated compound was superimposed with that of an authentic sample in the region 500-1500 cm^{-1}.

Alcoholic extraction of both plants

The air dried powdered plant material (50g) of each plant was extracted separately and successively with 70%alcohol at room temperature for 3 times. The resultant extracts were separately concentrated under reduced pressure at 30^{0}C. *Camelia sinesis* residue was waxy, dark brownish solid residue weighed 2.4g while that of *Rosumarium officinale* was powdered yellowish to light brown residue, of characteristic odour that weigh 3.5g .TLC analysis of *Camelia sinesis* residue on silica gel F percolated plates showed five spots using ether : $CHCl_3$(8:2) as solvent system and anisaldehyde/ H_2So_4 as spray reagent. R_f of these spots were: 0.13, 0.35, 0.75, 0.83 and 0.92. TLC analysis of *Rosumarium officinale* residue on silica gel F percolated plates showed six spots using ether : CHCl3 (8:2) as solvent system

and anisaldehyde/ H_2So_4 as spray reagent. R_f of these spots were: 0.21, 0.31, 0.52, 0.63, 0.89 and 0.96. Both extracts were kept as residue for antimicrobial activity test.

Aqueous extraction of both plants

The air dried powdered plant material (50 g) of each plants were extracted separately and successively with hot distilled water for 3 times. The resultant extracts were separately lyophilized to give dark greenish residue and yellow residue (3.5 & 3.1g) in case of green tea and rosemary leaves respectively. TLC analysis of both extracts using chloroform : methanol (8:2) gave five spots in case of green tea and six spots for rosemary extract after visualization with UV lamp and spraying with $FeCl_3$ on silica gel F plates.

Antimicrobial activity

Antimicrobial activity assay using cut plug method. Two gram negative bacteria (*Pseudomonas aeruginosa* ATCC 11921 and *Salmonella typhimurium* isolates), one gram positive bacteria *Staphylococcus aureus* ATCC 6538) were selected for antibacterial activity assay. Gentamycin was used as the positive antibacterial control. Three fungi (*Candida albicans* Lab isolated and *Aspergillus niger* NRRL599 and *Cryptococcus neoformans* ATCC 208821) were selected for the antifungal activity assay. Fluconazole was used as the positive antifungal control. Cut plug method recorded by Pridham, et al[21] was employed to determine the antimicrobial activity of the prepared extracts as following: Freshly prepared spore suspension of different tested microorganisms (0.5 mL of about 10^6 cells/mL) was mixed with 9.5 mL of melting sterile Sabouraud's dextrose medium (for fungi) or nutrient agar medium (for bacteria) at 45°C, poured on sterile Petri dishes, and left to solidify at room temperature. Regular wells were made in the inoculated agar plates by a sterile cork borer of 0.7 mm diameter. Each well was filled with 20 mg of each tested sample. Three replicates were made for each test, and all plates were incubated at 27°C for 72 h for fungi, and at 32°C for 24 h for bacteria. Then the average diameters of the inhibition zones were recorded in millimetres (mm), and compared for all plates.

MIC DETERMINATION FOR THE MOST EFFICIENT EXTRACTS AGAINST THE MOST SUSCEPTIBLE MICROORGANISMS

Half-fold serial dilutions were made for selected extracts and fractions in order to prepare concentrations of 6.25, 12.5, 25, 50 and 100 mg/mL in distilled water, zero concentration was considered as a negative control. A previously prepared pure spore suspension of the most susceptible microorganism (0.5 mL of about 10^6 cells/mL) was mixed with 9.5 mL of each

concentration in sterile test tubes, incubated at 27°C for 72 h for fungi, and at 32°C for 24 h for bacteria, then the optical density of growth was measured by spectrophotometer (Optima SP-300, Japan) at 620 nm for each incubated mixture. The growth-inhibiting effect was quantitatively determined by percentage of the surviving cells (% Optical density). Results were represented graphically, and the minimum inhibitory concentration (MIC) was recorded for each tested material.[22]The MIC value was determined as the lowest concentration of the sample at which the tested micro-organisms did not demonstrate any visible growth after incubation.

Statistical analysis

Results were expressed as means standard deviation of at least three independent experiments using the software InfoStat (2008). All data were analyzed by ANOVA followed by Tukey test. Differences were considered significant when $p < 0.05$.

RESULTS

GLC analysis of the unsaponifiable fraction of *Camelia sinesis* resulted in identification of the acyclic diterpene alcohol phytol as the major constituent (55.92 %).The higher alkane – tricosane- is the second leaf constituent (15.15 %). Squalene and stigmastan-6, 22- dien, 3, 5-dihydro are also representable contents with concentration of 5.81 and 3.61%respectively. Results are tabulated in Table 1.

GLC analysis of fatty acid methyl esters of *Camelia sinesis* resulted in the identification of both saturated and unsaturated fatty acids. The results are listed in Table 2. Palmitic acid is the major saturated fatty acid with percent concentrations of 35.70. The major unsaturated fatty acids are oleic acid and linoleic acid with percent concentrations of 20.58 and 9.01 respectively. Few concentration of the omega 9 fatty acid, brassidic acid (1.21%) was also detected.

In *Rosumarium officinale*, GLC analysis of the unsaponifiable fraction of petroleum ether extract identified the presence of mixture of terpenes in addition to steroids and some hydrocarbons. The results are listed in Table 3. The triterpene, α-amyrin was found to be the major constituent followed by the diterpene, eicosane with concentration percentage of 38.08 and 27.61 respectively. The steroids, ω- sitosterol, stigmasteryltosylate and stigmastan-3, 5-diene were detected in percent concentrations 4.24 , 2.04 and 1.4 respectively. Also, the triterpene, squalene and the meroterpene, ferruginol were also detected in 2.4 and 1.61 %

concentrations respectively. GLC analysis of fatty acid methyl esters identified the presence of 17 fatty acids. Fourteen of the seventeen are saturated ones .The results are listed in Table 4. Palmitic acid is the major saturated fatty acid followed by 4- methyloctanoic acid and 4-methylnonanoic acid with percent concentrations of 12.3, 9.95 and 5.6 respectively. The total percent of the unsaturated fatty acids were calculated as 48.53%. The major unsaturated fatty acid is 1, 14- eicosadienoic acid followed by Petroselinic acid with percent concentrations of 26.4 and 18.05%.respectively.

The isolated compound R1 is concluded to be α- amyrin by melting point, being superimposed with the authentic sample in the fingerprint region and giving one spot with mixed authentic sample in two different solvent systems.

Preliminary screening of the antimicrobial activities of the ethanolic, aqueous extracts of both plant leaves as well as their two similar mixtures in 1:1 ratio revealed positive inhibitory effect against all the tested human pathogens. All the inhibition zones were higher than that made by Gentamycin in case of bacteria and Fluconazole dealing with fungi. It was obvious that green tea extracts are more potent than rosemary ones. *Cryptococcus neoformans* is the less susceptible organism despite of giving inhibition zones equal to that of the potent antifungal Fluconazole. Mixing both similar extracts of both plants in 1:1 ratio showed no dramatic increase in potency as inhibition zones were either the mean of them or equal that of green tea extract alone except in case of the effect of mixture of aqueous extracts with *Pseudomonas aeruginosa.* Thus, quantitative evaluation of the antimicrobial activity of the most potent extracts showed the higher inhibition zones was done against the most susceptible organisms. The minimum inhibitory concentration (MIC) of the aqueous green tea extract against *Pseudomonas aeuroginosa, Staphylococcus aereus, Salmonella typhi* and *Candida albicans* was found to be 25 mg/ml. Results are presented in Figure 1. MIC of the 100% growth inhibition of the alcoholic green tea extract against *Pseudomonas aeuroginosa* was 25 mg/ml as shown in Fig 2. MIC of 1:1 mixture of aqueous extracts of both plants against *Pseudomonas aeuroginosa* was also 25 mg/ml as shown in Fig 3.

Table 1: GC/MS analysis results of the major representative compounds of the Unsaponifiable Matter of the petroleum ether extract of *Camellia sinesis* L. leaves

Retention time (min)	Identified compound	Molecular Formula	Percent area
36.67	Ar-tumerone	$C_{15}H_{20}O$	1.59
38.18	Tridecane	$C_{13}H_{28}$	1.57
41.72	Octacosane	$C_{28}H_{58}$	1.91
43.08	2-pentadecanone	$C_{15}H_{30}O$	2.93
48.34	Octadecane-2-methyl	$C_{19}H_{40}$	2.54
51.63	Phytol	$C_{20}H_{40}O$	55.92
54.39	Undecane 3,7 dimethyl	$C_{13}H_{28}$	2.15
58.68	Dodecanamide (lauramide)	$C_{12}H_{25}NO$	2.60
62.60	Heptadecane 2,6- dimethyl	$C_{19}H_{40}$	2.60
67.35	Stigmastan-6,22-dien,3,5-dihydro	$C_{29}H_{46}$	3.61
67.58	1- Iodo-2-methylundecane	$C_{15}H_{25}I$	2.22
70.1	Squalene	$C_{30}H_{50}$	5.81
72.24	Tricosane	$C_{23}H_{48}$	15.15

Table 2: GC/MS analysis results of the major representative compounds of the Saponifiable Matter of the petroleum ether extract of *Camellia sinesis* L. leaves

Retention time (min)	Identified compound	Molecular Formula	Percent area
27.72	1-propene-1,2,3 tricarboxylic acid	$C_6H_6O_6$	**8.84**
28.97	Nonanedioic acid	$C_9H_{16}O_4$	**11.25**
45.94	Palmitic acid	$C_{16}H_{32}O_2$	**35.70**
51.11	Linoleic acid (18:2)	$C_{18}H_{32}O_2$	**9.23**
51.35	Trans-13-octadecenoic acid(oleic acid)	$C_{18}H_{34}O_2$	**20.58**
52.21	Stearic acid	$C18H_{36}O_2$	**9.01**
62.61	Brassidic acid (22:1ω9)	$C_{22}H_{42}O_2$	**1.21**
67.36	9-Dodecenoic acid (12:1)	$C_{12}H_{22}O_2$	**1.21**
67.60	Undecane,3,7-dimethyl	$C_{13}H_{28}$	**1.61**
67.78	Tetracosanoic acid	C24H48O2	**0.82**
72.93	15-Methylpalmitic acid	$C_{17}H_{34}O_2$	**0.15**

Table 3: GC/MS analysis results of the major representative compounds of the Unsaponifiable Matter of the petroleum ether extract of *Rosmarinus officinalis* L. leaves.

Retention time (min)	Identified compound	Molecular Formula	Percent area
16.33	Endo-Borneol	$C_{10}H_{18}O$	1.28
17.39	α-Terpineol	$C_{10}H_{18}O$	0.45
36.32	Andrographolide	$C_{20}H_{30}O5$	1.1
41.71	1-Iodo-2-methylundecane	$C_{15}H_{25}I$	0.18
51.60	Phytol	$C_{20}H_{40}O$	0.84
57.29	Stigmasteryltosylate	$C_{36}H_{55}O_4S$	2.04

57.35	Ferruginol	$C_{20}H_{30}O$	1.61
62.20	α- amyrin	$C_{30}H_{50}O$	38.08
62.61	Hexadecane -1-chloro	$C_{16}H_{33}Cl$	1.3
66.78	Stigmastan-3,5-diene	$C_{29}H_{48}$	1.4
67.58	ω-sitosterol	$C_{29}H_{50}O$	4.24
69.30	Tricosane	$C_{23}H_{48}$	1.21
70.09	Squalene	$C_{30}H_{50}$	2.4
72.27	Eicosane	$C_{20}H_{42}$	27.61

Table 4: GC/MS analysis results of the major representative compounds of the Saponifiable Matter of the petroleum ether extract of *Rosmarinus officinalis* L. leaves

Retention time (min)	Identified compound	Molecular Formula	Percent area
8.39	Pentanoic acid	C5H10O2	1.5
14.28	Caprilic acid	$C_8H_{16}O_2$	1.25
27.72	9-Oxononanoic acid	$C_9H_{16}O_3$	4.08
30.48	Methyl-3-hydroxytetradecanoic acid	$C_{15}H_{30}O_3$	2.68
32.64	Pentadecanoic acid	$C_{15}H_{30}O_2$	2.4
39.01	Lauric acid	$C_{12}H_{24}O_2$	1.93
45.89	Pentadecanoic acid	$C_{15}H_{30}O_2$	1.28
45.89	Palmitic acid	$C_{16}H_{32}O_2$	12.3
51.08	1,14- eicosadienoic acid	C20H36O2	26.4
51.32	6- octadecenoic acid (Petroselinic acid)	$C_{18}H_{34}O_2$	18.05
52.18	Undecanoic acid	$C_{11}H_{22}O_2$	3.45
52.46	Nonanoic acid	C9 H18O2	1.25
54.38	heptadecanoic acid C17:0)	$C_{17}H_{34}O_2$	1.75
56.59	Capric acid	$C_{10}H_{20}O_2$	1.65
65.75	4-methylnonanoic acid	C6H12O2	5.6
71.25	Myristic acid	$C_{14}H_{28}O_2$	4.5
72.23	4- methyloctanoic acid	C9H18O2	9.95

FIGURES

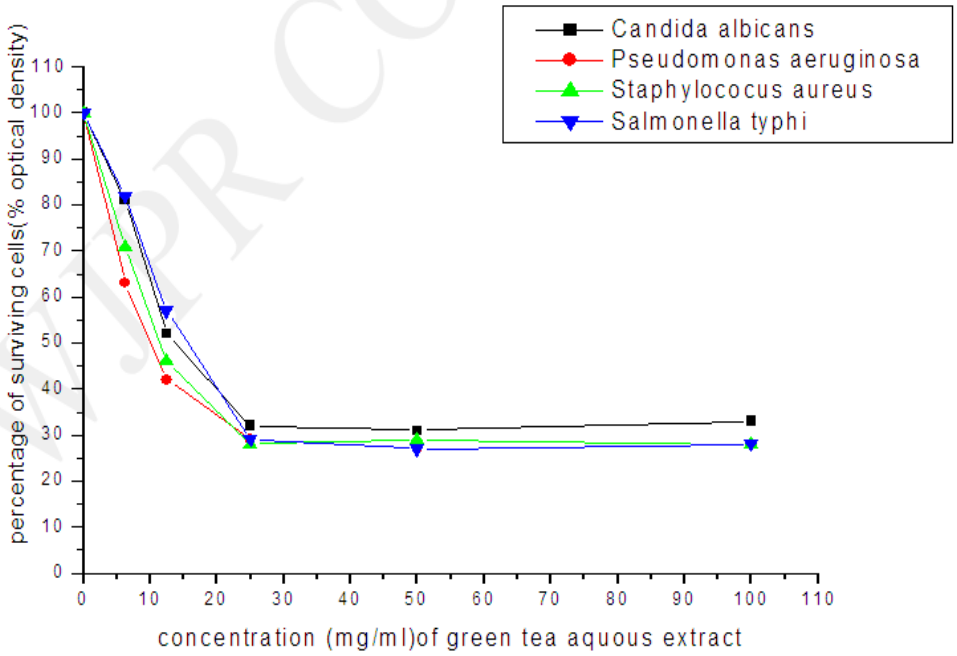

Fig.1 Antimicrobial activity of aqueous green tea extract against *Candida albicans,Pseudomonas aerugimosa,Staphylococcus aureus* and *Salmonella typhi*

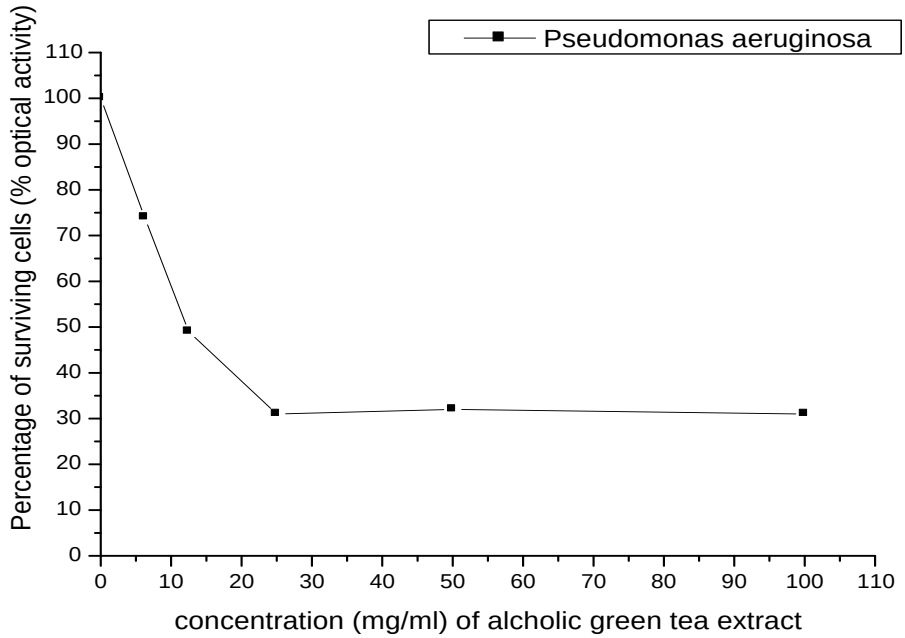

Fig 2: Antimicrobial activity of alcoholic green tea extract against *Pseudomonas aerugimosa.*

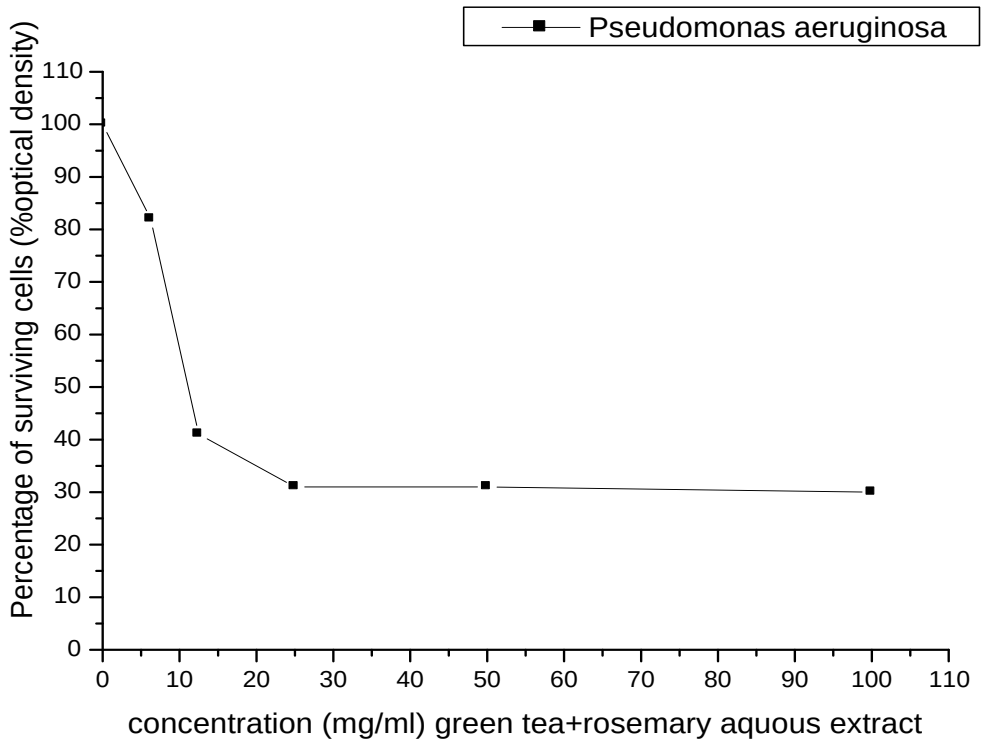

Fig 3: Antimicrobial activity of 1:1 mixture of green tea and rosemary aqueous extract against *Pseudomonas aerugimosa.*

DISCUSSION

A clear investigation of the lipoidal matter content of both plants have been detected for the first time in this work. Phytol (55.92 %) was detected in green tea. It is used as a precursor for synthesis of vitamin E and vitamin K.[23]

Palmitic acid is the major saturated fatty acid can be detected in both plants with much higher concentration in green tea (35.70%) than in rosemary (12.3%). On the other hand, rosemary despite of containing 14 saturated fatty acids from total 17 fatty acids, the percentage of the unsaturated three fatty acids is calculated as 48.53%. Steroids are represented in rosemary with nearly double (7.68 %) the percent in green tea (3.61%). These have been used as a precursor for the synthesis of cholesterol and other sterols with variable side-chains.[24]

Squalene is the vital part of the synthesis of all plant sterols. It is a constituent in both plants with nearly double the concentration (5.81%) in green tea as compared with rosemary (2.4%). In rosemary, α- amyrin is represented in a high concentration (38.08%) and thus been isolated and identified. Also, Ferruginol (1.61%) is the former compound in the pathway of carnosic

acid. Carnosic acid is a benzenediolabietane diterpene with anti-tumour, anti-diabetic, antibacterial and neuroprotective properties synthesis.[25] Its level was found to be dependent on the developmental stage of the plant.[26]

Petroselinc acid, the monounsaturatedomega-12 fatty acid, is detected in rosemary for the first time and brassidic acid a monounsaturatedomega-9 fatty acid, is detected in green tea. Concerning antimicrobial activity, the susceptibility of *Salmonella typhi* isolates and *Candida albicans* lab isolates in this study may provide a new plant source for safe and efficient anti bacterial drugs. Thus, the future use of these plants for the development of human antibiotics is highly promising.

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Isolated microbes were been supplied from Tanta Hospital University.

CONFLICT OF INTEREST STATEMENT

The author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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