Section A-Research paper

ISOLATION, CHARACTERISTIC OF BIOACTIVE COMPOUNDS FROM *AGARICUS CAMPESTRIS* AND ANTIOXIDANT PROPERTIES

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ABSTRACT

Agaricus campestris is a traditional Indian medicinal plant that exhibits biological activities, such as anti-diabetic activity, Antipyretic Activity, and Anti-inflammatory Activity. Not with standing little data is accessible regarding its antioxidant activity. The basis of this analysis is to study the antioxidant activity of a bioassay-guided fractionation and its active compounds/components. High-performance thin-layer chromatography (HPTLC) and preparatory high-performance liquid chromatography (pre-HPLC) were used to isolate compounds and to determine their structures through mass spectrometry (MS), nuclear magnetic resonance (^{1}H &^{13}C NMR) and Fourier transform infrared spectroscopy (FT-IR) spectroscopic analyzes. 3 compounds were isolated from fraction among them. Methoxydiazine (AC-1), afrormosin (AC-2), and candidol (AC-3) were reported from *Agaricuscampestris*. They were showed an excellent antioxidant activity potential at concentrations (100, 200, 300, 400 and 500µg/mL) compared. In conclusion, AEAC may have therapeutic potential for antioxidant effects. AC-1, AC-2, and AC-3 as an effective antioxidant activity.

Keywords: *Agaricus campestris;* by-products; vaporization; extraction techniques; bioactive compounds.

INTRODUCTION

Agaricus campestris (AC), commonly known as field mushroom is a species of fungi in the family Agaricaceae. It is found in fields and grassy areas after rain from late summer onwards worldwide. The cap is white, fine scales and is 3-5 centimeters in diameter [1]. Culinary uses of the meadow mushroom include it eating it sautéed or fried and included in salads. *AC* has history of use in treatment of ulcers, bed sores, slices of AC are applied to scalds, and burns. Aqueous extracts of *AC* enhance the secretion of insulin.

The mushroom derived chemicals were confirmed, such as the polyphenol concentrations of alcoholic extracts from entire AC [2].The reducing and non-reducing sugars are highest in AC [3]. Presence of Glycogen content is marked characteristic of Agaricus species. Qualitative analysis of carbohydrates by paper chromatography

showed the galactose was the main sugar present. Traces of ribose, arabinose, and rhamnose were detected. Moderate concentrations of glucose were present in AC [4].

Material and methods

Plant Materials

AC material was collected from Khammam District, Telangana. The plant specimen was identified by Prof. Rana Kausar, Dept. of Botany, Osmania University, Hyderabad, Telangana State. A specimen was deposited in their herbarium. After thoroughly washing the mushroom to remove any adhering soil or earthy matter, they were sliced into thin chips and dried in the shade at room temperature before being ground to an optimal coarse powder.

General experiment procedure

Bruker Optics (Germany) was used to obtain an infrared spectrum. The spectra ^{1}H NMR (400 MHz) and^{13}C NMR (100 MHz) was also registered on a 400 spectrometer Bruker advance, with $CDCl_3$ as solvent and TMS as internal normal. The same spectrometer was used for NMR experiments. HPTLC conducted 60F254 (Merck, Darmstadt, Germany) on a 5X10 cm plate coated with a 0.25 mm coating of silica gel. By employing a Camag (Muttenz, Switzerland), the samples were applied as 4 mm wide bands and 6mm apart. Thin-layer chromatography (TLC) of silica gel aluminum sheets (Merck Kiesel gel 60F254) was conducted on 0.20mm pre-coated. Spots were visualized with UV light (254nm) and sulphuric acid was sprayed with vanillin. Column chromatography with gravity was performed using Merck silica gel 60 (70–230mesh). HPLC Schimasze LC10 AD (Shimazu, Kyoto, Japanese).

Preparation of *AC* extract, isolation, and characterization of the active constituent

The collected herbs of *AC* were shade dried and fine-grained. AC powder was then extracted thrice at close temperature (50-60°C), with 90% CH_3OH. Throughout the extraction method, the solvent was modified for each 24h. The CH_3OH from the pooled extracts was then removed by distillation under reduced pressure at 50-60^{0}C for creating extracts of *AC* (AEAC). The extracts were subjected to preliminary phytochemical exploration and jointly for biological activity screening tests such as anti-bacterial activity (ABA).90% of CH_3OH extracts were chosen for more isolation. The extracted solutions were suspended in distilled water and then consecutive extracted thrice (2000mL×3) with $CHCl_3$ and EtOAc, CH_3OH, and H_2O extracts severally. All these fractions were screened for antioxidant activity. The potential active fractions were submitted on a silica gel column for more fractionation. These fractions were collected and combined after HPTLC analysis for yielding different fractions. The fractions possessing the potential pharmacological activity were consecutively re-chromatographed on a silica gel column. Such fractions were further accompanied by semi-preparative victimization of the HPLC 90% as a mobile phase CH_3OH solution to get the ultimately distilled compounds. Structural analysis established the purity of the isolated compound and was established by the interpretation of the spectral data (FT-IR, ^{13}C, ^{1}H- NMR, and MS) and further screened for its pharmacological activities [5-7].

Animal model and grouping:

At the age of two months, wistar rats of either sex (M or F) were subjected to an experimental study. Their body weights ranged between 200 and 250 g, and they were divided into six groups of six animals per cage. The animals were kept under standard aseptic laboratory conditions (12-hour light/dark cycle, 24 hours). The food is provided ad

libitum in the form of dry pellets and water. The ethics approval committee of the institute (926/PO/Re/S/06/CPCSEA) approved all of the animals. The study lasted four weeks.

Experimental setup

- Group I: Vehicle treated rats (1 mg/kg b.wt)
- Group II: Control (DS 100mg/kg b.wt, PO)
- Group III: DS (100 mg/kg b.wt, PO) + with simultaneous administration of Silymarin (100 mg/kg b.wt) orally for 28 days through gastric gavages.
- Group IV: Receive DS (100 mg/kg b. wt) + with simultaneous administration of AC-1 (2 mg/kg b.wt) orally for 28 days through gastric gavages.
- Group V: Receive DS (100 mg/kg b. wt) + with simultaneous administration of AC-1 (4 mg/kg b.wt) 28 days through gastric gavages.
- Group VI: Receive DS (100 mg/kg b. wt) + with simultaneous administration of AC-2 (2 mg/kg b.wt) orally for 28 days through gastric gavages.
- Group VII: Receive DS (100 mg/kg b. wt) + with simultaneous administration of AC-2 (4 mg/kg b.wt) 28 days through gastric gavages.
- Group VIII: Receive DS (100 mg/kg b. wt) + with simultaneous administration of AC-3 (2 mg/kg b.wt) orally for 28 days through gastric gavages.
- Group IX: Receive DS (100 mg/kg b. wt) + with simultaneous administration of AC-3 (4 mg/kg b.wt) 28 days through gastric gavages.

Free radical scavenging activity

Ohkawa et al. [8] investigated the DPPH radical scavenging activities of an AC-1, AC-2, AC-3. In brief, 1 mL of freshly prepared DPPH (2, 2-diphenyl-1-picrylhydrazyl) solution at 0.1 mM was mixed with 0.2 mL of each of the sample solutions. After 20 minutes at room temperature, the reaction mixture was vigorously shaken, and the absorbance at 517 nm was measured. A control sample with the same volume but no test compounds or reference antioxidants was prepared, and DMSO (dimethyl sulfoxide) was used as a blank. The positive control in all assays was the reference antioxidant BHT (butylated hydroxyl toluene). The radical scavenging activity was calculated by measuring the decrease in DPPH absorbance:

$$scavenging\ effect(\%) = \left[\frac{A\ control - A\ sample}{A\ control} \times 100\right]$$

Where $A\text{c}_{\text{ontrol}}$ is the absorbance of the control and A_{sample} is the absorbance of the extract or fractions or standard.

Assay of $FeCl_3$ Power

The FRAP assay was determined using the Benzie and Strain technique, with minor modifications [9]. It is determined by the sample's ability to reduce the Fe (III)-TPTZ (ferric tripyridyl triazine) complex to the ferrous tripyridyl triazine Fe (II)-TPTZ at low pH. Fe (II)-TPTZ has an intense blue colour at 575nm. 300 mM acetate buffer (pH 3.6), 10 mM TPTZ (2, 4, 6 tripyridyl S triazine) in 40 mM HCl, and 20 mM ferric chloride solution comprise the stock solutions. After mixing 25 mL of acetate buffer, 2.5 mL of TPTZ, and 2.5 mL of $FeCl_3.6H_2O$, the new working solution was ready, and the temperature was maintained at 35^0 C earlier than usual.In the dark,

different concentrations of AC-1, AC-2, AC-3 and BHT (10-50g/mL) were allowed to react with 2 mL of the FRAP solution for 30 minutes. At 575 nm, the absorbance was measured. The results are expressed in M Fe (II)/g and were calculated using aqueous $FeSO_4.7H_2O$ 20-100 μM as a calibration standard.

The *in-vitro* lipid peroxidation inhibition activity in rat liver homogenate

A 5 percent rat liver homogenate in cold TBS (Thiobarbituric acid) was prepared and 50µl of that was used in the assay. AC-1, AC-2, AC-3 (0.5g) was weighed and homogenized in 1ml of cold TBS. The assay used 50µl of it in aliquots. Ferrous sulphate was added to the assay media at a final concentration of 10µmoles to cause oxidation. Final volumes of up to 500µl were made in test tubes with cold TBS. Tests containing the respective AC-1, AC-2, AC-3 (50µl), liver homogenate (50µl), and TBS in a final volume of 500µl were prepared for each sample. The control tubes did not contain any pro-oxidants. A blank with no fungi extract, no homogeneous liver, but only $FeSO_4$ and TBS was also prepared to yield a final volume of 500µL. With the exception of the AC-1, AC-2, AC-3, an assay medium corresponding to 100 percent oxidant was prepared, and the volume with cold TBS was increased to 500µL. To make up the final volume of 500µL, the auto-oxidation-related experimental medium contained only liver homogenate and TBS. To avoid the reaction, all of the tubes were incubated for 1 hour at 37°C after which 500µL of 70% alcohol was applied to all of them.1 ml of 10% TBA was added to each tube before boiling for 20 minutes in a hot water bath. After the tubes had cooled to room temperature, they were centrifuged. After 500µL of acetone was added to the clear supernatants collected into tubes, the TBARS were measured in a spectrophotometer at 535nm [10].

Reduced glutathione estimation

A 20 percent homogeneous result was obtained by homogenizing 0.5g of sample in 2.5 ml of 5%TCA. To which 0.5 ml of homogeneous tissue was added to precipitate the protein 125µL of 25% TCA. The precipitated protein had been centrifuged at 1000rpm for 10 minutes. The homogenate was cooled on ice, and 0.1mL of supernatant was taken for estimation. For up to 1 mL, the supernatant was buffered with 0.2M sodium phosphate (pH 8.0). After 10 minutes, 2.0 mL of freshly prepared DTNB solution was added to the tubes, and the yellow colour intensity was measured at 412nm in a spectrophotometer [11].

Assessment of Liver Functions

The hepatoprotective effect of the extract was evaluated using liver potential, biochemical parameters for design, Alanine Amino Transferase (ALT), Aspartate Amino Transferase (AST) [21], Alkaline Phosphatase (ALP), Total Serum Bilirubin (SB), Lipid Peroxidation (LPO) as Malondialdehyde (MDA), and Glutathione [12, 13].

Statistical analysis

On each set of data, Bartlett's test was conducted to ensure that the variation in the set was homogeneous. In the case of a homogeneous set of results, an ANOVA was conducted to assess the treatment effects, and using Origin Pro 7.6 statistical software, Dunnett's test was used as applicable. This was transformed using the correct transformation in the case of heterogeneous data. The variance was calculated using a 5% significance level, the values were expressed as mean ± SEM, and the statistically significant $P< 0.05$ was used[14]

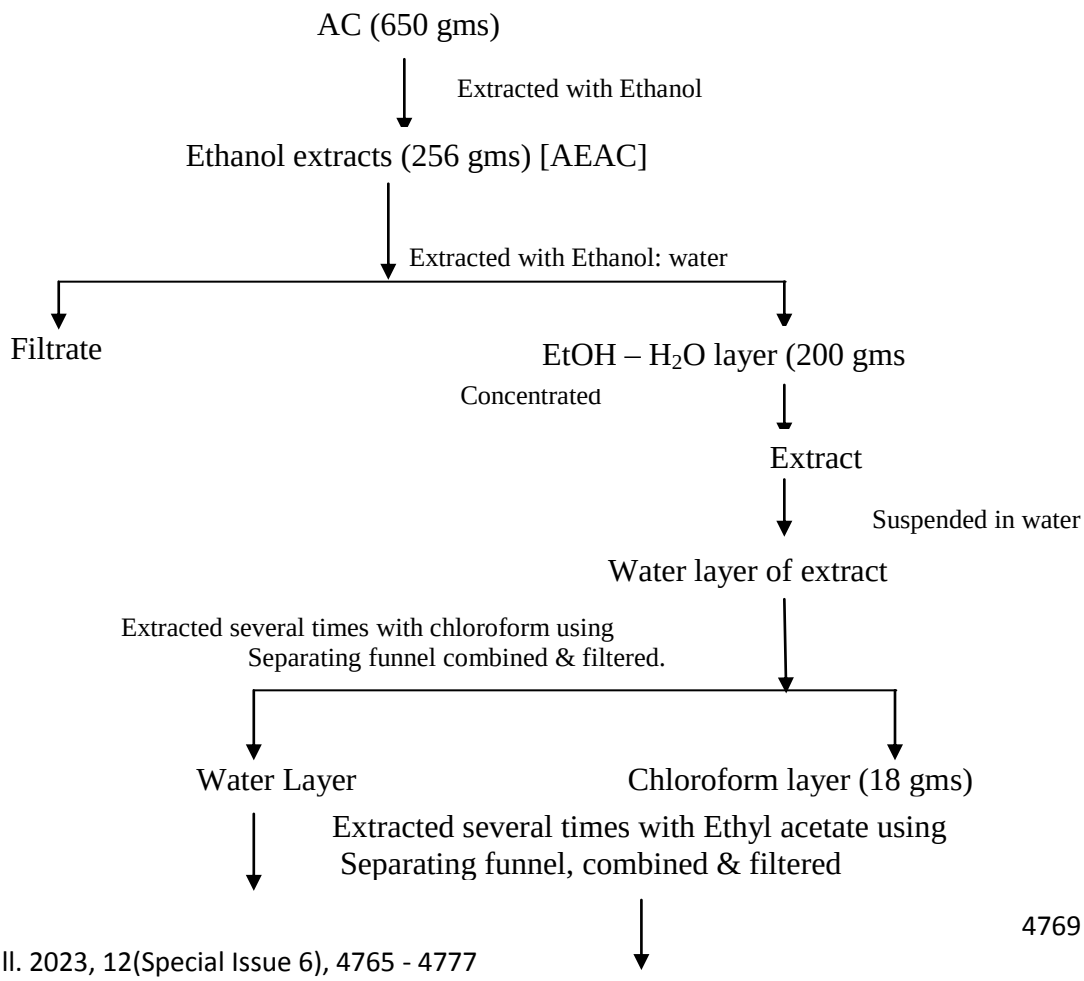
Results and discussion

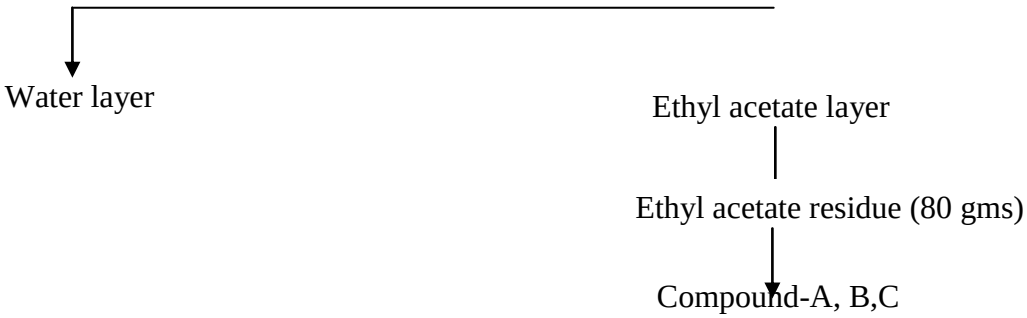
Isolation of *AC*

AC powder (650 g) were collected under shade and dried. The extracts used were prepared by taking 500 ml of EtOH equivalent to two portions using soxhlet apparatus. The filtrate was boiled up to 50-60^{0}C for 4-5hrs, until the concentrated residue was formed (AEAC). Powdered Drug is extracted using EtOH yielding a crude extract (AEAC 256gms). To separate two portions of EtOH from AEAC, one portion used pharmacological screening[15] (56gms), the second portion used chloroform and ethyl acetate extract fractionation (200gmsAEAC).Powdered Drug is extracted with EtOH resulting in a crude extract (200gms) of AEAC that the residue is suspended in water (1000ml) and fractionated with chloroform and ethyl acetate in vacuum, chloroform (18gms) of *AC* and ethyl acetate extract (80gms) of *AC* yields.[16] For preliminary qualitative analysis, all fractions were subjected, and the ethyl acetate fraction showed positive results for flavonoids (Flow chart-1). The fraction of ethyl acetate (25gms AC) was subjected to cover silica gel (60-12 mesh) and was eluted with chloroform: methanol was collected at different ratios by increasing polarity and 68 fractions. All fractions were subjected to TLC analysis and the fractions were combined having the same R_f value. 3 compounds compound-A (AC1), compound-B (AC2), and compound-C (AC3) were finely collected.

The isolated compound by structure was confirmed by interpreting the spectral data (FT-IR, ^{1}H-NMR, and MS) AC1, AC2, and AC3.

Flow chart-1: showing the isolation of chemical compounds from AC





Phytochemical Analysis:

Characterization with TLC and HPTLC:

Using thin layer chromatography and HPTLC analysis, characteristic identification features of all the fungi materials used in this study were established. From fig-1 to 5 the results of the different extracts on HPTLC were as shown. AC-1[Solvent system: Ethyl acetate-Methanol-Water (75:15:10)] R_f 0.58, AC-2 [n-Butanol-n-Propanol-Glacial acetic acid-Water (60:20:15:5)] R_f 0.69, AC-3 [n-Butanol-n-Propanol- Methanol-Water (60:30:5:5)] R_f 0.69 at 254 nm.

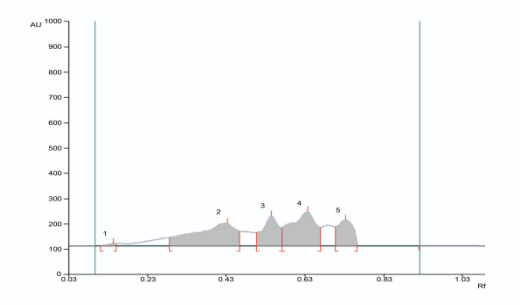


Figure-1: HPTLC fingerprints of solvent-1

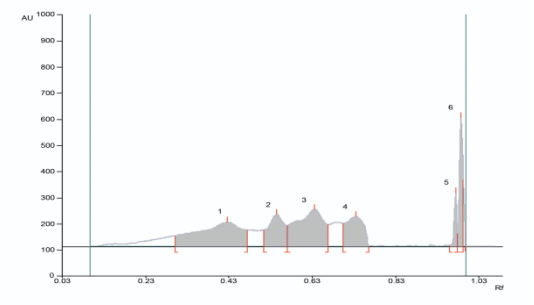


Figure-2: HPTLC fingerprints of AC-1

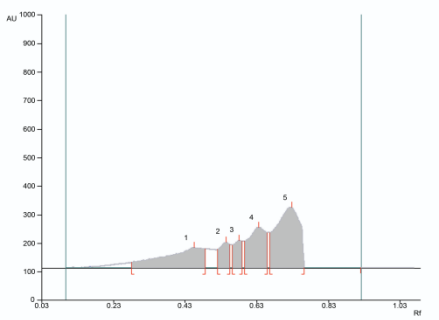


Figure-3: HPTLC fingerprints of AC-2

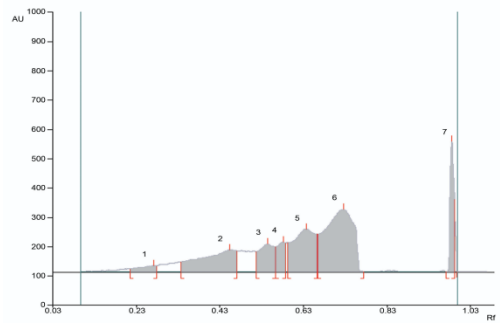


Figure-4: HPTLC fingerprints of AC-3

Figure 1-4: Densitogram of herbal Extract-determined by using CAMAG HPTLC analysis system

Spectral studies of isolated compounds of AC:

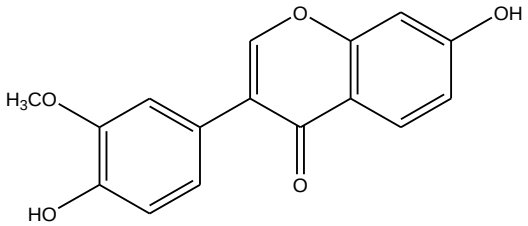
AC-1:

FT-IR: OH Stretch-3422: C=O aryl ketone-1636; C=C -1703; C-C-Stretch 1508, 1451; C-O-C-1276; OCH3-Stretch 1276, 2821.

NMR:δ 7.5,7.9 (4H, d,j7.6,2.0), δ 8.4,7.0(3H, s), δ 5.3,5.4 (2H, s,OH), 3.9 (3H, s,-OCH3).

MS:$C_{16}H_{12}O5$

Exact Mass: 286.08; Mol. Wt.: 286.28 m/e: C, 67.13; H, 4.93; O, 27.94



7-hydroxy-3-(4-hydroxy-3-methoxyphenyl)-4*H*-chromen-4-one

METHOXY DIAZINE

AC-2:

FT-IR: OH Stretch-3443; C=O aryl ketone-1630; C=C -1620; C-C-Stretch 1503,1138; C-O-C-1120,1273; Ar-CH 915, 846; OCH3-Stretch 2923,2657

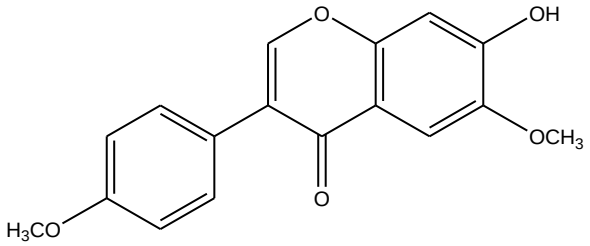
NMR: δ 7.4,6.9 (4H, d,j8.2,1.0), δ 6.8,7.9(3H, s), δ 5.9 (1H, s,OH), 3.84 (6H, s,-OCH3)

MS:$C_{17}H_{14}O_5$

Exact Mass: 300.1

Mol. Wt.: 300.31m/e:298.10(100.0%), 301.10 (18.6%), 302.11 (1.7%), 302.10 (1.0%)

C, 67.99; H, 5.37; O, 26.64



7-hydroxy-6-methoxy-3-(4-methoxyphenyl)-4*H*-chromen-4-one

AFRORMOSIN

AC-3:

FT-IR: OH Stretch-3338; C=O aryl ketone-1630; C=C -1618; C-C-Stretch 1530,1439; C-O-C-1120,1273; Ar-CH 915, 846

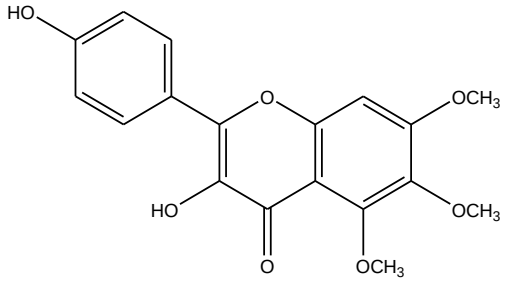
OCH3-Stretch 2923,2657

NMR: δ 7.9,8.4 (4H, d,j0.8,1.0), δ 6.6,5.8(3H, s,OH), δ 5.0 (1H, s), 3.4 (9H, s,-OCH3).

MS:$C_{18}H_{16}O_7$

Exact Mass: 344.11

Mol. Wt.: 344.33 m/e: 346.11 (100.0%), 347.11 (19.9%), 348.11 (3.3%) C, 62.42; H, 5.24; O, 32.34



3-hydroxy-2-(4-hydroxyphenyl)-5,6,7-trimethoxy-4*H*-chromen-4-one

CANDIDOL

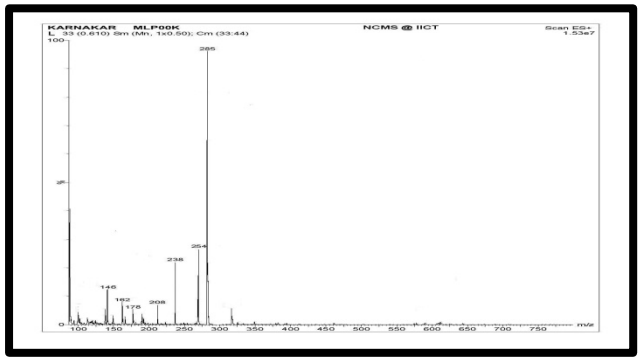


Figure-5: AC-1

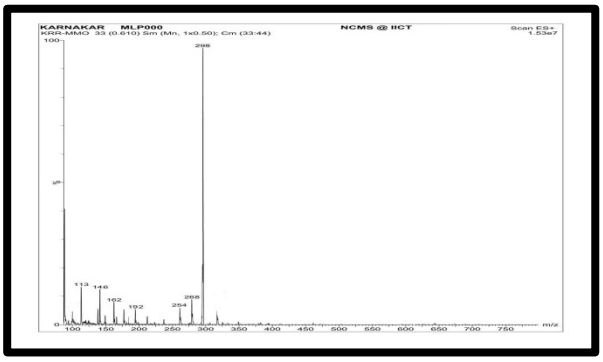


Figure-6: AC-2

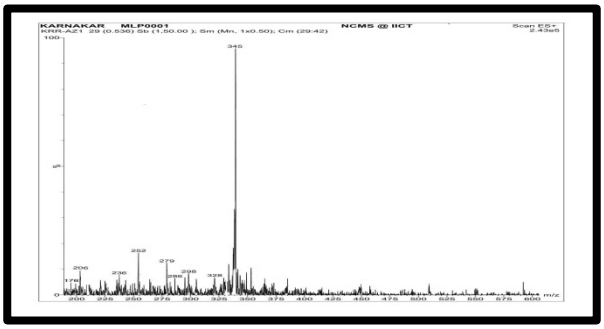


Figure-7:AC-3

Figure-5-8: MASS Spectral of AC-1, AC-2, and AC-3

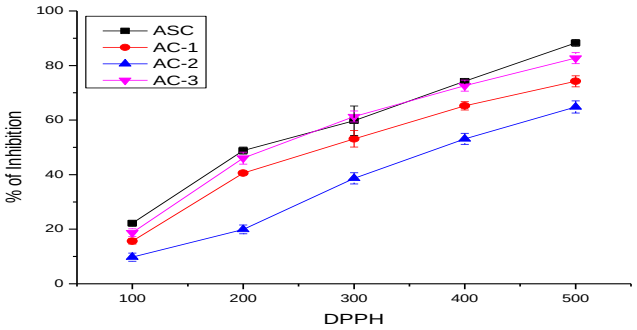
Preliminary phytochemical studies

The phytochemical constituents present in AC extracts were determined using the standard methods. Preliminary phytochemical alkaloids, flavonoids, tannins/phenols, and terpenoid show positive results.

Free radical scavenging activity

DPPH Radical Evaluation

Antioxidants are substances that can convert the stable radical (purple) DPPH to the non-radical form DPPH-H (yellow) and thus act as radical scavengers due to their hydrogen donation capabilities. Figure 8 depicts the DPPH scavenging activity results for all test samples. The scavenging activity of AEAC extract and ascorbic acid (ASC) increased as sample concentration (100-500µg mL^{-1}) increased. The IC_{50} values were determined by AC-1, AC-2, AC-3 and ASC to be 151.77 (Y= 0.283x-6.956), 104.35 (Y= 0.286x-20.082), 143.67 (Y= 0.309x-5.548), and 181.77µg mL^{-1} (Y= 0.2332x+7.61). Based on these findings, the AC-1 has been identified as an effective free-radical inhibitor as well as a primary antioxidant capable of limiting free-radical damage in the body.

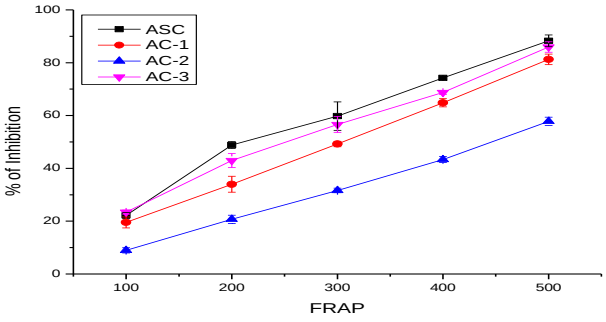


(ASC): Standard antioxidant; data represented as means ± SD (n = 3).

Figure-8: DPPH radical scavenging activity of AC- 1, AC-2, AC-3 and ASC

Antioxidant Power Reduction Assay (FRAP)

The ferric reduction / antioxidant power (FRAP) assay is widely used to assess the antioxidant component of dietary polyphenols. The presence of compounds that act by donating a hydrogen atom to break the free-radical chain[17] is usually associated with the presence of reduction properties. Figure 9 depicts the reduction potential of fungi extracts relative to ASC, a well-known antioxidant data. The IC_{50} values for AC- 1, AC-2, AC-3 extract and ASC were 123.27 (Y= 0.308x-11.958), 142.85 (Y=0.240X-15.628), 149.96 (Y= 0.301X-4.774), and 181.27g mL^{-1} (Y= 0.2346x+7.47), respectively.

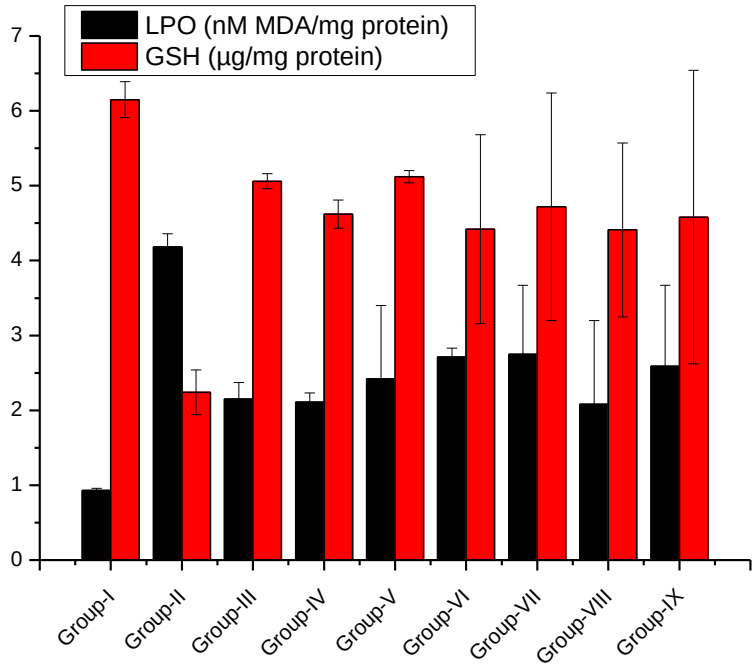


(ASC): Standard antioxidant; data represented as means ± SD (n = 3).

Figure 9: FRAP scavenging activity of AC- 1, AC-2, AC-3 and ASC

The *In-vitro* lipid peroxidation inhibition activity in rat liver homogenate:

There has been a significant increase in MDA substance and a decrease in GSH activities in inebriated animals. Pre-treatment with silymarin (100 mg / kg b.wt) and AC-1, AC-2, and AC-3 (2 and 4 mg / kg b.wt) effectively kept the expansion at MDA levels and transmitted them close to the normal level, while GSH levels[18] were increased overall ($P<0.01$), providing assurance against diclofenac toxicity. Figure -10 shows the results.



*Each value represents the mean ± SEM. n =6 number of animals in each group. [a]P<0.001 vs vehicle control, *P<0.05, **P<0.01, *** P<0.001, Compared to respective PCM treated control groups*

Figure -10: *In-vitro* lipid peroxidation inhibition and GSH activity

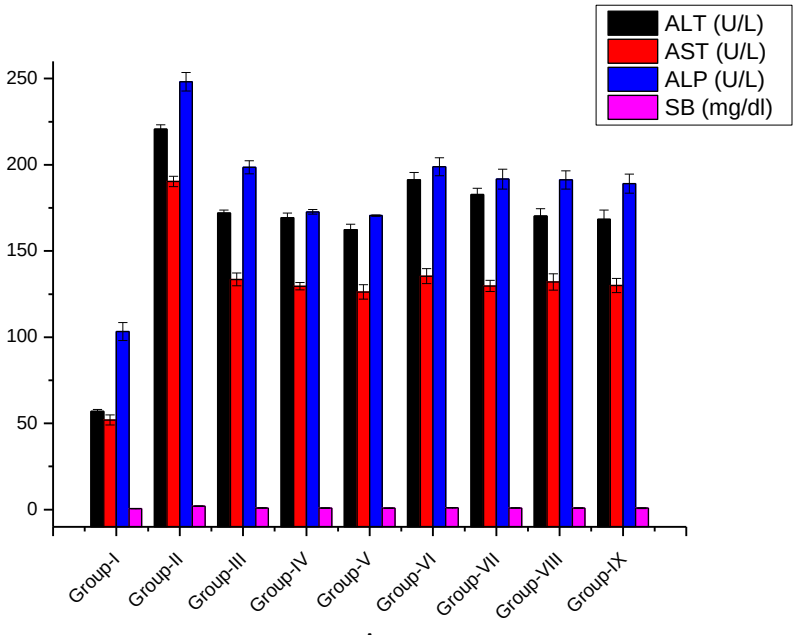
Hepatoprotective activity:

Diclofenac (DS) Induced Liver Toxicity

According to the assurance given by Silymarin (100 mg kg-1b.wt; p.o.), a well-known hepatoprotective specialist, crude alcoholic extraction of *Agaricus campestris* (AEAC) safeguarded the auxiliary uprightness of the hepatocellular film in a subordinate measurement manner as part of the study. DS is known to cause liver damage via cytochrome P_{450}. This metabolite depletes glutathione (GSH), causing cells to pass through. It is obvious that the AEAC concentrate will reduce all elevated levels of AST, ALT, ALP, and total serum bilirubin to normal levels, implying that plasma layer adjustment and hepatotoxin-induced hepatic tissue repair will be affected.

The similar viability of the concentrates tested results in their hepatoprotective movement, as shown in Figure -11.

Percentage of inhibition = 100 X (value of toxic control – value of test sample)/ (value of toxic control – value of control).



*Each value represents the mean ± SEM. n =6 number of animals in each group. [a]P<0.001 vs vehicle control, *P<0.05, **P<0.01, *** P<0.001, Compared to respective DS treated control groups.*

Figure -11: Effect of AEAC on ALT, AST, ALP and SB in DS induced liver toxicity in rats

DISCUSSION

The phytochemical screening revealed that the extracts of AC (white rot fungi) contains a wide range of alkaloids, flavonoids, phenols, steroids and terpenoids. The thin layer chromatography and HPTLC analysis,[19] characteristic identification of the extract done at A_{254} nm and Rf value of AC1, AC2, and AC3 were found to be 0.58, 0.69, 0.66 respectively in their solvent system. Spectral studies like FT-IR, NMR and MS were done for the AC and the following compounds were isolated such as AC-1 (methoxydiazine), AC-2 (Aformosin), and AC-3 (Candidol)

CONCLUSIONS

We isolated bioactive compound viz AC1 (*methoxydiazine)*, AC2 (*Aformosin*), AC3 (*Candidol*) were reported from *Agaricuscampestris*. On the basis of the above findings, it is concluded that the antioxidant activity potential is direct correlated with the content in phenols and flavonoids. They were showed an excellent antioxidant activity potential at concentrations (100, 200, 300, 400 and 500µg/mL) compared. In conclusion, AEAC may have therapeutic potential for antioxidant effects. AC-1, AC-2, and AC-3 as an effective antioxidant activity.

Conflict of Interest: None

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