ASIAN JOURNAL OF PHARMACEUTICAL AND CLINICAL RESEARCH

INNOVARE ACADEMIC SCIENCES Knowledge to Innovation

Vol 7, Suppl 2, 2014

ISSN - 0974-2441

Research Article

ANTIOXDATIVE ACTIVITIES OF WILD MACRO FUNGI *GANODERMA APPLANATUM* (PERS.) PAT.

NAGARAJ K*[1], N MALLIKARJUN[2], RAJA NAIKA[1], VENUGOPAL TM[2]

[1] **Department of P.G.Studies and Research in Applied Botany, Kuvempu University, Jnana Sahyadri, Shankaraghatta, Shivamogga, Karnataka, India.**

[2] **Department of P.G. Studies and Research in Microbiology, Sahyadri Science College (Autonomous), Shivamogga, Karnataka, India. Email:** nkuruni@gmail.com

Received: 16 March 2014, Revised and Accepted: 8 April 2014

ABSTRACT

The present research work was under taken to evaluate antioxidant activity and quantitative determination of phytoconstituents of various solvent extracts of wild macro fungi *Ganoderma applanatum* (Pers.) Pat. using *in vitro* models. The total phenolic content was estimated using Folin-Ciocalteu method and total flavonoid content was determined using aluminum chloride. *In vitro* antioxidant activity of solvent extracts were studied in different systems including DPPH scavenging assay, reducing power assay, metal chelating activity, superoxide radical assay and hydrogen peroxide activity along with standard. The results revealed that, the total phenolics and flavonoids were high and extracts showed good antioxidant capacity in DPPH radical scavenging assay compared to other *in vitro* models. The methanol extract can be considered as a valuable source of antioxidant products as the obtained values are more or less near to the used standard compounds. The present study concludes that methanolic extract of study species is effective in scavenging free radicals and has the potential to be powerful antioxidant. This potent antioxidant activity may be attributed to its high phenolic and flavonoid content.

Keywords: Antioxidant activity, *Ganoderma applanatum*, Radical scavenging, Phenolics, flavonoids.

INTRODUCTION

Free radicals are found to be a product of normal metabolism. Although oxygen is essential for aerobic forms of life, oxygen metabolites are highly toxic. As a consequence, reactive oxygen species (ROS) are known to be implicated in many cell disorders and in the development of many diseases including cardiovascular diseases, atherosclerosis, chronic inflammation etc [1-2]. Antioxidants that can inhibit or delay the oxidation of an oxidizable substrate in a chain reaction, therefore, appear to be very important [3]. Although organisms have endogenous antioxidant defences produced during normal cell aerobic respiration against ROS, other antioxidants are taken both from natural and synthetic origin. Synthetic antioxidants are widely used but their use is being restricted nowadays because of their antioxidants, without any undesirable effect, has increased greatly [4].

Antioxidant compounds have important ability to trap free radicals and thus inhibit the oxidative mechanisms that lead to degenerative diseases. Naturally occurring antioxidants can be found in whole grains, fruits, vegetables, teas, spices and herbs. Mushrooms have also been reported as organisms with antioxidant activity which is correlated with their phenolic and polysaccharide compounds [5-7].

The fungi of the genus *Ganoderma* are popular medicinal mushrooms, and they have been used widely in China, Japan and Korea over the past two millennia [8]. Traditionally, *Ganoderma* (Ganodermataceae) is highly valued as a folk herb and functional food due to its anti-tumor and other helpful pharmacological functions including anti-inflammatory, antiviral, antibacterial, anti-parasitic, kidney tonic, hepatoprotective, nerve tonic and sexual potentiator functions, as well as regulation of blood pressure, cardiovascular disorders and chronic bronchitis and immune enhancement [9]. However, the pharmacological efficacy and bioactive components in *Ganoderma* from different origins or hosts are significantly different [10-11] and the reasons leading to this phenomenon are still unclear.

Mushrooms are widely used for pharmaceutical purposes and as health foods for centuries in China, Japan and Korea. Many metabolites isolated from mushroom species have proven to be therapeutically active against all major infectious diseases [12]. Antioxidant activities of metabolites from mushrooms have attracted the interest of researchers for exploitation of the valuable resource. Mushrooms such as *Ganoderma* spp., *Antrodia camphorate*, *Agaricus blazei*, *Dictyophora indusiata*, *Grifola frondosa* showed excellent antioxidant activity [13].

Mushrooms produce a wide range of secondary metabolites with high therapeutic value [14]. Health promoting properties such as antioxidant, antimicrobial, anticancer, cholesterol lowering and immunostimulatory effects, have been reported for some species of mushrooms [15-16]. Both fruiting body and the mycelium contain compounds with wide-ranging antioxidant and antimicrobial activities [17]. The antioxidative and free radical scavenging properties of the phenolic content of mushroom methanol extracts have been reported, suggesting possible protective roles of these compounds, due to their ability to capture metals, inhibit lipoxygenase and scavenge free radicals [18].

Antioxidant supplements or antioxidant containing food may be useful in helping human body to reduce oxidative damage cause by free radicals. Beside the damage that free radicals can induce in biological systems, they are major culprits of food deterioration, loss of color, flavor and nutrition value. Synthetic antioxidants, used in the food industry as food preserving compounds, are being restricted due to their inherent risk of carcinogenicity [19]. Because of this critical problem, most consumers insist on natural food additives, such as extracts from fruits and vegetables, over synthetic ones. Therefore, isolation of natural antioxidants from plants or mushrooms for food applications and the enhancement of the antioxidant systems for the prevention of cellular oxidative damage via the consumption of antioxidant rich foods is of great interest.

Considering the growing interest for mushrooms, as dietary source

for human consumption and the demand search for natural antioxidants sources, objective of this study was to investigate antioxidant properties and quantitative determination of chemical constituents of wild macro fungi *Ganoderma applanatum* from Karnataka, India.

MATERIALS AND METHODS

Chemicals

Petroleum ether, chloroform, methanol, acetic acid, ammonium hydroxide, sodium carbonate, sodium chloride, sodium hydroxide, Folin-Ciocalteu reagent, aluminum chloride, quercetin, gallic acid, ascorbic acid, 1,1-Diphenyl-2-picrylhydrazyl (DPPH), potassium ferricyanide, trichloroacetic acid, ferrous chloride, ferric chloride, ferrozine, ethylelediaminetetraacetic acid (EDTA), Nitroblue tetrazolium (NBT), Nicotinamide adenine dinucleotide (NAD), phenazine methosulphate, diethyl ether, butanol were obtained from Hi- Media laboratory and Qualigen, India. All the chemicals used in this study including solvents were of analytical grade.

Sampling of macro fungi and Extraction

Wild growing basidiocarp of *G.applanatum* (Pers.) Pat. were collected from two distinct geographical regions ARF (Agumbe Reserve Forest) and SWLS (Shettihalli Wildlife Sanctuary), the site of collection at ARF is 13° 31′ 11.04″ N and 75° 05′ 18.43″ E and at SWLS is 13° 53′ 33 .27″ N and 75° 22′ 16 .30″ E. Identification was done by comparing their morphological and anatomical characteristics [20] and also through the electronic data on identification keys of mushrooms.

The mushroom samples were brush cleaned of attached soil, air dried to constant mass, chopped and pulverilized in electric grinder to a fine powder that was stored in a glass bottles, within paper sample bags in the dark at room temperature in desiccators prior to analysis. About 500 gm of dried powder were packed in a soxhlet apparatus and extracted using the polarity difference of various organic solvents at 60^0 C, in order by petroleum ether, chloroform, methanol and distilled water.

Phytochemical Screening

Determination of alkaloids

Alkaloids were quantitatively determined according to the method of Harborne [21]. Two hundred ml of 10 % acetic acid in ethanol was added to 5 g powdered fungal sample, covered and allowed to stand for 4 h. The filtrate was then concentrated on a water bath to 1/4 of its original volume. Concentrated ammonium hydroxide was added drop wise to the extract until the precipitation was completed and the whole solution was allowed to settle. The collected precipitates were washed with dilute ammonium hydroxide and then filtered. The residue was dried and weighed. The alkaloid content was determined using the formula:

% Alkaloid= (Final weight of sample / Initial weight of extract) X 100

Determination of tannins

Tannin determination was done according to the method of AOAC [22] with some modifications. One fifth gram (0.20 g) of the sample was added to 20 ml of 50 % methanol. This was shaken thoroughly and placed in a water bath at 80^0 C for 1 h to ensure a uniform mixing. The extract was filtered into a 100 ml volumetric flask, followed by adding 20 ml of distilled water, 2.5 ml of Folin Denis reagent and 10 ml of 17% aq. $Na_2CO_3$was also added and thoroughly mixed together. The mixture was made up to 100 ml with distilled water, then mixed and allowed to stand for 20 min. The bluish green color developed at the end of the reaction mixture of different concentrations ranging from 0-10 ppm. The absorbance of the tannic acid standard solutions as well as sample was measured after color development at 760 nm using the AJI-C03UV-VIS spectrophotometer. Results were expressed as mg/g of tannic acid equivalent.

Determination of saponins contents

The determination of saponins was done following the method of [23]. Five grams of fine powder was dispersed in 50 ml of 20% v/v ethanol prepared in distilled water and the mixture was heated over hot water bath at 55^0 C for 4 h with continuous stirring. The residue collected after filtration was re-extracted with another 50 ml of 20 % ethanol and reduced to 20 ml over hot water bath at boiling temperature. The concentrated solution obtained was shaken vigorously with 10 ml of diethyl ether in a separating funnel; the aqueous layer was collected for purification process and repeated. 20 ml of butanol was added to the filtrate and then washed with 10 ml of 5 % w/v aqueous sodium chloride. The whole mixture was heated to evaporation on hot water bath and later oven dried at 40^0 C to a constant weight. The percentage saponins content of the sample was calculated using the formula.

% Saponins = (Weight of final filtrate/ Weight of sample) X 100

Determination of steroids contents

Steroid content of the plant sample was determined using the method described by [24]. A portion of 2 ml was taken from a solution of 2.5 g of powdered fungal material prepared in 50 ml of distilled water after vigorous shaking for 1 h. The extract solution was washed with 3 ml of 0.1 M NaOH (pH 9) and later mixed with 2 ml of chloroform and 3 ml of ice cold acetic anhydride followed by adding two drops of concentrated H_2SO_4 cautiously. The absorbance of both sample and blank were measured spectrophotometrically at 420 nm.

Determination of total phenolic content

The total phenolic content in the fungal extract was determined by a colorimetric assay method based on Folin and Ciocalteu procedure described by [25]. About 0.5 ml of ethanolic extract was mixed with 2.5 ml Folin and Ciocalteu reagent (diluted 1:10 %) followed by 2 ml of sodium carbonate (7.5% v/v) solution. The absorbance was then measured at 765 nm after incubation at 30° C for 90 min. Estimation of the phenolic compounds was carried out in triplicate. Results were expressed as gallic acid equivalents.

Determination of total flavonoid content

The total flavonoid content was calculated by the method developed by [25]. About 0.5 ml of ethanolic extract was mixed with 2 ml of distilled water and subsequently with 0.15 ml of $NaNO_2$ solution (15 %). After 6 min, 0.15 ml of aluminum chloride solution (10 %) was added and allowed to stand for 6 min. To the resultant solution 2 ml of sodium hydroxide solution (4%) was added. Immediately water was added to bring the final volume to 5 ml. The mixture was thoroughly mixed and allowed to stand for 15 min. Absorbance was determined at 510 nm versus distilled water as blank. Concentration of sample was detected from standard quercetin calibration curve and calculated in mg quercetin equivalent.

Determination of *in vitro* antioxidant activity

DPPH scavenging assay

The method of Shen *et al*. [26] was used for the determination of scavenging activity of DPPH radical in different concentrations of various solvent extracts and Ascorbic acid. DPPH (0.005%) was used as the free radical. Equal volume of different concentrations of various solvent extracts and DPPH were vortexed thoroughly and left in dark at room temperature for 30 min. The absorbance was measured spectrophotometrically at 517 nm. The scavenging ability of the extract on DPPH was calculated using the equation.

DPPH scavenging activity (%) = [(A-B)/A] X 100

Where A is the absorbance of DPPH + methanol; B is the absorbance of DPPH + sample extract or standard.

Reducing power assay

The reducing power of the extract was evaluated by following the methodology of [27]. Various concentrations of different solvent extracts were mixed thoroughly with the mixture of 2.5 ml of 0.2 mM

phosphate buffer (pH 7.4) and 2.5 ml of potassium ferricyanide. The resulting mixture was incubated at 50^0 C for 20 min, followed by the addition of 2.5 ml of trichloroacetic acid (10% w/v) and the mixture was centrifuged at 3000 rpm for 10 min. The upper layer of the solution was collected and mixed with 2.5 ml of distilled water and later with 0.5 ml of ferrous chloride (0.1 % w/v). The absorbance was measured at 700 nm against a blank sample. Increased absorbance of the reaction mixture indicated higher reducing power of the extract.

Metal chelating activity

Metal chelating capacity of extracts was measured according to the method described by [28]. One ml of different concentrations of extract was added to 0.05 ml of 2 mM ferric chloride solution. The reaction was initiated by the addition of 0.2 ml of 5 mM Ferrozine and the mixture was shaken vigorously. After 10 min, the absorbance of the solution was measured at 562 nm against blank. All readings were taken in triplicate and EDTA was used as the standard. The percentage inhibition of ferrozine- Fe^{2+} complex was calculated by following equation.

Metal chelating activity (Inhibition of Ferrozine-Fe^{2+} complex) = $([A_0-A_1)/ A_0] \times 100$

Where A_0 was the absorbance of control and A_1 was the absorbance of extract or standard.

Hydrogen peroxide activity

A solution of hydrogen peroxide (2 mM/l) was prepared in a phosphate buffer (pH 7.4). The hydrogen peroxide concentration was determined spectrophotometrically from absorption at 230 nm with molar absorbtivity of 81M/cm. Each extract (100 µg/ml) was added to the hydrogen peroxide solution (0.6 ml). The absorbance of hydrogen peroxide at 230 nm was determined after 10 min against a blank containing phosphate buffer without hydrogen peroxide [29].

Superoxide radical scavenging activity

The reaction mixture contained Nitroblue tetrazolium (0.1 mM) and Nicotinamide adenine dinucleotide (0.1 mM) with or without sample to be assayed in a total volume of 1 ml of Tris-HCl buffer (0.02 M, pH 8.3). The reaction was started by adding Phenazine methosulphate (10 µM) to the mixture and change in the absorbance was recorded at 560 nm. The percent inhibition was calculated against a control without test sample [30].

Statistical analysis

Antioxidant activities like DPPH radical scavenging activity, reducing power activity, metal chelating activity, hydrogen peroxide activity and superoxide radical scavenging activity were estimated in triplicate determinations. Data were analyzed using Microsoft Excel 2007 and ez Annova version 0.98. Estimates of mean, standard deviation for aforesaid parameters were calculated.

RESULTS AND DISCUSSIONS

Phytochemical Screening

The quantitative determination of phytochemicals of methanol extract of *G.applanatum* were depicted in table 1, it shows that the macrofungi containing a good amount of phenols and flavonoids followed by steroids and tannins, and a very low amount of alkaloids and saponins.

Table1: The phytochemical constituents of methanol extract of wild macro fungi *G.applanatum* (Pers.) Pat.

Sl No.	Phytochemical constituents	Amount of compound (%)
01	Alkaloid	7.52±0.01
02	Tannin	12.82±0.02
03	Saponin	5.91± 0.01
04	Steroids	13.45±0.01
05	Total Phenols (µg/mg)	71± 0.02
06	Total Flavonoids (µg/mg)	45± 0.01

The phytochemical analysis indicated that the macrofungi contains significant amounts of phenolic compounds such as total phenolic and flavonoids. These classes of compounds were responsible for antioxidant and free radical scavenging effect of mushrooms [31-32]. The wild macro fungi *G.applanatum* is rich source of phytoconstituents containing phenols, terpenoids, flavonoids, saponins, steroids, alkaloids and glycosides [33].

DPPH radical scavenging activity

The photometric evaluation of the antioxidant capacity of different extracts of *G.applanatum* from ARF and SWLS regions showed good antioxidant capacity (Figure 1). Significant decrease was observed in the DPPH radical activity due to the scavenging ability of the extracts.

The IC_{50} values of methanol, aqueous (hot water), chloroform and petroleum ether extract of ARF sample was found to be 47.13 µg/ml, 53.76 µg/ml, 94.33 µg/ml and 119.04 µg/ml respectively, and of SWLS sample was found to be 52.44 µg/ml, 60.24 µg/ml, 105.63 µg/ml and 135.13 µg/ml respectively. Among all different solvent extracts, methanol extract showed higher degree of scavenging activity and had slightly higher the IC_{50} value of the standard ascorbic acid (39.89 µg/ml). Compared to SWLS sample, ARF sample showed lower IC_{50} value indicating higher free radical scavenging activity. A lower IC_{50} value indicates a higher free radical scavenging activity.

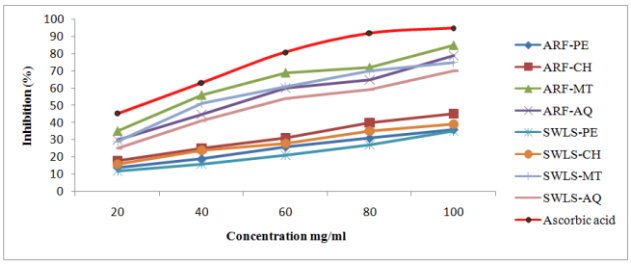

Fig.1: DPPH radical scavenging activities of different solvent extracts of *G.applanatum* from ARF and SWLS regions.

ARF-PE = Petroleum ether extract of *G.applanatum* from ARF region

ARF-CH = Choloroform extract of *G.applanatum* from ARF region

ARF-MT = Methanol extract of *G.applanatum* from ARF region

ARF-AQ = Aqueous extract of *G.applanatum* from ARF region

SWLS- PE = Petroleum ether extract of *G.applanatum* from SWLS region

SWLS-CH = Choloroform extract of *G.applanatum* from SWLS region

SWLS- MT = Methanol extract of *G.applanatum* from SWLS region

SWLS-AQ = Aqueous extract of *G.applanatum* from SWLS region

The DPPH is a free radical and accepts an electron or hydrogen radical to become a stable dimagnetic molecule. The antioxidant present in the extracts reacts with DPPH free radical solution and converts into reduced form either by donating hydrogen atom or transferring electron followed by proton. The efficacies of antioxidants are often associated with their ability to scavenge stable free radicals [34]. This oxidation reaction is visually noticeable as a change in color from purple to yellow which can be measured quantitatively at 517 nm [35]. It has been shown that the scavenging effects on the DPPH radical increase sharply with the increasing concentration of the samples and standards to a certain extent [36] and hence are said to be strongly dependent on the extract concentration.

At 6.4 mg/ml, the methanolic extract from three oyster mushrooms scavenged DPPH radical by 81.8%, whereas scavenging effects of extracts from other commercial mushrooms were 42.9-69.9%. In addition, at 1 mg/ml, methanolic extracts from black and red ear mushrooms scavenged DPPH radical by 96%, whereas those from

snow and ear mushrooms scavenged DPPH radical by 94.5% at 0.4 mg/ml and 95.4% at 3 mg/ml, respectively [37].

Reducing power assay

The reductive ability of sample extract was determined by measuring its ability to transform Fe^{3+} to Fe $^{2+}$. The reducing power was confirmed by the changes of yellow color of the test solution to various shades of green and blue depending on the concentration of the fungal extract. The reducing power of the extract and standard ascorbic acid increased with an increase in concentration. The methanol extract showed significant reductive ability followed by aqueous, chloroform and petroleum ether extract.

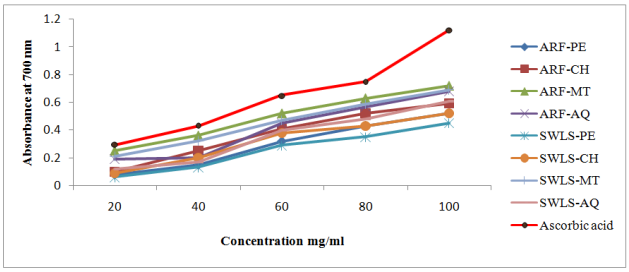

Fig.2: Reducing power of different solvent extracts of *G.applanatum* from ARF and SWLS region.

Ferrous ion, which commonly exists in food systems, is well known as an effective pro-oxidant component. Phenolic compounds can chelate pro-oxidant metal ions, thus preventing free radical formation from these prooxidants species [38].

The reducing power of medicinal mushrooms might be due to their hydrogen-donating ability as described by Shimada *et al.* [39]. Accordingly, medicinal mushrooms might contain a higher amount of reductone, which could react with radicals to stabilize and terminate radical chain reactions. Reducing powers of methanolic extracts from two strains of winter mushrooms were 0.52 and 0.65 at 10 mg/ml, whereas reducing powers of 0.62 and 0.85 were observed with extracts from two strains of shiitake at 10 mg/ ml [40].

Metal chelating activity

The Fe2+ chelating activity of ARF and SWLS sample showed potent chelating power. As observed in DPPH assay, the percentage of metal chelating activity was determined to be sample concentration dependent. The methanol extract showed good metal chelating power followed by chloroform, aqueous and petroleum ether extract in both ARF and SWLS samples. The IC_{50} values of methanol, chloroform, aqueous and petroleum ether extract of ARF sample were found to be 50.84 µg/ml, 54.74 µg/ml, 58.13 µg/ml and 69.12 µg/ml respectively. Whereas the IC_{50} value of standard, EDTA was found to be 44.24 µg/ml, indicating that methanol extract showed good activity compared to standard.

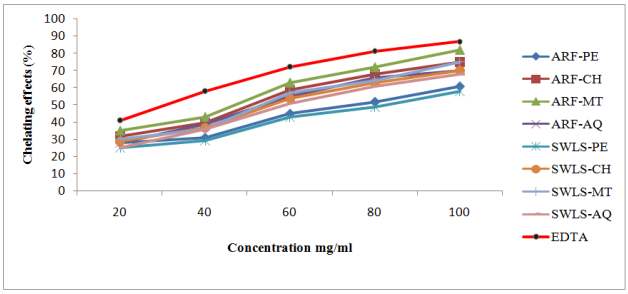

Fig.3: Metal chelating activities of different solvent extracts of *G.applanatum* from ARF and SWLS regions.

Iron is an essential mineral for normal physiological activity of the human body, but excess can cause cellular damage and injury. The ferrous ions are the most effective pro oxidants in food systems, the good chelating effect would be beneficial and removal of free ion from circulation could be a promising approach to prevent oxidative stress induced disease [41]. Ferrous ion reacts with ferrozine forms violet color Ferrozoin-Fe^{2+} complex. Chelating compounds present in the extract prevent the formation of Ferrozoine-Fe^{2+} complex, which leads to decrease in the intensity of violet color [42]. The methanol and chloroform extracts were found to have the remarkable metal chelating activity.

H_2O_2 scavenging activity

The ability of different solvent extracts at various concentrations from 20-100 µg/ml to scavenge H_2O_2 were determined. The Ascorbic acid was used as standard antioxidant and IC_{50} value was found to be 41.20 µg/ml. In this *in vitro* assay also, the methanol extract of both ARF and SWLS sample showed moderate scavenging effect on H_2O_2, having IC_{50} values of 104.89 µg/ml and 112.78 µg/ml respectively. The other extracts were showed very less effect compared to standard and methanol extract.

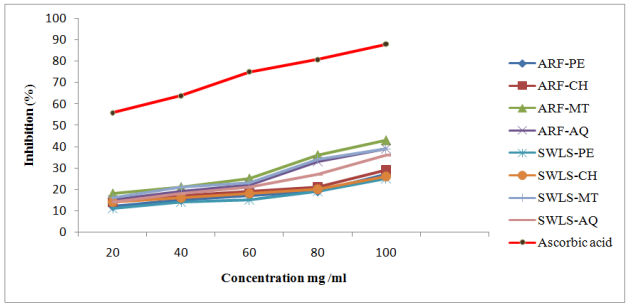

Fig.4: Hydrogen peroxide radical scavenging activities of different solvent extracts of *G.applanatum* from ARF and SWLS regions.

Hydrogen peroxide is formed *in vivo* by enzymatic oxidation processes. It may go through the cellular membrane, having significant oxidation abilities. Although not toxic in itself, its negative role results from the ability to generate hydroxyl radicals at cellular level leading to oxidative damage of DNA [43]. Hydrogen peroxide absorbs the ultraviolet radiation at 230nm. The addition of scavenger containing extract to the H_2O_2 solution caused a fast decrease in the concentration of hydrogen peroxide, Which is monitored at 230nm [44]. The biological value of the methanolic extract from *G.applanatum* comes from the high inhibiting ability of the hydrogen peroxide and was increased on increasing concentration from 20µg/ml, to 100 µg/ml. This conclusion is strengthened by the low value of IC_{50}, 104.89 µg/ml, as compared with that of the ascorbic acid, 41.21 µg/ml.

Super oxide radical scavenging activity

The super oxide radical scavenging activity of both samples were increased with increasing concentration. At a concentration of 100 µg/ml, the IC_{50} value of methanol extracts of ARF and SWLS sample were found to be 111.94 µg/ml and 120 µg/ml respectively. The petroleum ether extract showed very poor super oxide radical scavenging activity in both samples compared to chloroform and aqueous extracts. The IC_{50} value of standard, Ascorbic acid was found to be 38.56 µg/ml.

The IC_{50} values of both ARF and SWLS samples were found to be less for DPPH scavenging activity followed by metal chelating activity, hydrogen peroxide activity and superoxide scavenging activity.

Antioxidant properties of methanolic extracts of three species of medicinal mushrooms in Taiwan- *G.lucidum*, *G.tsuage* and *Coriolus verscicolor* were studied. Results showed that *G.lucidum* and *G.tsuage* were higher in antioxidant activity, reducing power, scavenging activity and chelating abilities was attributed to their total phenolic content. Analysis of proximate composition, total phenols and antioxidant activity of methanolic extracts of three wild edible mushrooms (*Agaricus* sp., *Boletus* sp. and *Macroleptia* sp.) from North of Mexico and two commercial sp. (*Agaricus bisporus* white strain and brown strain) showed that wild mushrooms had higher

phenolic content and antioxidant capacity than the commercial species [50].

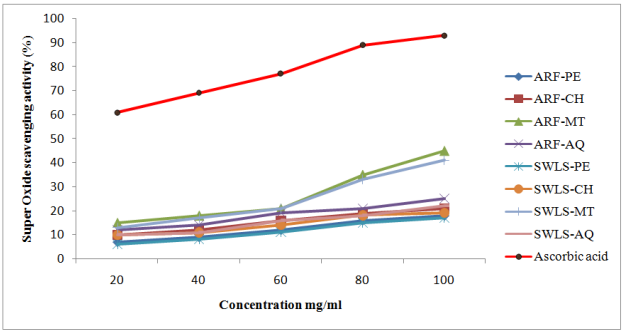

Fig.5: Super oxide radical scavenging activities of different solvent extracts of *G.applanatum* from ARF and SWLS regions.

Superoxide anions are the major ROS generated by spermatozoa [45]. Superoxide anions can be generated either from a membrane-associated NADPH oxidase [46-47] or as a result of electron leakage from mitochondrial electron transport [48]. Superoxide anions are ancestor of active free radicals and may react with biological macromolecules leading to tissue damage [49].

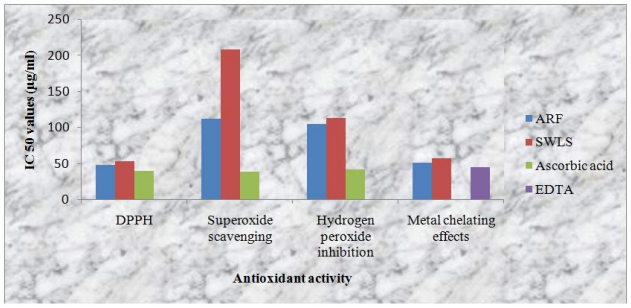

Fig. 6: IC_{50} values of Methanol extract of ARF and SWLS samples.

CONCLUSION

The results from various free radical scavenging systems reveals that the methanolic extract of wild macro fungi *Ganoderma appalantum* has significant antioxidant activity having potential source of natural antioxidants that could be of great importance for the treatment of radical related diseases. This potent antioxidant activity may be attributed to its high phenolic and flavonoid content. Future work on the identification, isolation and structural characterization of the active components will be the goal of our further investigations and these compounds will definitely serve as a good phytotherapeutic agent.

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