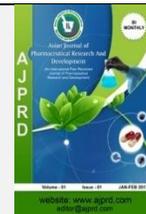


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Research Article

Assessment of *In-Vivo* Antioxidant Potential of Hydro-Alcoholic Extract and Ethyl Acetate Fraction of *Aerva Javanica* Linn. Flowering Tops

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ABSTRACT

Aerva javanica (Amaranthaceae) is a grey coloured woolly perennial tomentose shrub. Its traditional and folklore usage motivates further investigation on its pharmacognostic parameters and pharmacological potential. Hydro-alcoholic extract (AJCE) was prepared from flowering tops of *A. javanica*. In order to work further on activity guided fractions, ethyl acetate (AJEAF) fraction was prepared. Therefore, in order to establish its antioxidant potential, *in-vivo* effect on LPO, GSH, SOD and catalase activity was determined. For comparison, silymarin and *Centella asiatica* extract (CAE) were used as standard antioxidant compound/extract. Lipid peroxidation in term of MDA content expressed as nM/mg, which was 82.18 and 67.39 for AJCE with increasing doses of complete hydro-alcoholic extract (AJCE represented as AJCE-1 and AJCE-2) and 51.65 for AJEAF in contrast to 40.64 nM/mg for standard silymarin and 46.81 nM/mg for standard CAE. GSH content was determined as 3.12, 3.82 and 4.56 µg/mg wet tissue in contrast to 5.59 for standard silymarin and 4.42 for standard CAE. Superoxide scavenging was expressed as SOD U/mg wet tissue, determined as 7.26, 9.16 and 9.91 U/mg wet tissue for AJCE-1 (250 mg/kg *i.p.* b.w), AJCE-2 (500 mg/kg *i.p.* b.w), and AJEAF respectively in comparison to silymarin (10.11) and CAE (46.81 U/mg wet tissue). Catalase activity expressed as µM of H₂O₂ decomposed / min / mg wet tissue was determined as 0.61, 0.72 and 0.78 respectively for AJCE-1 (250 mg/kg *i.p.* b.w), AJCE-2 (500 mg/kg *i.p.* b.w), and AJEAF. Results indicated the SOD values and total antioxidant power of DEE and EAF fractions even better than standard ascorbic acid which expressed the prospective potential of fractions (DEE and EAF) against metabolic disorders.

Keywords: Patharphori, Tris, OECD, TBARS, Malondialdehyde, Silymarin

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INTRODUCTION

Folklore usage of herbs in various ailments motivates research of traditional drugs in modern system. Indigenous medical system is much more explored to develop drugs from plants.¹ Traditional use of *Aerva javanica* flower tops in is the basis of present study. Chopra (1956) reported its traditional use as demulcent, diuretic,

anthelmintic and also in headache. Swellings were reported to be removed by administration of plant decoction.²⁻³ *Aerva javanica* Linn. (Amaranthaceae) also known as 'Patharphori', is a grey colored woolly, perennial, suffruticose, hoary-tomentose, erect to scandent dioecious conspicuous under shrub, 0.6- 1 m tall.⁴⁻⁵ Phyto-chemical standardization and *in-vitro* antioxidant potential of aerva extracts were determined by Swarnkar et al.⁶⁻⁸ The present study was undertaken to evaluate the extract and its fractions

for their involvement in scavenging of oxidative radicals *in-vivo*. In this order, their *in-vivo* effect on LPO, GSH, SOD and catalase activity was evaluated.

MATERIALS AND METHODS

Materials: Absolute ethanol, acetate buffer (pH 3.6) (SD finechem), silymarin (Himedia), *Centella asiatica* extract (Indian herbs), DMSO (Rankem), Malondialdehyde (MDA) (Himedia), methanol, sodium dodecyl sulfate (SDS) (Rankem), sulphuric acid (Rankem), Thio barbituric Acid (TBA), trichloro acetic acid (TCA) (Rankem), Tris-HCl buffer (16 mM, pH 8.0), Ellman's reagent (Himedia).

Collection and extraction: *Aerva javanica* flowering tops were collected from forests of Jhalana in periphery of Jaipur, Rajasthan and authentication was done at "Department of Botany, University of Rajasthan, Jaipur" (Voucher specimen no. RUBL211644) (Authentication certificate Ref. no.: Bot/2017/5424 dated 13/02/2017).

Hydro-alcoholic (50%) extract was prepared from air dried plant materials using maceration method. Fractionation was carried out by first defatting and then with solvents of increasing polarity (dielectric constant). Solvents used for this purpose were petroleum ether, diethyl ether, ethyl acetate, benzene, acetone, and ethanol. Whole hydro-alcoholic extract (AJCE) and ethyl acetate fraction (AJEAF) were further used to assess antioxidant potential.⁹

Selection and preparation of animals and doses: Male wistar rats (180-220 g) were used for the present study. Animals were kept in well ventilated animal house maintained at standard environmental conditions (temperature 25±2°C relative humidity: 55-65% and 12 h light/dark cycle) at Department of Pharmacy, Banasthali Vidyapith, Rajasthan. Animals were provided with standard diet with complete access to water during entire experiment. The present protocol was approved by Institutional animal ethical committee (IAEC) of Banasthali Vidyapith, Rajasthan (Approval no. **BV/3632/2017-2018**). All the animal handling, maintenance and procedures were carried out in accordance to CPCSEA guideline.⁹⁻¹⁰

Toxicity studies: Acute toxicity of AJCE and AJEAF was determined in wistar rats, in accordance to OECD-423 guidelines. Overnight fasted group of rats (n=3) were administered with graded doses (50 -2000 mg/kg, *p.o.*) of extracts respectively. After administration, rats were observed for alteration in behavior of animals, gait abnormality, signs of nervous manifestations, discomfort if any, up to 14 days with special attention during first 4 hours.⁹⁻¹¹

In-vivo antioxidant evaluation

After one week of acclimatization rats were divided into various groups of control (0.5% w/v sodium CMC), treatment and standard and received treatment for 8 days¹³. Silymarin (25mg/Kg body weight *p.o.*) and *Centella asiatica* extract (CAE) (500mg/Kg body weight *p.o.*) were used as standard. *Centella asiatica* being an established herb having antioxidant potential, was selected as herbal standard extract, to compare activity of extracts in study. Extract treatment groups were divided as AJCE-1 (low dose 250mg/Kg body

weight *p.o.*) AJCE-2 (500mg/Kg body weight *p.o.*) and AJEAF (250mg/Kg body weight *p.o.*). All treatment groups received CCl₄ in liquid paraffin (1:2) (1.0 ml/kg i.p.) once in every 72 h along with treatment.

Tissue sample preparation: For lipid peroxidation (LPO), glutathione (GSH) and superoxide dismutase (SOD) assay, after 24 h of the last dose, animals were euthanized and livers were separated for further studies. 1 g of tissue was taken from each liver collected, washed out with normal saline and soaked in tissue papers. Collected liver tissues were then homogenized in 10 ml of 0.15 M tris buffer (pH-7.4) and supernatant was collected by centrifugation at 3000 g at 4°C for 30 min. Supernatants were then used for glutathione, lipid peroxidation and superoxide dismutase assays.

In order to prepare tissue sample for catalase assay, 900 mg of tissue sample was taken from each liver collected from experimental rats, washed in normal saline soaked in tissue paper. The tissues were then homogenized in 3.0 ml M/150 phosphate buffer (pH-7.0) and supernatant was collected after centrifugation at 3000 g at 4° C for 1 hr. The supernatant was used for the assay.

Determination of the Lipid Peroxidation (LPO)

Lipid Peroxidation (LPO) being an autocatalytic process, is a common consequence of cell death. Inflammation, cancer and toxicity of xenobiotics and aging are being caused due to the peroxidative tissue damage resulted by LPO. One of the end product of this lipid peroxidation process is malondialdehyde (MDA), formed during oxidative degeneration. It is a product of free oxygen radicals, and used as a lipid peroxidation indicator. LPO was determined in homogenate by measuring the amounts of malondialdehyde (MDA).¹⁴

1 ml of tissue homogenate was mixed well with 1 ml of normal saline (0.9% w/v) and 2.0 ml of (10% w/v) TCA. It was then centrifuged for 10 min at room temperature at 3000 g to separate proteins. Supernatant was withdrawn and 2 ml of it was added with 0.5ml (1.0% w/v) thiobarbituric acid (TBA) and heated at 95°C for 60 min. Pink colour of MDA was generated. Optical density (OD) of the pink coloured samples was measured at 532 nm using UV spectrophotometer.

LPO activity resulted in lipid peroxides and expressed in terms of nM of MDA/mg wet tissue. The concentration of thiobarbituric acid reactive substances (TBARS) was calculated using the molar extinction coefficient of MDA (1.56×10^5 mol/L/cm) using the formula, $A = \Sigma CL$, where A = absorbance, Σ = molar coefficient, C = concentration, and L = path length. The results were expressed in nmol/mg of protein.¹⁵⁻¹⁶

Determination of Superoxide Dismutase (SOD)

Marklund and Marklund devised the assay method of superoxide dismutase (SOD) activity (17). Tris-EDTA: 49.78 mM Tris in distilled water, 0.0012 mM EDTA in distilled water; pH 8.5; Pyrogallol- 2mM in distilled water. 2.8 ml Tris-EDTA was mixed with of 100µl pyrogallol was taken as blank and taken in the cuvette scanned for 3 min at 420 nm wavelength. Then same process was followed with 2.8 ml

Tris-EDTA mixed with of 100µl pyrogallol and 50µl of liver homogenate. Enzyme activity was defined in terms of equivalents of SOD units. One SOD unit, as expressed by Units/mg protein/min, is the amount of SOD enzyme inhibiting the rate of pyrogallol auto oxidation by 50%.¹³ The enzyme unit can be calculated by using the following equations:

$$\text{Rate (R)} = \frac{(OD_{final} - OD_{initial})}{3 \text{ min}}$$

$$\% \text{ of inhibition} = \frac{(OD_{blank} - R)}{OD_{blank}} \times 100$$

$$\text{Enzyme unit (U)} = \frac{\% \text{inhibition}}{50} \times \text{common dilution factor}$$

where, 50% inhibition \equiv 1 U of enzyme activity

Determination of Catalase activity

Catalase enzyme breaks down the H₂O₂. Measurement of catalase activity is therefore based on this principal. 10 µl of either sample was mixed with 3.0ml of H₂O₂ in phosphate buffer (M/15 phosphate buffer; pH- 7.0). Change in optical density was observed till it changes by 0.05 observed at 240 nm in UV spectrophotometer. Observations were made against blank. For blank an enzyme source was used in hydrogen peroxide (H₂O₂) free phosphate buffer (0.16 ml of hydrogen peroxide is 30% w/v and was diluted by phosphate buffer to 100ml). Enzyme was added and its absorbance was observed at 240nm; Δt was observed till 0.45 OD. If Δt goes greater than 60 seconds, enzyme samples were used with concentration and the process was repeated. Observations were made at every 3 second interval.¹⁸ One unit enzyme activity is the amount of catalase enzyme that at 25°C liberates half the peroxide oxygen in 100 seconds from hydrogen peroxide (H₂O₂) solution of any concentration. CAT activity is expressed in term of moles of hydrogen peroxide per minute as units per mg using following formula:

$$\begin{aligned} \text{Moles of H}_2\text{O}_2 \text{ consumed/min (units/mg)} \\ = \frac{2.3}{\Delta t} * \ln \left(\frac{E_{initial}}{E_{final}} \right) * 1.63 * 10^{-3} \end{aligned}$$

Where, E = optical density of sample at 240nm, Δt = time required for a change in absorbance till 0.45

Determination of reduced Glutathione (GSH)

Activity of glutathione was assayed as reduced glutathione (GSH) as per the method of Ellman¹⁹. Reduction in glutathione was determined spectrophotometrically by estimation of Dithiobis-(2-nitrobenzoic acid) (DTNB) reduced by thiol (-SH) groups, expressed as microgram per milligram (µg/mg) wet tissue. 0.1 ml of tissue sample was taken with 2.4 ml of EDTA (0.02 M) solution. Mixture was kept on ice bath for 10 min. To the mixture, 2 ml of distilled water and 0.5 ml of trichloro acetic acid (TCA) (50 % w/v) were added. This mixture was ice chilled in ice bath for 10-

15 min, then centrifuged for 15 min at 3000 g. To 1 ml of supernatant was withdrawn and 2.0 ml of 0.4M Tris buffer was added to it. Then 0.05 ml of Ellman's reagent (0.01M DTNB in methanol) was added to it and vortexed thoroughly. Mixture was scanned for OD (without much delay i.e. within 2-3 min after the addition of Ellman's reagent) at 412 nm in UV spectrophotometer against a reagent blank.

RESULTS AND DISCUSSIONS

Acute oral toxicity: Acute toxicities of extracts were determined on female wistar rats using OECD guidelines 423.¹¹ Results of acute toxicity revealed that extracts were safe up to 2000 mg/kg and did not cause any signs of toxicity in rats. No mortality and neurological, behavioural and additional toxicity symptoms were observed with any of selected doses.

Based on observations, it was evident that there was no reduction in alertness, spontaneous motor activity, reactivity to sound and touch, body and limb tone. Respiration, urination, pupil size and pineal, corneal and righting reflexes were found normal for all 14 days of study. Abnormal signs pertaining to toxicity such as ataxia, body tremors, convulsions, lacrimation, salivation, diarrhoea, writhing, pilo-erection, sedation, coma, cyanosis etc. were not observed in all groups during experimental tenure of 14 days. Maximum tolerated dose (MTD) of extract was determined as 2000mg/kg body weight.

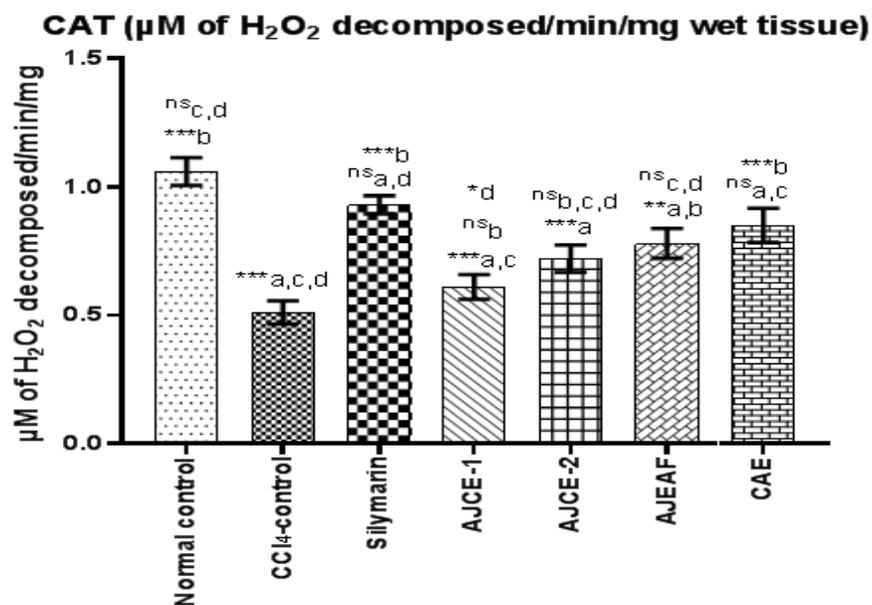
Selection of dose: On the basis of results of acute toxicity studies, two doses i.e. 250 mg/kg body weight and 500 mg/kg body weight (1/8 and 1/4 of MTD) for extract were selected for further pharmacological studies. For fractions only 1/8 of MTD was selected as dose equivalent to 250 mg/kg body weight.

Antioxidant potential of AJCE extract and its ethyl acetate fraction was shown *in-vivo* using male wistar rats. Endogenous enzymes that affect oxidation process in body are lipid peroxidase (LPO), superoxide dismutase (SOD) and catalase (CAT). Level of endogenous enzymes affecting oxidation process was estimated in tissues. Methods used include LPO, SOD and CAT assay. Level of glutathione (GSH) was also estimated. Silymarin (25mg/kg) and *Centella asiatica* extract (CAE- 250 mg/kg) were used as standard. After 8 days of treatment with extracts and standards, estimation of levels of LPO, SOD, CAT and GSH was carried out in homogenized liver tissues. LPO level was determined by estimating level of MDA (Okawa, 1979), SOD level by using method described by Marklund (1974), CAT level by method used by Sumner (1947) and GSH by Ellman's method (1959) using UV-Vis spectrophotometer. All results are expressed as mean \pm S.E.M.

Table 1: *In-vivo* antioxidant assay- effect of extracts on various enzymes and non-enzymatic oxidative systems

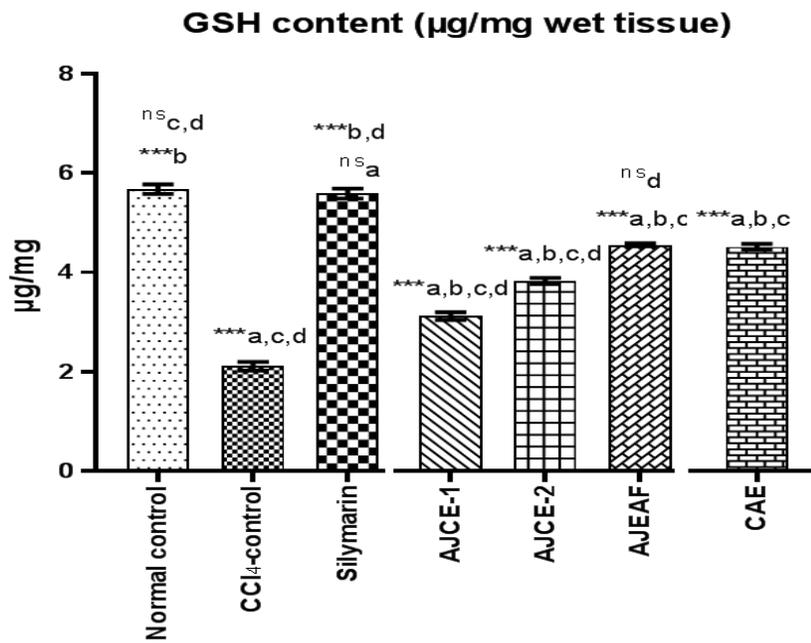
Treatment	Lipid Peroxidation [MDA content] (in nM/mg)	SOD (U/mg wet tissue)	CAT (μM of H_2O_2 decomposed / min / mg wet tissue)	GSH content ($\mu\text{g}/\text{mg}$ wet tissue)
Normal control	21.21 \pm 0.976	10.29 \pm 0.135	1.06 \pm 0.054	5.68 \pm 0.096
CCl ₄ control	100.43 \pm 2.467	6.89 \pm 0.115	0.51 \pm 0.045	2.11 \pm 0.084
Silymarin	40.64 \pm 1.242	10.11 \pm 0.138	0.93 \pm 0.036	5.59 \pm 0.097
AJCE-1	82.18 \pm 1.231	7.26 \pm 0.117	0.61 \pm 0.048	3.12 \pm 0.074
AJCE-2	67.39 \pm 1.786	9.16 \pm 0.154	0.72 \pm 0.053	3.83 \pm 0.059
AJEAF	51.65 \pm 1.289	9.91 \pm 0.143	0.78 \pm 0.058	4.56 \pm 0.031
CAE*	46.81 \pm 1.313	9.87 \pm 0.123	0.85 \pm 0.067	4.52 \pm 0.054

Treatment groups received CCl₄ in liquid paraffin (1:2) (1.0 ml/kg i.p.) once in every 72 h along with treatment. Dosing was done daily for 8 days.



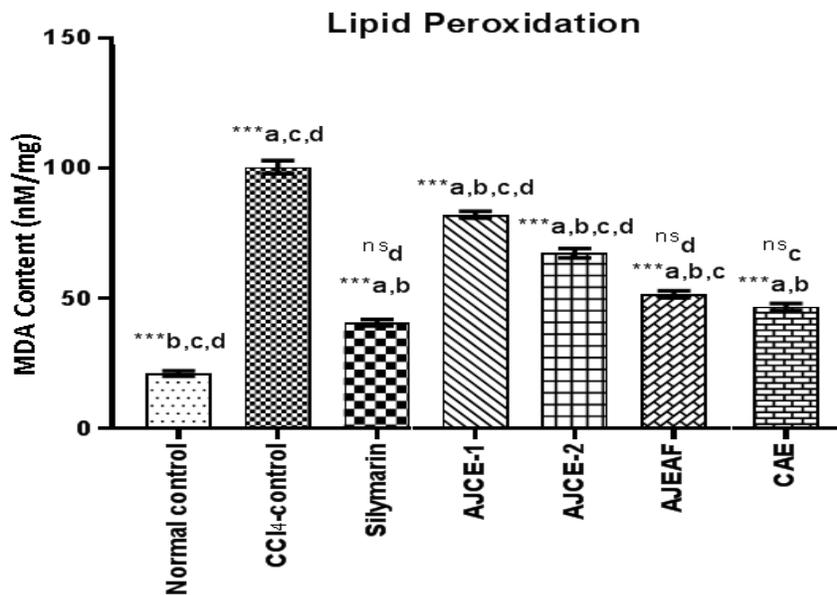
Results analyzed statistically using 1-way ANOVA followed by post hoc Tukey's multi-comparison test. Results compared with control (a), CCl₄ control, standard silymarin (c) and CAE (d) herbal standard with all groups. Significance level represented as * (p<0.05), ** (p<0.01), *** (p<0.001), ns- no significant different.

Figure1: *In-vivo* antioxidant activity- effect on Catalase activity



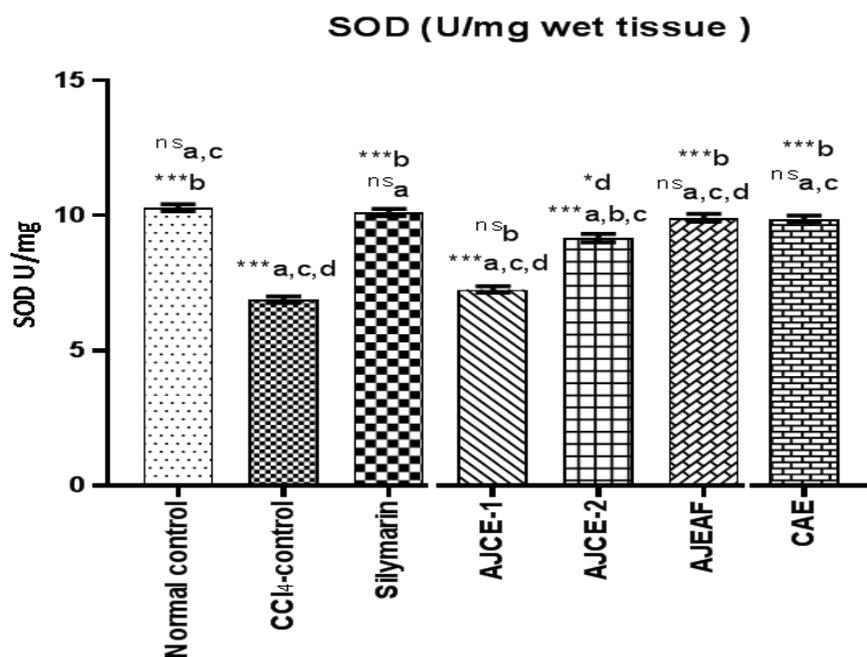
Results analyzed statistically using 1-way ANOVA followed by post hoc Tukey’s multi-comparison test. Results compared with control (a), CCl₄ control, standard silymarin (c) and CAE (d) herbal standard with all groups. Significance level represented as * (p<0.05), ** (p<0.01), *** (p,0.001), ns- no significant different.

Figure 2: *In-vivo* antioxidant activity- effect on GSH activity



Results analyzed statistically using 1-way ANOVA followed by post hoc Tukey’s multi-comparison test. Results compared with control (a), CCl₄ control, standard silymarin (c) and CAE (d) herbal standard with all groups. Significance level represented as * (p<0.05), ** (p<0.01), *** (p,0.001), ns- no significant different.

Figure 3: *In-vivo* antioxidant activity- effect on lipid peroxidation activity



Results analyzed statistically using 1-way ANOVA followed by post hoc Tukey's multi-comparison test. Results compared with control (a), CCl₄ control, standard silymarin (c) and CAE (d) herbal standard with all groups. Significance level represented as * (p<0.05), ** (p<0.01), *** (p,0.001), ns- no significant different.

Figure 4: *In-vivo* antioxidant activity- effect on Superoxide dismutase (SOD) activity

On comparing with control, an improvement in catalase level was seen with AJCE (0.61 and 0.72 μ M of H₂O₂ decomposed / min / mg wet tissue respectively with increasing doses) and AJEAF (0.78 μ M of H₂O₂ decomposed / min / mg wet tissue) including standards. Low dose AJCE (250 mg/kg b.w.) could not elevate the CAT to a significant level. Overall trend show an increase in CAT level with increase in dose. AJEAF showed better results than mother extract in any dose.

An impressive improvement in glutathione peroxidase (GSH) level was seen with mother extracts and fraction, though AJEAF was the best performing one with 4.56 μ g/mg wet tissue GSH level when compared with CCl₄ intoxicated control. Same order was followed in elevation of GSH level when compared with standards- silymarin and CAE. Dose dependant elevation in GSH level was seen with extract.

LPO level was determined by determining MDA level in tissues. An increase in LPO level is indication of increased oxidation. Therefore, decrease in LPO level in terms of MDA is an indication of reduced oxidative stress. In the present study, there was a significant increase in MDA level in CCl₄ intoxicated control group. AJCE mother extract and its fraction AJEAF showed dose dependant decline in MDA level with best performing fraction AJEAF with 51.65 nM/mg MDA level. Similar results were obtained when compared with standards- silymarin (40.64 nM/mg) and CAE (46.81 nM/mg).

Similar results were obtained with AJ complete hydroalcoholic extract (7.26 and 9.16 U/mg wet tissue with increasing doses) and fraction- AJEAF (9.91U/mg wet tissue) with significant improvement in SOD levels when compared with CCl₄ intoxicated control. A dose dependant

improvement was seen within extracts. Results show same trend when compared with standards- silymarin and CAE.

In general, all extracts were found beneficial in improving CAT, GSH, LPO and SOD levels. Ethyl acetate fraction shown superior activity. Potential of extracts to reduce or inhibit generation of free radical is important, as CCl₄ induces liver lesion by elevating levels of free radical. Therefore a suitable radical scavenger can provide protecting against detrimental effect of CCl₄ by not only scavenging radicals but also by elevating levels of endogenous antioxidant mechanisms.²⁰⁻²¹

An increase in level of lipid peroxidation is a measure of structural functions due to membrane damage or alterations. MDA, being one of the end products of lipid peroxidation in liver tissue, is used as marker as its level was found high in CCl₄ control group. Increased MDA level implies to failure of antioxidant defense mechanisms against free radicals leading to tissue damage.²²⁻²³ Treatment with extracts and Silymarin significantly reversed these changes.

Our body has an effective defense system against free radical induced damage. It consists of a set of endogenous antioxidant enzymes, two of the key components of which is catalase (CAT) Superoxide dismutase (SOD). Regarding non-enzymic antioxidants, reduced glutathione (GSH) is a critical determinant of tissue susceptibility to oxidative damage.²⁴⁻²⁵ It is an intracellular reductant which is extensively found in cells. It protects cells against electrophilic attacks by xenobiotics such as free radicals peroxides. In the present study it is observed that CCl₄ depletes GSH concentration in the rat livers. Extracts and Silymarin treatment reverses this effect, which may be due to de novo GSH synthesis or GSH regeneration.

Better activity of ethyl acetate fraction of hydro-alcoholic extract might be attributed to the presence of high levels of polyphenolic components, as depicted by the phytochemical investigation done in pharmacognostic evaluation section of the present study.²⁶

CONCLUSION

From the results of present study, this can be concluded that extracts in study possess powerful antioxidants which are more firmly distributed in diethyl ether and ethyl acetate fractions. The possibility of counteracting oxidative stress by a pool of proper antioxidants plus an appropriate diet, mainly in patients whose blood antioxidant deficiencies can be easily

rebalanced, may have real health benefit and represent a promising way of inhibiting the progression of disease.

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Declaration of Interest Statement

The authors report no declarations of interest.

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