J. Bangladesh Acad. Sci., Vol. 42, No. 2, 129-136, 2018
DOI: https://doi.org/10.3329/jbas.v42i2.40040

PHENOLIC CONTENT ANALYSIS AND EVALUATION OF ANTINOCICEPTIVE, ANTIOXIDANT, ANTI-INFLAMMATORY POTENTIAL OF *ALTERNANTHERA PUNGENS* KUNTH

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ABSTRACT

The present study evaluated the analgesic, anti-inflammatory and antioxidant activities of *Alternanthera pungens* kunth.The ethanolic extract of *Alternanthera pungens* (EAP) showed analgesic effects by exerting ameliorated reaction time 40.79% and 44.74% at a dose of 250 and 500 mg/kg,respectively, whereas standard was 41.78% by the hot plate test. Results of writhing inhibition were statistically significant ($p < 0.05$) when compared to control. In addition to that, anti-inflammatory effects were also evaluated against denaturation of protein and the result was significant. Scavenging activity of EAP was 80.95% at the concentration of 800µg/mL. Moreover, HPLC-DAD analysis of EAP was also carried out to determine the presence of phenolic constituents & noted the presence of epicatechin,vanillin, *p*-coumaric acid etc. These studies inferred that EAP possesses analgesic, anti-inflammatory and antioxidant potential which might be due to presence of phytochemicals like flavonoids, tannins and phenolic compounds.

Keywords: EAP, analgesic, anti-inflammatory, antioxidant, phenolic compounds.

INTRODUCTION

Medicinal plants play an appealing role in alleviation of several human ailments. Many civilizations and cultures have a confirmed credence on these cures to treat their health related issues. Plants are the rich genesis of the active chemical ingredients such as polyphenols and flavonoids, glycosides, alkaloids, and tannins (Škrovánková *et al.* 2012). Traditional medical practitioners use a wide range of medicinal plants for treatment of various ailments in Bangladesh. Bangladesh is rich in flora and fauna; more than five hundreds potential medicinal plants are present here, having traditional uses (Ghani, 2002). Most often, a desired biological response is not due to the presence of one component, but the presence of a medley of bioactive plant components. Medicinal plants are a rich source of antioxidants, which has the ability to ameliorate oxidative damage in tissues and prevents degenerative diseases such as cardiovascular diseases, cancer, diabetes and aging (Gutteridge and Halliwell, 2010; Ndhlala *et al.* 2010). Despite the progress that has occurred recently in the development of pain therapy, there is still need for effective and potent anodyne, especially for the treatment of chronic pain (Calixto *et al.* 2000). The existing synthetic therapeutic agents are also costly and not easily available to the rural people of the developing country. On the other hand, plant derived preparations are achieving much importance in therapeutic applications owing to cheap in price, easily available and have modicum side effects. In this manner, the use of traditional medicine and medicinal plants in most developing countries is widely observed in the treatment of various diseases, including prevention and management

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of pain, inflammation and other oxidative stress associated diseases such as cancer, atherosclerosis, aging and neurodegenerative ailments (Beg *et al.* 2011; Jain *et al.* 2014). The genus Alternanthera, under the family of Amaranthaceae is a medicinally important plant which contains volatile constituents, essential amino acids, flavonoids, glycosides and steroids (Khatun *et al.* 2012). The common name of *Alternanthera pungens* Kunth is khaki weed which is distributed in Central and South America, some tropical countries including Bangladesh (Wild, 1995). It is a perennial herb and its stem is hairy, 10-50 cm long, prostrate and occasionally develop roots from the nodes. Leaves are green and are generally 0.5 to 4.5 cm long and 0.3 to 2 cm wide (Naidu, 2012). Traditionally, this plant is used as painkiller, for stomachache, swelling and nasopharyngeal infections and also reported for lactation stimulus in veterinary (Burkill, 1985).

However, the scientific validation of EAP for the use in inflammation and pain related disorders have not been reported earlier. The present paper deals with rudimentary phytochemical composition, antioxidant and anti-inflammatory activities of EAP.The EAP was also subjected to HPLC-DAD analysis for determining the presence of phenolic constituents.

MATERIALS AND METHODS

Preparation of extracts

The plant was recognized by its local appellation and garnered from Sitakunda, Chittagong, Bangladesh. The plant sample after authentication was deposited (Accession No. DACB 40178) at the Bangladesh National Herbarium, Mirpur, Dhaka. Whole plants of *A.P* were first powdered, then extracted (72h) with ethanol in a ratio of 1:6. The filtered extract was subjected to evaporation on rotary evaporator (Bibby RE-200, Sterilin Ltd., UK). The crude extracts were collected and preserved at $+4^0$ C for *in vitro* and *in vivo* experiments.

Phytochemical Analysis

Qualitative: The freshly prepared crude extract was qualitatively analyzed for the identification of chemical constituents. The tests were performed by the method described by Harborne and Sazada *et al.* and in each test 10% (w/v) solution of the extract was taken, unless otherwise mentioned in individual test (Evans, 1989). Different tests, including alkaloids by Dragrendroff's reagent, flavonoids by HCl, carbohydrates by Molisch's reagent, terpenoids by Salkowski method and reducing sugar by Fehling's solution were carried out.

Quantitative assay: Total phenolic as well as flavonoid contents were quantified by the standared procedures.

Total Phenolic contents (TPC)

The total phenolic content of of EAP was determined using Folin-ciocalteu reagent with minor modifications (Yu *et al.* 2002). Estimation of TPC was carried out as mg of gallic acid equivalents (GAE) per gram of dry extract/fraction by using gallic acid standard curve, $y = 0.003x + 0.204$; $R^2 = 0.956$.

Total Flavonoid content (TFC)

Total flavonoid was determined using the aluminum chloride calorimetric method described by Wang and Jiao (Wang and Jiao, 2000). The Total content of flavonoid compounds in EAP was determined in quercetin equivalents using the quercetin standard curve, $y = 0.003x + 0.109$; $R^2 = 0.915$.

In vitro Antioxidant assay

DPPH Free Radical Scavenging Assay:

1,1-diphenyl-2-picrylhydrazyl (DPPH) is a reactive free radical and acts as oxidizing agent.

The scavenging of DPPH free radical is indicated as deep violet color being turned into pale yellow or colorless (Braca *et al.* 2001). Radical Scavenging activity was calculated using the following equation :

$$\%\ Inhibition = \left(1 - \frac{Absorbance\ of\ Sample}{Absorbance\ of\ Control}\right) \times 100$$

High Performance Liquid Chromatography (HPLC) DAD analysis

Detection and quantification of selected phenolic compounds in EAP were determined by HPLC-DAD analysis as described by Jahan *et al*. (2014) with some modifications. It was carried out on a Dionex UltiMate 3000 system equipped with a quaternary rapid separation pump (LPG-3400RS) and photodiode array detector (DAD-3000RS).

Evaluation of Analgesic Activity:

Young Swiss-albino mice aged 4-5 weeks, mean weight 25-30 gm was used for the experiment. Experiments in which animal involved were conducted according to the UK Home Office Regulations (UK Animals Scientific Procedures Act 1986) and the 'Principles of Laboratory Animal Care' (National Institutes of Health publication no. 86-23, revised 1985).

Hot plate test method

The hot plate test method was performed to evaluate the analgesic activity in accordance with the method explicated previously with exiguous modification (Mahomed and Ojewole, 2004). The animals were placed on Eddy's hot plate at a temperature of 55±1ºC. After oral administration of the samples, the reaction time was recorded when animals licked their fore or hind paws, or jumped prior to and 0, 30, 60, 120,180 and 240 min. The antinociceptive response latency was recorded.

Acetic acid induced writhing method

The analgesic activity of the EAP was also evaluated using acetic acid induced writhing method in mice (Ahmed *et al.* 2004). After intra-peritoneal administration of acetic acid solution, number of writhing they made were viewed.

In vitro anti-inflammatory activity test against denaturation of protein

The anti-inflammatory activity of EAP was studied by using inhibition of albumin denaturation technique which was studied according to Mizushima *et al*. followed with minor modifications (Mizushima and Kob, 1968). Percentage inhibition of protein denaturation was calculated from the control applying the following equation:

%inhibition = [{Abs of control−Abs of test/Abs of control} X 100

Statistical analysis

The analgesic data are expressed as the mean ± SEM analyzed by one-way analysis of variance (ANOVA) and Dunnett's *t*-test was used as the test of significance. P value<0.05 was considered as the minimum level of significance.

RESULTS

Phytochemical analysis

Phytochemical screening tests ascertained the presence of various bioactive compounds. Most eminent of them are tannins, alkaloid, flavonoids, saponins, gums, terpenoids, phenols and resins. It is shown in Table 1. Total phenolic as well as flavonoid contents were 12.81±0.15

Table 1. Bioactive compments of EAP evaluated by phytochemical screening

Entityin EAP	Tannins	Flavonoids	Saponins	Gum	Steroids	Alkaloids	Terpenoids	Phenol	Resin
Results	+	+	+	+	-	+	+	+	+

(+) indicates presence and (–) indicates the absence of the respective phytochemical.

mg GAE/g extracts and 17.18 ±0.25 mg QE/g extracts respectively.

HPLC-DAD analysis for determination of phenolic compounds

Chromatography segregated phenolic compounds of EAP as shown in Table 2.

Analgesic activity

Hot plate test

EAP has increased reaction time of heat sensation in mice at a dose of 250 and 500 mg/kg with great momentous (Table 3). In the 3rd hr of study, EAP has enhanced the reaction time 40.79 and 44.74% at a dose of 250 and 500 mg/kg respectively whereas standard was 41.78% that is nearest to EAP. The results were found to be statistically significant ($p<0.01$).

Table 2. Contents of polyphenolic compounds in the ethanol extract of *Alternanthera pungens* (n=5)

Polyphenolic Compounds	**Ethanol extract of *A. P.***	
	Content (mg/100 g of dry extract)	% RSD
Epicatechin (ECA)	83.55	1.88
vanillin (VL)	4.14	0.06
p-coumaric acid (PCA)	5.55	0.09
Trans-ferulic acid (FA)	17.36	0.54
ellagic acid (EA)	11.49	0.18
myrecctin (MC)	8.32	0.11

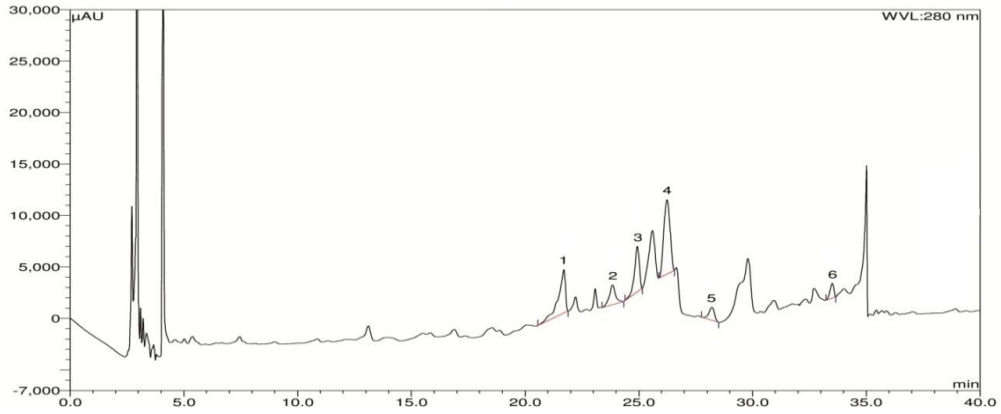

Fig 1. HPLC chromatogram of ethanol extract of *Alternanthera pungens*.Peaks: 1, (–)-epicatechin; 2, vanillin; 3, *p*-coumaric acid; 4, *trans*-ferulic acid; 5, ellagic acid; 6, myricetin.

Experimental compounds in EAP resulted in identifying, including (-) -epicatechin (ECA), vanillin (VL), *p*-coumaric acid (PCA), *trans*-ferulic acid (FA), ellagic acid (EA) and myricetin (MC) and copious amount of (-)-epicatechin (ECA) and *trans*-ferulic acid (FA) (Fig1).

Table 3. Analgesic activity of the ethanol extract of *Alternanthera pungens* (hot plate method)

Treatment Group	Drug reaction time				
	0 hour	**1 hour**	**2 hours**	**3 hours**	**4 hours**
Control	8.04±1.21	11.06±1.83	10.22±1.054	10.86±1.41	9.7±1.35
Standard	12.76±0.8	18.16±0.73 (29.74)	20.96±1.44*** (39.2)	21.92±1.53*** (41.78)	16.06±1.402 (20..54)
***EAP* 250 mg/kg**	7.91±0.67	10.28±.356 23	12.26.48±1.1** 35.23	13.36±1.399*** 40.79	9.78±0.769** 19.12
***EAP* 500 mg/kg**	8.46±1.46	12.02±1.084 29.62	14.20±0.480*** 40.42	15.31±0.237*** 44.74	11.4±1.37 25.79

SEM = Standard error of Mean, n = number of mice. All values are mean ± SEM, n = 5. The minimum value of $p < 0.05$ was considered significant. *$p < 0.05$, **$p < 0.01$, *** $p < 0.001$ as compared with control group.

Acetic acid induced writhing method

The prohibition of licking response in mice shown in Fig. 2. The oral administration of both doses of EAP had momentous ($p<0.001$) attenuation on acetic acid-induced abdominal writhes in mice. The percent prohibition of writhing response showed by the extract was 59.81 and 70.1 at 250 and 500 mg/kg doses, respectively.

In vitro antioxidant assay

DPPH contains an odd number of electrons, responsible for the absorbance at 517nm. The DPPH radical scavenging activity of EAP and ascorbic acid (standard) are represented in Fig 3.

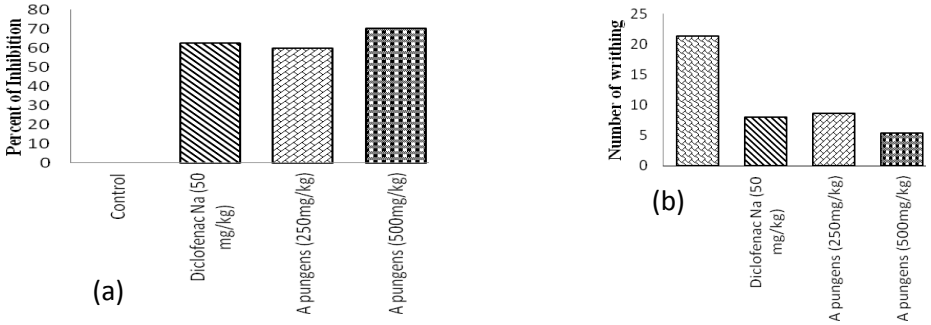

Fig 2. Effect of EAP of acetic acid-induced writhing in mice. (a) Percent inhibition of pain, (b) Number of writhing. Data are represented as the mean ± SEM, (n = 5); **$p<0.001$ considered statistically significant as compared to control.

The scavenging activity of EAP (80.95%) was analogous to that of standard ascorbic acid (92.02%) at the concentration of 800 µg/mL.

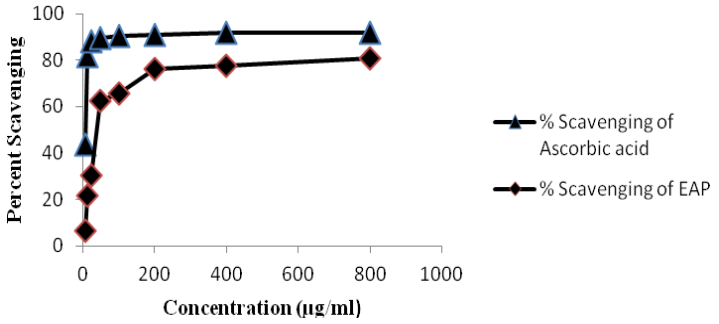

Fig 3. DPPH scavenging activity of EAP and ascorbic acid (standard)

Anti-inflammatory assay

EAP (100-500µg/mL) showed significant inhibition of denaturation of egg albumin in a concentration dependent manner (Fig 4).

The most prominent inhibition of 63.14% at 500 µg/mL concentration of EAP was observed by HPLC-DAD analysis. Earlier investigations suggested that the use of acetic acid induced writhing and hot plate tests for the evaluation of peripheral and centrally acting analgesic drugs respectively (Koster *et al*. 1959).

The anodyne activity was evaluated by writhing

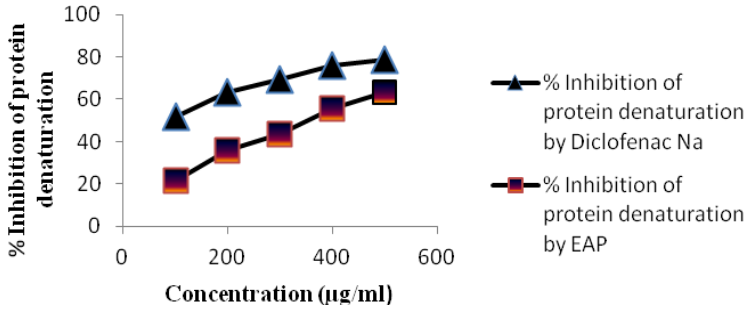

Fig 4. Percent inhibition of protein denaturation by diclofenac sodium and EAP

while standard diclofenac sodium showed inhibition of 78.69% at the same concentration.

DISCUSSION

The study reports the activity of EAP on a discrete experimental animal model of ache and inflammation. Antioxidant properties of EAP were also estimated with the help of DPPH method. EAP inhibited the nociception produced by hot plate and writhing induced by acetic acid with greater momentous. It ascertained the presence of anti-inflammatory phenolic compounds (e.g. epicatechin) in EAP test which has been reported to be useful for investigation of peripheral antinociceptive activity and carried out as a chemical pain model (Abdollahi *et al.* 2003; Golshani *et al.* 2004). The hot plate test was performed as a thermal pain model which is widely known as useful for study of the central mechanism of analgesic activity. Diclofenac sodium increased the pain threshold with greater moments throughout the observation period of 1-3h. The EAP showed a dose dependent, significant antinociceptive activity in both animal models of pain. Acetic acid believed to increase the PGE_2 and $PGF_{2\alpha}$ in

peritoneal fluid (Krasteva *et al.* 2008). However, in two models of evaluation, the EAP showed analogous results with diclofenac sodium. Therefore, the analgesic activity shown in two models of pain indicated centrally and peripherally mediated antinociceptive properties of EAP.

In the current study, the protein denaturation bioassay was chosen for *in vitro* assessment of the anti-inflammatory property of EAP. Denaturation of the tissue proteins is one of the well-documented reason of inflammation and arthritic disorders. Production of auto-antigens in certain arthritic diseases may be due to denaturation of proteins *in vivo (*Opie, 1962; Umapathy *et al.* 2010*)*. It is already proved that conventional NSAID's like Diclofenac sodium not only acts by the inhibition of endogenous prostaglandin production by blocking the COX enzyme but also by prevention of denaturation of proteins. Hence, using agents that can impede protein denaturation would be worthwhile for anti-inflammatory drug development. *In vitro* anti-inflammatory effect of EAP was estimated against denaturation of egg albumin. The results are summarized in Fig 4. The present findings exhibited a concentration-dependent inhibition of protein (albumin) denaturation by EAP as well as diclofenac sodium at the same concentration range of 100-500 µ/mL. So, it can be inferred that EAP has potential anti-inflammatory effects as compared with standard (diclofenac sodium). So, the secondary metabolites like phenolic compounds and tannins, which were found in initial phytochemical screening might be responsible for this activity.

The EAP exhibited significant antioxidant activity. The results suggest that EAP might serve as a potent therapeutic agent for scavenging of free radicals and the maintenance of pathological conditions caused by oxidative stress. The observed antioxidant effect of the plant extract could be assigned to the presence of polyphenolic compounds. The results are in line with the previous studies which indicated the free radical scavenging activity of plant extracts correlated with the phenolic content (Omoruyi *et al.* 2012; Jain *et al*. 2014).

CONCLUSION

In conclusion, EAP displayed analgesic, anti-inflammatory and antioxidant activity and supported the traditional use of this plant in pain relief. Further study is needed to identify the active compounds present in this extract and to elucidate the mechanisms involved in its analgesic properties.

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(Received revised manuscript on 13 August 2018)