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COMPARATIVE PHYTOCHEMICAL STUDY, ANTIOXIDANT CAPACITY AND ANTIMICROBIAL ACTIVITY OF DIFFERENT PROPAGATED CALLUS OF *ECHINACEA PURPUREA* AGAINST ITS LEAF EXTRACTS

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ABSTRACT

Biotechnology has revolutionized the field of biological sciences worldwide. Importance of technological advances has created a renewed interest about its applications. The aim of this study is to use the biotechnology for propagation of *Echinacea purpurea* callus with high value secondary metabolitesin Egypt for medicinal use. In this work we evaluate total polyphenol, flavonoids and polysaccharides contents of *Echinacea purpurea* propagated callus using different media againstitsleaf extract. Also determined the most important potential active compounds, caffeic acid derivatives, as caffeic acid, chlorogenic acid and chichoric acid in all tested extracts. Investigate their antioxidant capacity and antimicrobial activity as a comparative study. The results showed that propagated callus (7) using 1.0 auxin has the highest total ploypheno1s and flavonoids content while callus (6) using 0.50 2,4-Dichloro phenoxy Acetic Acid (2,4D) +0.25 kinetin (k) has the highest total polysaccharides content. Chicoric acid in Echinacea extract was 22 mg/g and in callus (7) was 9.6 mg/g while chlorogenic acid was 12.25 mg/g in callus (7) and 8.5 mg/g in Echinacea extract. Echinacea extract showed the highest antioxidant activity followed by Callus (7). All tested extracts have antimicrobial activity. It could be concluded that the possibility of propagation of *Echinacea purpurea* secondary metabolites using biotechnology with valuable activity for medicinal use.

KEYWORDS: Biotechnology, *Echinacea purpurea* callus, flavonoids, chichoric acid, antioxidant activity.

INTRODUCTION

Echinacea purpurea (L.) family Asteraceae, the most widely cultivated medicinal plant in this species, has gained considerable attention because of its increasing economic value and use as a medicinal plant.[1] The genus Echinacea is found in the United States and in south central Canada.[2] Echinacea species have been reported as important medicinal plants for the treatment of many diseases as colds, toothaches and wound infections.[3] The most important potential active compounds in *Echinacea purpurea* are polysaccharides, caffeic acid derivatives (especially cichoric acid), alkamides, glycoproteins[4], flavonoids, essential oils and polyacetylenes.[5] Using of cichoric acid as a biological marker for *Echinacea purpurea* extract is may be due to its immune stimulant activity and it's probably the most active compound in the extract.[6] Echinacea purpurea posse's antioxidative, antibacterial, antiviral and antifungal activities, but most of all its effects on various immune parameters.[7] It has been used in AIDS therapy[8], also it is used as chemo-preventive for infectious diseases in both upper and lower respiratory systems.[9] Plant cell culture is an alternative source for the production of high value secondary metabolites.[10] For preparing tissue culture medium the percentage of auxin to cytokinin will resulting in an unorganized growing and dividing mass of callus cells. Callus are classified as compact or friable.[11] Friable calluses can be used to generate cell suspension cultures.[12] *Echinacea purpurea* extract found in many commercial products which are among the most popular herbal medicines for the treatment of colds, flu and immunostimulant in Egyptian market. Cultivation of *Echinacea purpurea* in Egypt has some environmental limitation and so in vitro leaf regeneration using tissue culture technique in order to produce callus with high value phytochemical constituents is deem necessary.

The objective of this work is to develop an in vitro regeneration method for *Echinacea purpurea* from

leaves explants. Compare the phytochemical constituents, antioxidant and antimicrobial activities between the developed callus generations against mother Echinacea plant.

PLANT MATERIAL

Seeds of *Echinacea purpurea* (L.) were obtained from the Egyptian local market of herbs (Haraz). *Echinacea purpurea* powder leaf extract was obtained from Utopia Company, Egypt.

CHEMICALS

The solvents used (methanol, ethanol and sulphuric acid) were obtained from Adwic, Egypt. Acetonitrile, orthophosphoric acid of HPLC grade were purchased from Sigma-Aldrich (Steinheim, Germany). The de-ionized water was obtained from NODCAR, Egypt. AlCl3 and phenol were purchased from Win lab, Australia. Sodium carbonate was purchased from El-Nasr chemicals, Egypt. Dettol was purchased from Reckitt Benckiser, England. Clorox (5.25%NaOCL) was purchased Nile company, Egypt. Sucrose was purchased from top chem. company. Phytagel was purchased from Duchefa Biochemie, Netherlands. Tween 20 was purchased from biochemie company, Egypt. MS (Murashige and Skooge, 1962) was purchased from caisson laboratories united states of America. Dimethylsulfoxide (DMSO) was obtained from Chemlimited (Mumbai, India) and nutrients broth was obtained from Britinia, Argentina. Gallic, caffeic acid, chlorogenic acid, chichoric acid and quercetin were purchased from Sigma Chemical Company, St. Louris, Mo, USA. Glucose was supplied from Lobachemie, India

REAGENTS

Folin-Ciocalteu reagent (Phosphomolybdotungstic reagent) was purchased from LobaChemie, Mumbai-India and DPPH (2,2-diphenyl-1-picrylhydrazyl radical) purchased from Sigma Chemical Company, St. Louris, Mo, USA.

SEED STERILIZATION

Echinacea purpurea seeds were washed several times with commercial detergent and tap water then treated with 10% Dettol for 10 secondsfollowed by 70% ethanol for 10-15 seconds and 40 ml 20% Clorox + 60 ml distilled water for 20 minutes. Tween 20 (2 drops/100 ml sterilization solution) were add to all sterilization treatments at the end of sterilization period. Each treatment was rinsed 3 times with 200 ml sterile distilled water (5, 10 and 15 minutesrespectively). Each treatment was planted in ten sterile jars (350 ml)containing 25 ml of MS[13] basal medium + 3.0% sucrose + 0.20% phytagel, eachjar containing ten seeds.

The pH value was adjusted to 5.7-5.8. The different treatments were planted under Laminar Airflow hood and incubated for 3 weeks in a growth chamber under 25+2°C and 1500 Lux white artificial light using cool white fluorescent lamps for 16 hours light/ 8 hours dark of 24 hours cycle. In the end of incubation period (3 weeks), the germination percentage was recorded.

CALLUS INDUCTION

In this study induction of callus was achieved using *Echinacea purpurea* leaves as explant. In order to propagate callus MS medium was used with different concentrations of 2,4-Dichlorophenoxy Acetic Acid (2,4-D) (D) and cytokinin (kinetin) (K). Where C1 (1.0D+0.50K sub.3), C2 (1.0D+0.25K sub.3), C3 (1.0D+0.25 K sub.2), C4 (1.0D+0.250 sub.1), C5 (0.50D sub.1), C6 (0.50D+0.25K sub.1) and C7 (1.0D sub.1).

Scalpel was used to cut the sterilized explants individually into 2-5 mm pieces. Four segments were inoculated in each jar which containing sterile MS with different concentrations and different combination of growth factors. Incubation for 3 weeks at 16 hours light and 8 hours dark at 25+2°C.

TEST ORGANISMS

Pseudomonas aeruginosa, *Staphylococcus aureus* 43300, *micrococcus luteus* and *E.coli* were supplied from the Microbiology Department, Applied research for medicinal plant center (NODCAR), Egypt. Tested organisms were inoculated on nutrient agar slant for 24 hours at 37 ^{0}C.

SAMPLE PREPARATION

Preparation of Echinacea extract

One gram of Echinacea powder extract was accurately weighed and extracted with 25 ml 70% ethanol using soxhlet for 3 hours, filtrated, concentrated and adjusted the volume to ten ml in volumetric flask.

Preparation of callus extracts for phytochemical analysis

One gram of each callus (which contains 5±0.5% mg dry weight) was ground separately using mortar and pestle with 25 ml methanol, leave it in shaker 24 hours then in freezer 24 hours, then filtrated, concentrated and adjusted the volume to ten ml in volumetric flask.

Preparation of standard solutions for HPLC analysis

Five mg of chicoric acid, caffeic acid and chlorogenic acid standards were separately dissolved in five ml methanol.

Preparation of calibration curve of gallic acid, quercetin and glucose standard

A stock solution of gallic acid and quercetin standard in methanol (10 mg/10ml) and glucose standard in hot water (10 mg/20ml) were prepared separately then its aliquots were transferred in a series of ten ml volumetric flasks in varying fractions and their volumes were made to prepare different standard dilutions to be determined using UV technique.

PHYTOCHEMICAL STUDY

The different propagated Echinacea callus extracts (from C1 to C7) and Echinacea leaf extract were tested for their total polyphenolic, flavonoids and polysaccharides contents also caffeic acid, chlorogenic acid and chichoric acid were quantitavely determined using HPLC technique.

Determination of total polyphenolic

SPECORD 210 PLUS UV/Visible spectrophotometer (Analytik Jena AG, Germany) was used. Where one ml from each callus and Echinacea solutions was added separately to 5 ml of Folin-Ciocalteu reagent diluted ten folds, after 3 to 5 minutes, 4 ml of 7.5% Na_2CO_3 solution was added to the mixture. The absorbance at 765 nm was taken after 30 minutes as well as for gallic acid standard. All determinations were performed in triplicates.[14] Blank consisted of Folin-Ciocalteu reagent (5ml), methanol (1ml) and 7.5% Na_2CO_3 solution (4 ml).

Determination of total flavonoids

0.5 ml of each callus, Echinacea extract solutions and quercetin standard were mixed separately with 2.25 ml of distilled water followed by addition of 0.15 ml of 5% $NaNO_2$ solution. After 6 min, 0.3 ml of 10% $AlCl_3.6H_2O$ solution was added and allowed to stand for another 5 min before 1 ml of 1M NaOH was added. The mixture was mixed well with vortex. Blank was carried out at the same conditions without addition of the sample. The absorbance was measured immediately at 510 nm.[15]

Determination of total carbohydrates

0.2 ml of the callus extract, Echinacea extract solutions and working standard were mixed separately with 0.2 ml phenol reagent (five % phenol in water), then one ml concentrated H2SO4 was added to the solutions and left undisturbed for ten minutes before shaking vigorously then measured after 30 minutes.Blank was carried out at the same conditions without addition of the sample.[16]

HPLC analysis of caffeic acid derivatives

The quantitative analysis of caffeic acid derivatives (mainly caffeic acid, chlorogenic acid and chichoric acid) in Echinacea extract and different prepared callus using HPLC chromatographic technique was carried out.[17] Agilent HPLC (USA) and UV detector equipped with sampler TML was used. All analyzed compounds were separated on C18 column (250x4.6mm i.d., 5µm particle size) at ambient temperature; the mobile phase was mixture of 0.1% orthophosphoric acid pH 2 / acetonitrile in the ratio of 60:40 v/v, pumped at flow rate of 1 ml/min and the UV detector was set at 330nm.

ANTIOXIDANT ACTIVITY

The 1, 1-diphenyl-2-picryl hydrazyl radical (DPPH) was used for determination of free radical scavenging activity of the extracts.[18] Different concentrations of *Echinacea purpurea* extract (100 to 1200 mg/ml, in 70% ethanol) and callus extracts from C1 to C7 (0.025 to 4 mg/ml, in 70% ethanol) were added separately, at an equal volume (2.5 ml) to an ethanolic solution of DPPH (0.3 mmol/L, 1 ml). After 30 min at room temperature, the absorbance of the plant extract with DPPH was measured at 517 nm on a spectrophotometer and converted into the percentage antioxidant activity using the following equation: DPPH scavenging effects (%) = 100 – [(A0 - A1/A0) x 100]

Where A_1 is the absorbance of the extracts containing DPPH and A0 is the absorbance of DPPH solution plus ethanol. The IC50 values were calculated.

ANTIMICROBIAL ACTIVITY

Agar well diffusion method

Screening of extracts as antibacterial was performed using the method of agar-well diffusion.[19] Where 10 ml of Mueller Hinton Broth was used (18 hour culture at 37°C). Using solution of sterile saline, the cultures were prepared nearly 105 CFU/ml. 5 hundred microliters of the suspensions were poured over the plates containing Mueller-Hinton agar using a sterile cotton swab to get an equal microbial growth on control and test plates. The extract were prepared in 10% aqueous dimethyl-sulfoxide with Tween 80 (0.5% v/v for easy diffusion) and sterilized by filtration through a 0.45µm membrane filter. Cups of 5 micrometers in diameters were done in the agar plates using sterile cork porer. Under a sterile condition, cups were filled with 50 µL of the respective extracts and placed on the agar surface. The control used was a standard disc containing gentamicin. All petridishes were stamped with sterile parafilm to avoid evaporation of the test samples. The plates were left at room temperature for 30 min, and then they were incubated at 37°C for 18 h (as there was no change in the inhibition up to 24 h). After that the zone of inhibition was measured with a caliper. After inoculation, plates leaved at 37°C for 2 hours to assure full spread of active substance in agar as mentioned in USP guidelines. Studies were performed in triplicate, and mean value was calculated and recorded.

RESULTS AND DISCUSSION

CALLUS INDUCTION

Induced callus using MS medium with different concentrations of both (2,4-D) auxin and cytokinin (kinetin) revealed that C1 with weight equivalent to 9.03 gm was the best as showed in table (1) and figure (1).

Table (1): Results of weight of different propagated callus.

CALLUS NO.	WEIGHT (GM)
C1	9.03
C2	8.4
C3	8.57
C4	7.73
C5	3.2
C6	0.71
C7	3.4

PHYTOCHEMICAL STUDY

Total polyphenolic and flavonoids content

Results of the quantitative determination of total phenolic acids as gallic acid and total flavonoids as quercetin in different propagated callus and Echinacea extracts revealed that C7 showed higher polyphenols and flavonoids content as shown in table (1) and in figure (2a and 2b).

Total polysaccharides content

Results of the quantitative determination of total polysaccharides as galactose in different propagated callus and Echinacea extracts reveled that C6 showed higher polysaccharides content as shown in table (2) and in figure (2c).

Table (2): Phytoconstituents of different propagated callus extracts.

Extract / Callus	Total Polyphenols	Total flavonoids	Total polysaccharides
	(mg/100mg)		
C1	0.464	2.08	0.011
C2	0.544	3.98	0.036
C3	0.432	3.52	0.040
C4	0.36	3.82	0.10
C5	0.412	4.06	0.37
C6	0.462	2.42	1.44
C7	1.521	6.26	0.91
Echinacea extract	2.26	8.6	14.2

HPLC analysis

The quantitative analysis of chicoric acid, chlorogenic acid and caffeic acid in different propagated callus extracts against Echinacea extract using HPLC technique was carried out and the results revealed that chlorogenic acid in C7 was 19% while in Echinacea leaf extract was 7% and Chicoric acid in C7 was 23% while in Echinacea leaf extract was 35% as represented in table (3) and in figure (3).

Table (3): HPLC analysis of chicoric acid, caffeic acid and chlorogenic acid standards in Echinacea and C7 extracts.

Compound	Sample	R_t	%	Concentration
Chicoric acid	C7	5.1±0.02	23%	9.6 mg/gm
	Echinacea extract		35%	22 mg/gm
Caffeic acid	C7	2.5±0.02	6%	3.01mg/gm
	Echinacea extract		11%	9.1 mg/gm
Chlorogenic acid	C7	2.2±0.02	19%	12.25 mg/gm
	Echinacea extract		7%	8.5 mg/gm

ANTIOXIDANT ACTIVITY

The percentage of DPPH radical-scavenging activity was plotted against the extracts concentrations to define its concentration which necessary to decrease DPPH radical concentration to 50% as shown in figure (4). The IC50 value was measured to evaluate the antioxidant activity of selected extracts as shown in figure (5) and the results revealed that Echinacea extract showed the highest antioxidant activity followed by C6 and C2as shown in table (4).

Table (4): IC50 values of gallic acid, Echinacea extract and different propagated callus extracts.

Compounds /Extracts	IC50 (mg/ml)
Gallic acid	0.012
Echinacea extract	0.034
C1	0.198
C2	0.189
C3	0.191
C4	0.2
C5	0.33
C6	0.176
C7	0.321

ANTIMICROBIAL ACTIVITY

Results of the screening of antimicrobial activity of Echinacea purpurea and prepared callus extracts against *E. coli*, *S. aureus*, *P. aeruginosa* and *M. luteus* revealed that *P. aeruginosa* was the most susceptible strain inhibited by Echinacea extract while *S. aureus* was the least affected. C5 showed the most effect on *E. coli* while C6 was on *p. aeruginosa* and C7 showed the most

effect on *S. aureus* while C1 was on *M. luteus* as shown in Table (5).

Table (5): Antimicrobial activity of Echinacea and different propagated callus extracts against the different tested strains.

Tested organisms / Extract	*E. coli*	*p. aeruginosa*	*S. aureus*	*M. luteus*
	Inhibition zone diameter			
Echinacea extract	22 mm	24 mm	20 mm	19 mm
C1	20 mm	18 mm	19 mm	23 mm
C2	19 mm	20 mm	20 mm	16 mm
C3	18 mm	19 mm	17 mm	21 mm
C4	21 mm	22 mm	20 mm	19 mm
C5	25 mm	23 mm	21 mm	20 mm
C6	23 mm	24 mm	20 mm	22 mm
C7	22 mm	21 mm	23 mm	21 mm

Figure (1): Callus 7 induction.

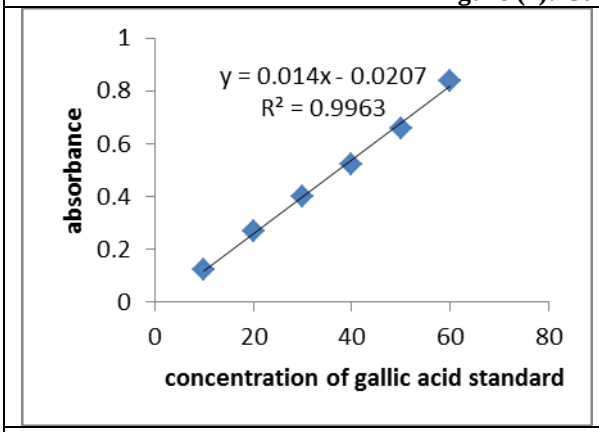


Figure (2a): Calibration curve of gallic acid standard.

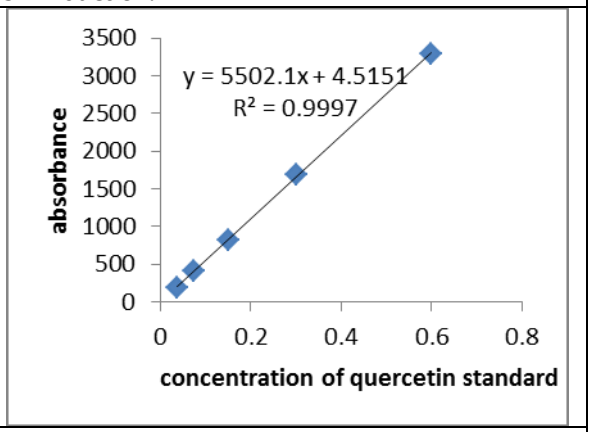


Figure (2b): Calibration curve of quercetin standard.

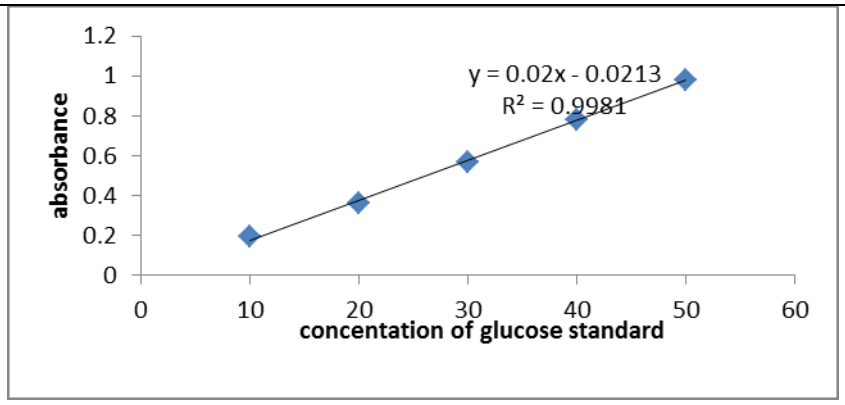


Figure (2c): Calibration curve of glucose using UV technique.

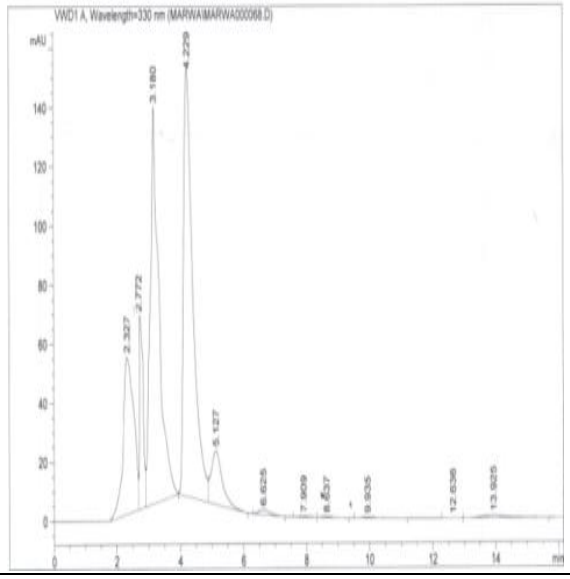


Figure (3a): HPLC chromatogram of Echinacea extract.

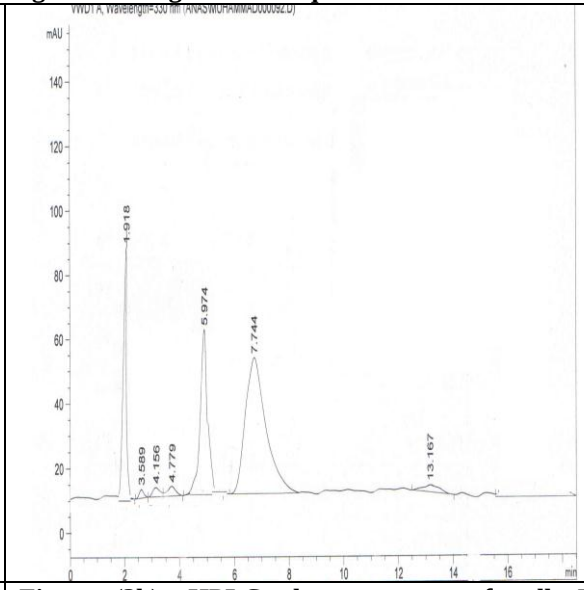
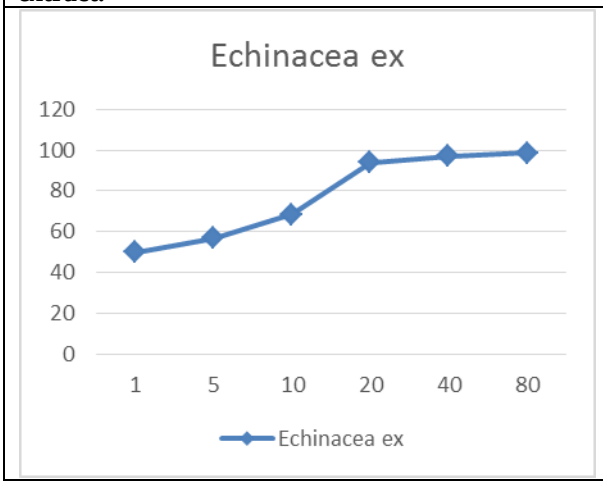
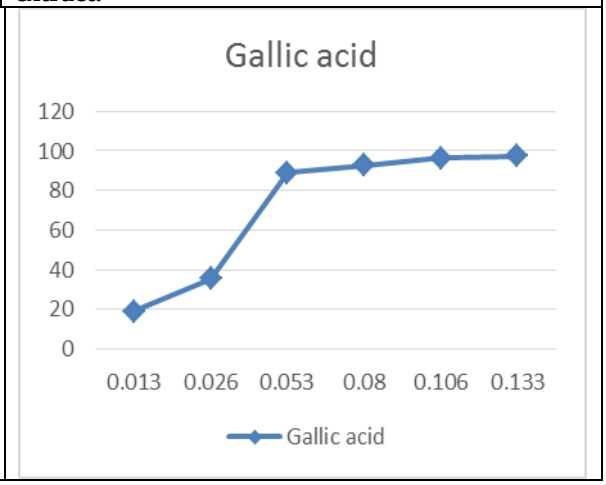


Figure (3b): HPLC chromatogram of callus7 extract.





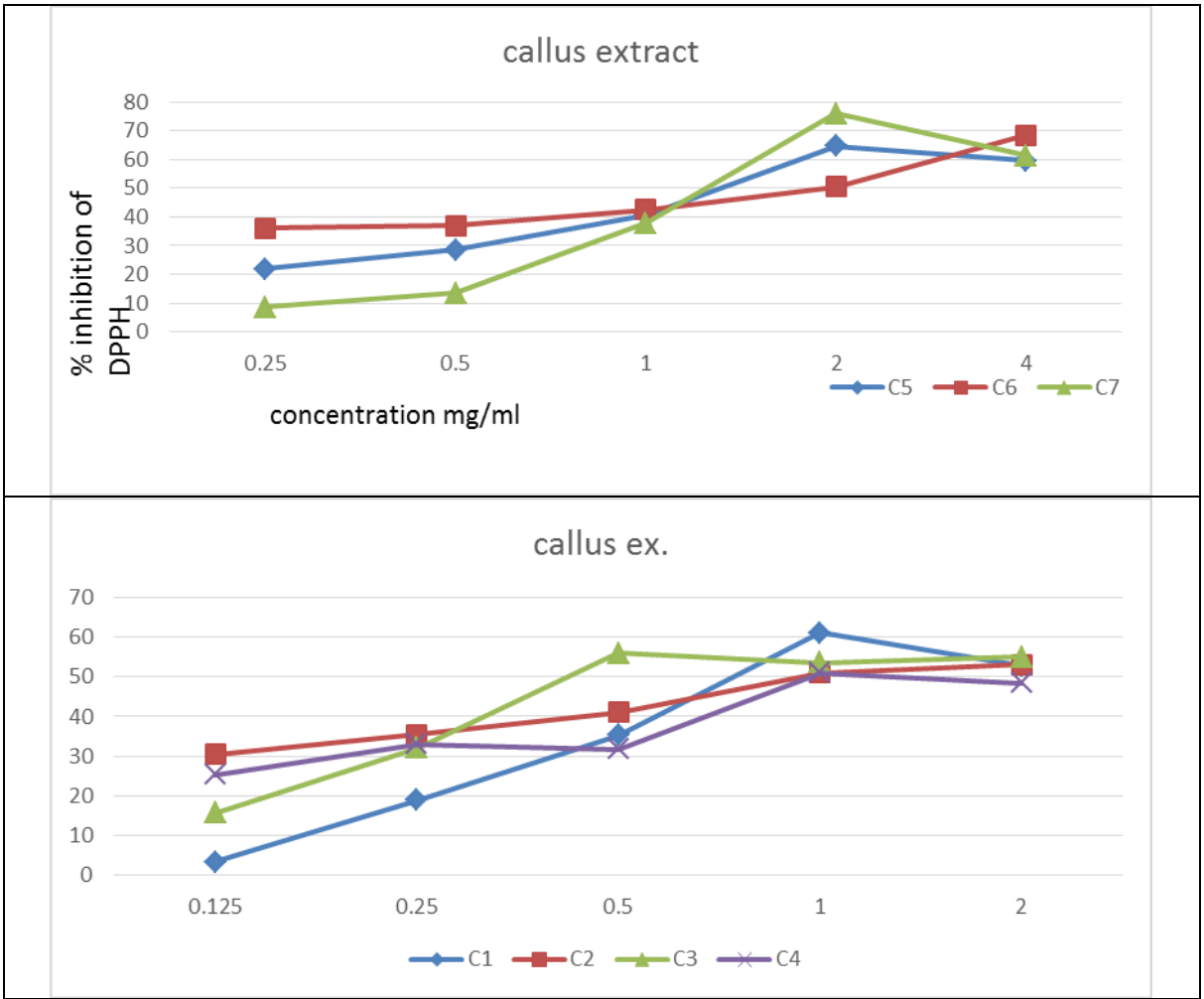


Figure (4): DPPH radical-scavenging activity of gallic acid, Echinacea and callus extracts.

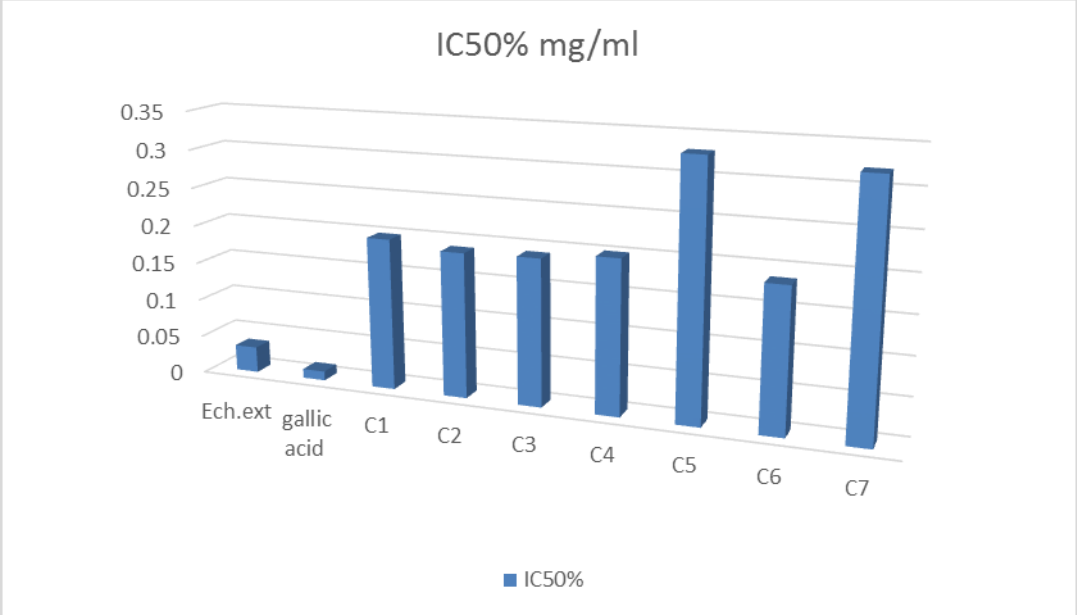


Figure (5): IC50 values of DPPH radical-scavenging activity of gallic acid, Echinacea and callus extracts.

CONCLUSIONS

All propagated callus extracts has polyphenols, flavonoids and polysaccharides content. Propagated callus (7) using 1.0 auxin media has the highest total ploy phenols and flavonoids content while callus (6) using (0.50auxin +0.25kinetin) has the highest total

polysaccharides content. Chicoric acid and chlorogenic acid was identified in all callus extracts. Where the content of chlorogenic acid in C (7) was higher than the reference extract while chicoric acid was lower. All propagated callus extracts showed antioxidant and antimicrobial activity where C (6) was the highest following the Echinacea extract. It could be concluded that the possibility of propagation of callus of *Echinacea purpurea* with valuable secondary metabolites using biotechnology for medicinal use.

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