IN VITRO EVALUATION OF TOTAL PHENOLICS AND ANTIOXIDANT ACTIVITIES OF *WITHANIA SOMNIFERA, ECLIPTA PROSTRATA* L AND *GOSSYPIUM HERBASCEUM* L

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ABSTRACT

Traditional medicinal plant, *Withania somnifera, Eclipta prostrata* and *Gossypium herbasceum* were analyzed for reducing power ability as an antioxidant using the 1,1-diphenyl-2-picrylhydrazyl (DPPH) assay and for total phenolics using the Folin-Ciocalteu method. The results of the analysis show that *W. somnifera, E. prostrate* and *G. herbascium* have percentage antioxidant activity (AA%) of 98.12±0.50, 96.45±0.45 and 90.62±0.35 respectively. *W. somniferam* and *E. prostrate* show higher while *Gossypium herbasceum* less activity than those of Gallic (92.92±0.55) and Ascorbic acids (94.81±0.56) used as standards. *W. somnifera, E. prostrata* and *G. herbascium* also show high reductive potential of 0.99, 0.94 and 0.90nm at the same concentration with Gallic acid whose reductive potential was 0.93nm. The total phenolic determination shows that *W. somnifera, E. prostrate* and *G. herbascium* have the phenolic content of 14, 12 and 11mg/g GAE respectively. The results of this analysis reveal the fact that plants are rich sources of natural antioxidant.

Key words: Antioxidant activity, DPPH, Folin-Ciocalteu, medicinal plants, *W. somnifera, E. prostrate* and *G. herbasceum*.

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INTRODUCTION

Antioxidants prevent the damage done to cells by free radicals-molecules that are released during the normal metabolic process of oxidation such as reactive oxygen free radicals species (ROS), reactive hydroxyl radicals (OH), the superoxide anion radical (O_2), hydrogen peroxides (H_2O_2) and peroxyl (ROO). Free radicals generate metabolic products that attack lipids in cell membranes or DNA. These are associated with several types of biological damage; DNA damage, carcinogenesis and cellular degeneration related to aging and also contribute to diseases like heart disease and arthritis (Ozcan et al., 2009; Habila et al., 2010). Antioxidants protect unsaturated fats in the body from oxidation by peroxides and other free radicals. Antioxidants that inhibit enzyme-catalyzed oxidation include agents that bind free oxygen (reducing agents), such as Ascorbic acid (vitamin C), and agents that inactivate the enzymes, such as citric acid and sulfites (Habila et al., 2010). The phytochemical evaluations of plants which have a suitable history of use in folklore have often resulted in the isolation of principles with

remarkable bio-activities (Afolabi et al., 2007). Studies around the world have identified many new plant constituents with antioxidant activity, among these are the polyphenols (Kahkonen et al., 1999; Habila et al., 2010).

The antioxidant activity of polyphenols has been reported to be mainly due to their redox properties, which can play an important role in neutralizing free radical and quenching oxygen or decomposing peroxides. Phenolic compounds from medicinal herbs and dietary plants include phenolic acids, flavonoids, tannins, stilbenes, curcuminoids, coumarins, lignans, quinones, and others (Huang et al., 2010). Polyphenols of plant origin like catechins exert anticarcinogenic, antimutagenic and cardioprotective effects, which is attributed to their free radical scavenging activity (Habila et al., 2010). It is an established fact that polyphenolic compounds possess remarkable antioxidant activities which are present quite commonly in the plant (Wolfe et al., 2008; Nahak and Sahu, 2010).

Nowadays, some toxicological doubts have been casted on synthetic antioxidants due to their adverse side effects and people are more concerned about food safety and quality. Thus, attention is now increasingly paid to the development and utilization of more effective and non-toxic antioxidants of natural origin (Kusirisin et al., 2009; Servili et al., 2009). In the recent years, food scientists and nutrition specialists agree that antioxidant enriched fruits and vegetables, consumed daily contribute to reducing risks of certain diseases including cancer and cardio and cerebro-vascular diseases (Liu et al., 2000; Guorong et al., 2009). They can scavenge radicals by inhibiting initiation and breaking chain propagation or suppressing formation of free radicals by binding to the metal ions, reducing hydrogen peroxide and quenching superoxide and singlet oxygen. So they are supposed to play an important role in the prevention of these diseases (Walia et al., 2010).

Eclipta prostrata L (Family-Asteraceae) is a common plant and has great traditional reputation of being used as a medicinal agent abundantly grows throughout subcontinent. The plant is used for several human illnesses like kidney and liver weakness, inflammatory conditions, ophthalmic and digestive disorders. It is also regarded as the best remedy for hair in Ayurvedic medicines and act as haematinic, diuretic and anthelmintic (Kirthikar and Basu, 1998).
The extract of the plant has the ability to act as an antidote for snake venom (Melo et al., 1994). Previous studies on this plant proved its usefulness in modification of immune function, cytological responses, serine proteinase inhibition, lipid lowering and liver function (He et al., 1992; Lans, 2001; Konarev, 2002; Kumari et al., 2006). Thiophenes, triterpenoids, coumestanes and flavonoids have been reported as constituents of Eclipta species (Wagner et al., 1986; Singh and Bhargava, 1992; Yahara et al., 1997). Triterpenoid saponins isolated from this plant have been indicated as antimicrobial, immunosuppressant, anti-guardian and anti-venom potentials (Liu et al., 2000; Pithayanukul et al., 2004; Sawangjaroen et al., 2005). Phytochemically, *E. prostrata* is rich in wadeoloctone, eclalbasaponin, stigmasterol and luteolin-7-glucoside. Wagner et al., (1986) further reported the effectiveness of the 5-lipoxygenase inhibition of wedelolactone isolated from *Eclipta alba* L. and *Wedelia calendulacea* Less in *in-vitro* porcine-leukocytes test system (Arunachalam et al., 2009).

In fact, Eclipta species have been reported to exert diverse biological activity including hepatoprotective, anti-inflammatory, antihemorrhagic, antihyperlipidemic and antihyperglycemic activities (Wagner et al., 1986; Melo et al., 1994; Kumari et al., 2006; Lee et al., 2009)
Withania somnifera (Solanaceae) is popular as a home remedy for several diseases and human requirements (Umadevi, 1996). The chemical composition; pharmacological and therapeutic efficacies of *W. somnifera* have been suggested that it is a rich source of bioactive compound. Root contains several alkaloids; withanolides, a few flavanoids and reducing sugars (Ganzera et al., 2003; Senthilnathan et al., 2006).

Gossypium herbaceum L has been widely used in the production of food and medicine. It is not only a valuable source of vitamins but an excellent pain reliever. Gossypin, an active compound has both analgesic and anti-inflammatory activities. The leaves are a good source of vitamin E. Seed extract has often been used in cooking as a substitute for sesame oil. Cottonseed oil is still used today in cooking oil, salad oil, and shortening. In addition, cottonseed oil can be found in many soap products, as it helps with producing thicker, longer-lasting soap suds. Plant root bark extract, has been used to stimulate irregular

menstrual cycles, ease childbirth by strengthening contractions and induce miscarriages when necessary. It has further been indicated to treat the symptoms of menopause, such as hot flashes. The decoction of root bark is effective in treating urinary disorders and decoction from leaves of the plant is used for treating headaches and fever. In addition, the seed oil and leaves are helpful when applied to snake bites; stings; and skin conditions, like poison ivy and warts (Batur et al., 2008).
The above mentioned data clearly indicating, wide application of *Withania somnifera, Eclipta prostrata* and *Gossypium herbasceum* in traditional medicinal practices, folkloric system. Therefore we planned out to evaluate the total phenol and free radical scavenging activity of these medicinal plant drugs and their aqueous and ethanol extracts.

MATERIALS AND METHODS

Plant material and preparation of extracts

Withenia somnifera root, aerial parts of *Eclipta prostrate* L and seed of *Gossypium herbasceum* were purchased from local herb shops in Bahawalpur-Pakistan and identified by expert taxonomist. The sample were preserved (voucher # WS. 061-12-02-2010, EP. 062-12-02-2010 and GH. 063-12-02-2010) at the herbarium of Pharmacognosy Section, Faculty of Pharmacy and Alternative Medicine, The Islamia University of Bahawalpur-Pakistan, for future reference. Shade-dried aerial parts of above plants were ground to fine powder (75 micron). Aqueous and ethanol extracts (500g/1.0L) were prepared, concentrated in a rotary evaporator (Laborota, Heidolph, Japan) at 37^{o}C and stored at 4^{o}C before their evaluations (Zaman and Rehman, 2010).

DPPH free radical scavenging activity

DPPH (1, 1-diphenyl-2-picrylhydrazyl, Sigma-Aldrich) scavenging activity was determined according to the method described by Mensor et al., (2001). Different concentrations (10, 25, 50, 125 and 250µg/ml in methanol) of the test drugs/extracts and standard; Gallic and Ascorbic acids (Fluka) were prepared separately. DPPH solution (1.0ml, 0.3M) was added to 2.5 ml solution of drugs/extracts and standard after 20min incubation period at room temperature in the dark, the absorbance of the resulting mixture was read at 518 nm. The percentage Antioxidant Activity (AA%) was calculated using the expression below:
AA% = 100 - [(Abs sample/nAbs control) × 100]

The absorbance of the control (nAbs) which was prepared by adding methanol (1.0ml) to the extract solution (2.5ml) without DPPH, while the positive control was prepared by adding 1.0ml of DPPH solutions to 2.5ml of Ascorbic and Gallic acids (Habila et al., 2010).

Determination of reducing power ability

According to the method of Qyaizu, (2006) test sample (1.0 ml, 250 µg/ml) was mixed with 2.3 ml phosphate buffer (0.2M, pH6.6) and 2.5 ml of 1% potassium ferricyanide (K3[Fe(CN)6]) (BDH). The mixture was incubated at 37°C for 20min. 2.5ml Trichloroacetic acid (10%, Merck) was added to the mixture and centrifuged for 10min at 1000rpm, the supernatant (2.5ml) was mixed with 2.5ml of distilled water and 0.5ml of 0.1% $FeCl_3$. After incubation for 10min, the absorbance was read at 700nm.

Determination of total phenolic contents

The total phenolic contents of the test agents were determined by Folin-Ciocalteu method (Yu et al., 2002). To each sample solution (1.0ml) and standard (Gallic acid) solution was added 5.0ml of Folin-Ciocalteu (Sigma-Aldrich) and 1.5ml Sodium carbonate (20% w/v). The mixture was shaken thoroughly and allowed to stand for 2h in the dark at room temperature, after which absorbance was read at 765nm. The phenolic content was calculated as Gallic aid (mg/g) equivalents (Walia et al., 2010).

Statistical analysis

All the experiments were done in triplicates. The values are given as mean ± S.E.

RESULTS AND DISCUSSION

The present study analyzes the reducing power ability as an antioxidant using the 1,1-diphenyl-2-picrylhydrazyl (DPPH) assay and for total phenolics using the Folin-Ciocalteu method of traditional medicinal plants of *Withania somnifera, Eclipta prostrata* and *Gossypium herbasceum*.

Free radical and antioxidant activity

The results of the DPPH radical scavenging activity of crude drug; *W. somnifera* (SW), *E. prostrata* (EP) and *G. herbasceum* (GH) show very high percentages antioxidant activity; 98.12±0.50, 96.45±0.45 and 90.62±0.35 (respectively) at the concentration of 250µg/ml. The aqueous extracts of *W. somnifera* (AWS) and *E. prostrate* (AEP) (99.62±0.47, 97.13±0.42 respectively) while ethanol extract of *G. herbasceum* (EGH) (92.81±0.49) also show very high percentage antioxidant activity (Table 1). Ethanol extract of *W. somnifera* (EWS) exhibits the highest radical scavenging activity followed by its aqueous extract (AWS) *i.e.* 72.40±0.31 at 250µg/ml concentration. In comparison AWS shows less scavenging activity to EWS at all the concentrations (10-250µg/ml) under test. *E. prostrata,* ethanol extract (EEP) shows 97.13±0.42, a highest scavenging activity followed by its aqueous extract (AEP) with 42.17±0.32. Comparatively EEP shows markedly high scavenging activity to AEP at all the concentrations (10-250µg/ml) under test. The result reveals the highest radical scavenging activity of ethanol extract of *G. herbasceum* (EGH) of 92.81±0.49 followed by aqueous extract of *G. herbasceum* (AGH), 40.53±0.16 at the concentration of 250µg/ml. Further EGH exhibits markedly greater values at all the concentrations (10-250µg/ml) in comparison to AGH. In overall comparison of the aqueous extracts, AWS shows maximum radical scavenging activity followed by AEP and minimum activity with AGH. While ethanol extracts show maximum radical scavenging activity with EGH followed by EWS and least with EEP. The comparison of all the crude drugs and their extracts reveals highest antioxidant activity with EWS *i.e.* 99.62±0.47. EWS shows greater antioxidant activity than standard/reference solution of Ascorbic (94.81±0.56) and Gallic (92.92±0.55) acids. WS, EP and EEP also exhibit greater antioxidant activity than standard/reference. Table 1 shows the comparative study of radical scavenging activity between the crude drugs and their extracts with respect to Ascorbic and Gallic acids as standard.
DPPH is a relatively stable Nitrogen centered free radical that easily accepts an electron or hydrogen, it react with suitable reducing agents as a results of which the electrons become paired off and the solution losses color depending on the number of electrons taken up (Blois, 2001). The result shows that SW, EP and GH extracts may have hydrogen donors thus scavenging the free radical DPPH. The results of the reductive potential of the plant extract show that, WS, EP, GH, AWS, AEP, AGH, EWS, EEP and EGH show reductive potential of 0.91, 0.89, 0.77, 0.31, 0.28, 0.26, 0.93, 0.85 and 0.81nm respectively as compared to the Gallic acid used as standard (0.88nm) (Figure 1). The high reductive potentials indicate that the plants have redox properties which allow them to act as reducing agents, hydrogen donors or oxygen quenchers (Rice-Evans et al., 1998).

The result further indicates a significant variation in the yields of SW, EP and GH extracts using aqueous and ethanol solvents. The yield of extracts using water and ethanol in case of SW remain 4.58g and 5.13g respectively. Likewise AEP, EEP, AGH and EGH yield 3.37, 5.71, 3.46 and 5.79g respectively. The variation in yield may be due to the polarity of the solvents *i.e.* water and ethanol.

Ethanol has been proven as effective solvent to extract phenolic compounds (Siddhuraju and Becker, 2003). In the present study, the values of ethanolic extracts remain higher than those of aqueous ones. Among solvents used in this study ethanol has showed the best effectiveness extracting phenolic components. Ethanol is preferred for the extraction of antioxidant compounds mainly because its lowers toxicity (Karadeniz et al., 2005). The data is in-agreement to the findings of Nahak and Sahu, (2010).

Phenol content and antioxidant activity

It has been reported that phenols are responsible for the difference in the antioxidant activity of the plant (Cai et al., 2004). They exhibit antioxidant activity by inactivating lipid free radicals or preventing decomposition of hydroperoxides into free radicals (Pokorny et al., 2001; Pitchaon et al., 2007). Phenolic compounds are considered to be the most important antioxidative components of herbs and other plant materials, and a good correlation between the concentrations of plant phenolic and the total antioxidant capacities has been reported (Ozcan et al., 2009; Nahak and Sahu, 2010).

Table 1. The percentage antioxidant activity of *Withania somnifera, Eclipta prostrate, Gossypium herbasceum* and their extracts

Test solution	**Concentration (µg/ml)**				
Sample/standard	10	25	50	125	250
Ascorbic acid	71.52 ±0.51	81.23 ±0.59	85.02 ±0.50	93.66 ±0.65	94.81 ±0.56
Gallic acid	66.25 ±0.34	75.22 ±0.45	81.81 ±0.46	91.60 ±0.45	92.92 ±0.55
Withania somnifera	82.13 ±0.27	90.78 ±0.28	93.16 ±0.38	94.56 ±0.45	98.12 ±0.50
Aq. Ext. of *W. somnifera*	22.52 ±0.15	50.18 ±0.19	68.76 ±0.25	71.34 ±0.30	72.40 ±0.31
Eth. Ext. of *W. somnifera*	83.51 ±0.25	91.24 ±0.33	94.72 ±0.45	95.39 ±0.45	99.62 ±0.47
Eclipta prostrate	79.43 ±0.17	87.12 ±0.18	90.67 ±0.21	93.34 ±0.23	96.45 ±0.45
Aq. Ext. of *E. prostrate*	29.58 ±0.05	37.45 ±0.19	40.13 ±0.18	41.55 ±0.21	42.17 ±0.32
Eth. Ext. of *E. prostrate*	81.03 ±0.20	90.37 ±0.22	92.08 ±0.27	94.29 ±0.25	97.13 ±0.42
Gossypium herbasceum	76.21 ±0.25	80.07 ±0.50	85.00 ±0.35	89.23 ±0.23	90.62 ±0.35
Aq. Ext. of *G. herbasceum*	26.62 ±0.10	27.86 ±0.19	31.09 ±0.19	32.78 ±0.18	40.53 ±0.16
Eth. Ext. of *G. herbasceum*76.	53 ±0.24	80.34 ±0.21	85.83 ±0.11	91.60 ±0.21	92.81 ±0.49

The total phenolic content varies markedly among SW, EP and GH. The contents of total phenolic compounds in crude drugs and their extracts are presented in Table 2. The results are reported as Gallic acid equivalents (GAE, µg/g). The highest concentration of total phenol is 160µg/g present in the WS. The aqueous and ethanol extracts of WS show 50µg/g and 140µg/g of phenol contents respectively. EP, AEP and EEP exhibit lesser phenol contents *i.e.*120, 40 and 90µg/g respectively than WS and corresponding

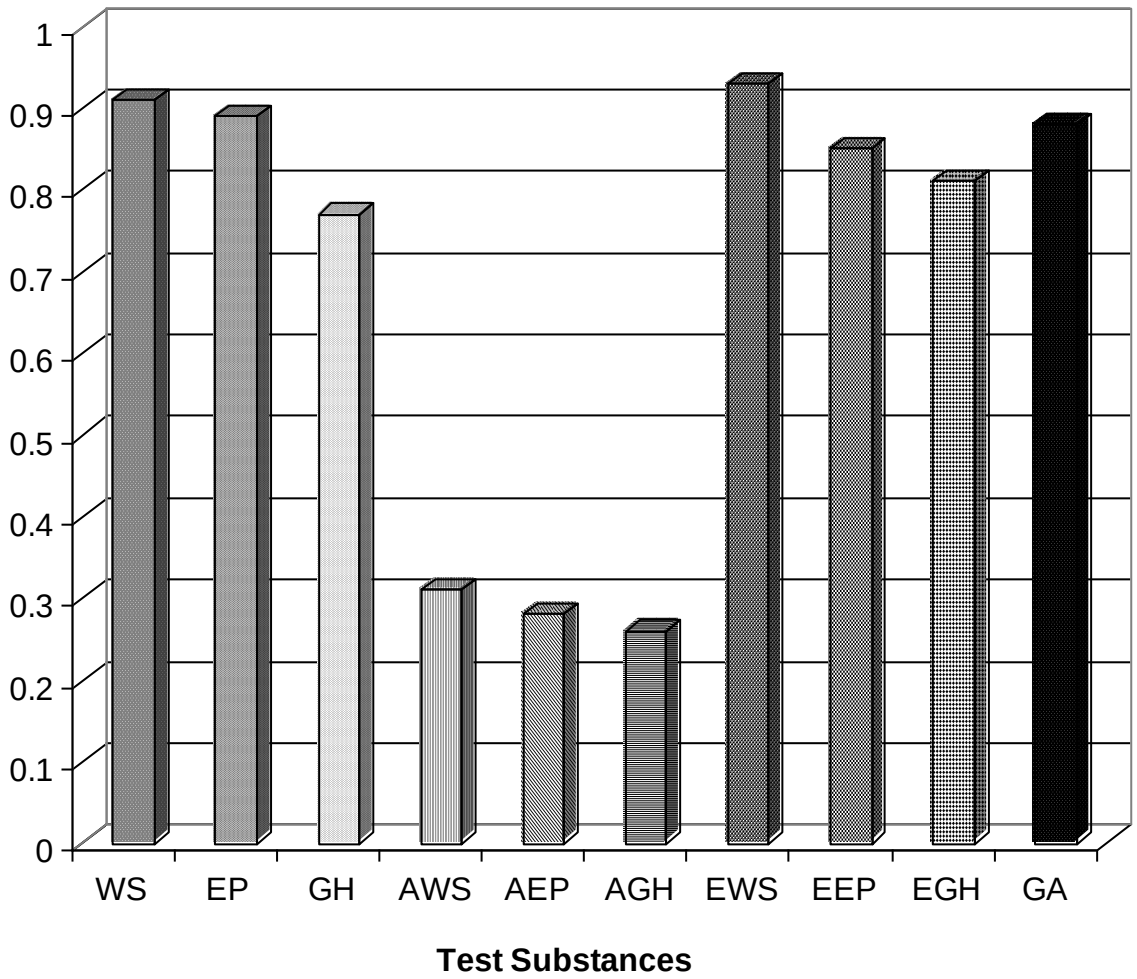
extracts. GH exhibits 110µg/g of phenol contents while its extracts show; AGH, 50µg/g and EGH, 100µg/g phenolic contents.

Table 2. The determination of total phenolics of *Withania somnifera, Eclipta prostrate, Gossypium herbasceum* and their extracts

Sample/Standard	Concentration (mg/g eq. to plant drug)	Mean absorbance
Gallic acid	0.05	0.346 ± 0.06
	0.10	0.614 ± 0.05
	0.15	0.955 ± 0.06
	0.20	1.248 ± 0.08
	0.25	1.407 ± 0.07
W. somnifera	0.16	1.0136 ± 0.09
Aq. Ext. of *W. somnifera*	0.05	0.346 ± 0.05
Eth. Ext. of *W. somnifera*	0.14	0.887 ± 0.08
Eclipta prostrate	0.12	0.740 ± 0.05
Aq. Ext. of *E. prostrate*	0.06	0.400 ± 0.08
Eth. Ext. of *E. prostrate*	0.09	0.560 ± 0.06
Gossypium herbasceum	0.11	0.682 ± 0.06
Aq. Ext. of *G. herbasceum*	0.05	0.346 ± 0.03
Eth. Ext. of *G. herbasceum*	0.10	0.614 ± 0.06

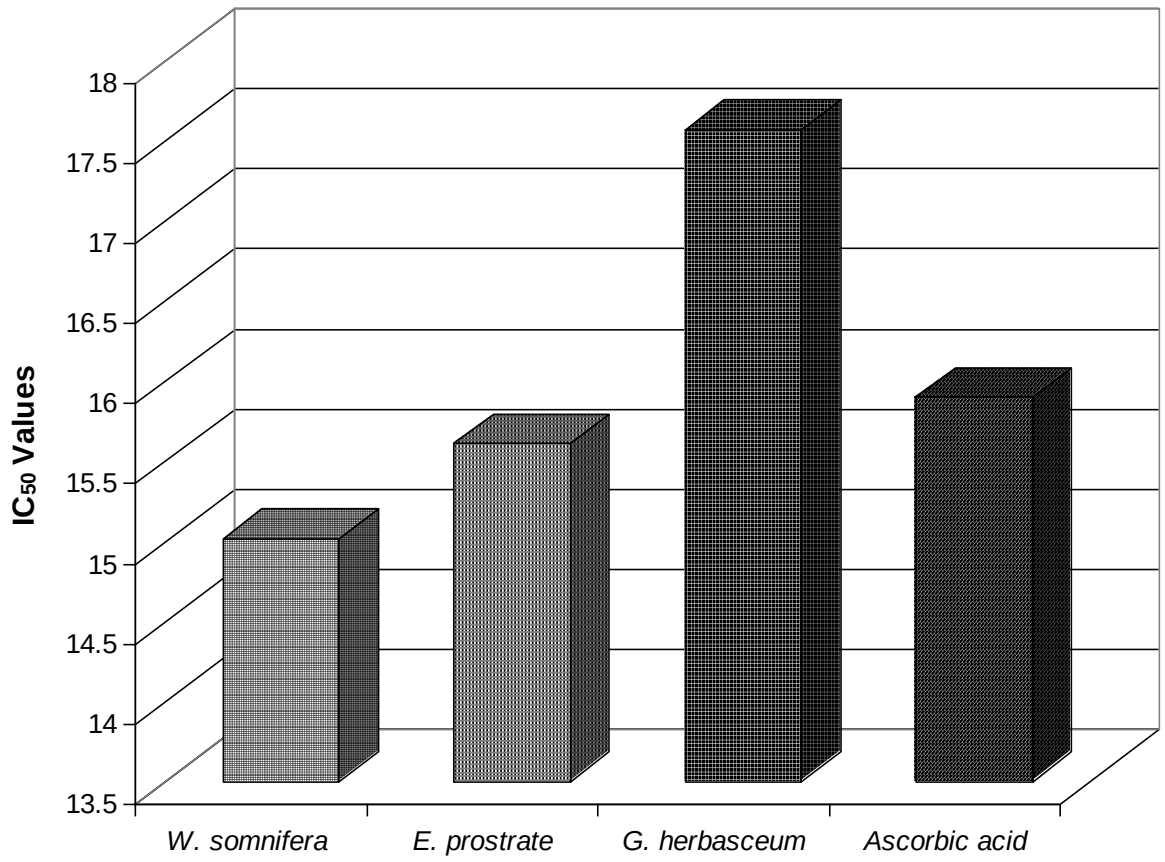
In the present study it is observed that the crude drugs exhibit the higher amount of phenol content in comparison to their respective extracts. The total phenol content in the ethanol extract obtained from WS showed highest amount of phenol content *i.e.* 140µg/g followed by EGH *i.e.* 100µg/g and EEP *i.e.*90µg/g. Similarly the aqueous extract from EP show highest amount of phenol content *i.e.* 60µg/g followed by AWS *i.e.* 50µg/g and AGH *i.e.* 50µg/g. Much higher antioxidant activity of the alcoholic preparation have given evident assumption, is more useful than the aqueous one in medical approach (Pietta et al., 1998). High percent of yield and high value of phenol content in ethanolic extracts show that phenolic constituents must be responsible for such properties (Nahak and Sahu, 2010). The finding is in-agreement with the data of Goncalves et al., (2005); Olabinri et al., (2009); Nahak and Sahu (2010). A positive correlation between total phenolic content and antioxidant activity may be present in experiment. Some studies have demonstrated a correlation between phenolic content and antioxidant activity (Yang et al., 2002). The correlation between total phenolic content and antioxidant capacity in the plant samples is possible owing to the presence of following factors: the antioxidant activity observed in plant extracts may be due to the presence of phenolic compounds or polyphenols or flavonoids or tannins (Wang et al., 2009; Nahak and Sahu, 2010).

Figure 1: Reducing power of *Withania somnifera, Eclipta prostrate, Gossypium herbasceum* and their extracts



WS: *Withania somnifera*, EP: *Eclipta prostrata*, GH: *Gossypium herbasceum*, AWS: Aqueous extract of *Withania somnifera*, AEP: Aqueous extract of *Eclipta prostrata*,
AGH: Aqueous extract of *Gossypium herbasceum*, EWS: Ethanol extract of *Withania somnifera*, EEP: Ethanol extract of *Eclipta prostrate*, EGH: Ethanol extract of *Gossypium herbasceum*, GA: Gallic acid.

Figure 2: IC_{50} values of ethanol extracts of *Withania somnifera, Eclipta prostrate* and *Gossypium herbasceum*



IC_{50} Value

IC_{50} value is defined as the concentration of substrate that causes 50% loss of the DPPH activity and was calculated by linear regression mentioned of plots of the percentage of antiradical activity against the concentration of the tested compounds. Figure 2 shows the reports of IC_{50} values in WS, EP and GH. It shows that there is no IC_{50} value in aqueous extraction of crude drugs. Only ethanolic extract of WS, EP and GH show the IC_{50}. In comparison to extracts, EWS show lower IC_{50} value *i.e.* 15.02 followed by EEP *i.e.* 15.61 and EGH *i.e.* 17.57. A linear relationship between the reciprocal of IC_{50} value and the total polyphenols content of test drug is observed in this study, indicating that increasing the polyphenols content strengths the antioxidant activity. This finding is similar to that reported by Katsube et al., (2004); Nahak and Sahu (2010).

CONCLUSION

The study concludes that *Withania somnifera, Eclipta prostrata and Gossypium herbasceum* have antioxidant potential, corresponding to the amount of total phenolic content of the plant samples. Hence the plants are potential source of natural antioxidant which could be useful in physiological and pathological medicine and of great interest to food manufacturing industries.

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