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In vitro antioxidative potential of *Artemisia indica* Willd

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ABSTRACT

There is enough evidence, including epidemiological studies indicating the relationship between the plant antioxidants and reduction of chronic diseases in animals and humans. This paper is the first report regarding the evaluation of *in vitro* antioxidative potential of *Artemisia indica* Willd. extracts (AEs), a perennial herb found in western Himalayas and many other parts of world, traditionally utilized as medicine and food for livestock and humans. The dried and powdered aerial parts were Soxhlet extracted with methanol (AME), ethanol (AEE) and 50% hydro-methanol (AHME), to compare the extraction efficacies of these solvents. These respective crude extracts were then subjected to DPPH, H_2O_2, NO scavenging and total antioxidant capacity (TAC) assays. For scavenging assays, various concentrations of each extract in triplicate, were tested with Quercetin as the standard. All AEs exhibited a significant DPPH, H_2O_2 and NO scavenging activity in a concentration-dependent manner. Lower to higher order of the IC_{50} value of all three scavenging assays were AME<AEE<AHME. TAC was expressed as ascorbic acid equivalents (AAE), with AME giving comparatively the highest TAC. Methanol proved to be better solvent system (as compared to ethanol and 50% hydro-methanol) in context to the antioxidative activity of *A. indica*, as AME gave consistently better results. Therefore, choice of extraction solvent is an important criterion regarding selecting a specific biological activity. The *in vitro* antioxidative potential of various AEs as evident by these assays may well be attributed to the presence of various polyphenolic compounds, as we have earlier reported (elsewhere) that *A. indica* possess a good amount of TPC and TFC. In gist, *A. indica* has a significant potential to be developed/used as a natural source of antioxidant not only as a food supplement for animals and humans, but also as an antioxidant additive in packaged food and animal feed industries.

Key words: Antioxidants, *Artemisia indica* Willd, *In vitro* radical scavenging assays, Plant extracts

The associated areas of oxidative stress and antioxidants have been of research interest since ancient times. A reasonable body of knowledge and evidence exists in many traditional and ethno-medicinal systems around the world, notably in Ayurveda and Chinese traditional medicine. Both these topics have been gaining tremendous amount of interest among scientists, health specialists and public at large, due to the exponential increase in environmental pollution, consequential of the population explosion and related industrial and commercial over-activities.

Reactive oxygen species (ROS), formed as a result of normal metabolic activity in organisms and due to exogenous sources as well, are involved in a variety of biological phenomenon and are well recognized for playing a dual role as deleterious and beneficial species (Nose 2000). Free radicals and ROS have been implicated as inducers of a plethora of cellular and tissue pathogenesis, leading to numerous human and animal diseases including cancers, ischemic heart disease, atherosclerosis, Alzheimer's disease, Parkinson's disease, aging, rheumatoid arthritis, skin disorders and drug induced peptic ulcers (El-Sayed *et al.* 1998, Aruoma 2003, Gulcin *et al.* 2004).

Many studies suggested that endogenous antioxidants, or exogenous antioxidants present in diet, can function as free radical scavengers and improve human and animal health (Aruoma 2003). There is enough evidence, including epidemiological studies indicating the relationship between the plant antioxidants and reduction of chronic diseases (Liu 2003). Medicinal plants are an integral part of the diverse traditional medical practices in the Himalayan region and are highly valued both in folk medicine and in codified traditional medical systems, such as Ayurveda and Chinese traditional medicine. In recent times, there has been growing interest in exploiting the biological activities of various Ayurvedic medicinal herbs, owing to their natural origin, cost effectiveness and lesser side effects (Ruwali *et al.* 2015).

A medicinal plant genus *Artemisia* (Asteraceae), named in honour of 'Artemis' the Greek goddess of chastity, popularly known as 'Sage Brush' or 'Worm wood', has been used extensively in folk medicine and as food by many cultures since times immemorial (Ruwali *et al.* 2015). The ethnoveterinary usage of *Artemisia* species are well

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documented since long, throughout world including India, China, Japan, Pakistan, several middle-east and African countries. Considerable literature is available affirming their use in livestock including poultry as anthelmintic, antiprotozoal for blood parasite (both intra- and extra-cellular), anticoccidial, acaricidal, anti-spasmodic, diuretic, cholegogue, against veterinary tumors and sarcomas and as fodder for ruminants (Katsuyama 1994, Lans *et al.* 2007, Mohamed *et al.* 2010, Breuer and Efferth 2014, Joshi *et al.* 2016).

Artemisia is a diverse and economically important genus and it has more than 500 species reported in the world and out of which about 47 species, are found in India (Shah 2014). In India, *Artemisia indica* Willd. (hereinafter *A. indica*) vernacularly known as '*Titepati'*, is a perennial herb found in the western Himalayas and is one of the most utilized locally as a traditional medicinal plant, especially in the Kumaun hills (Uttarakhand, India), though, surprisingly, is also one of the lesser studied with meagre information available on its phytochemical analysis and antioxidative profiles (Ruwali *et al.* 2015). A fair number of reports confirm the use of *A. indica* as a culinary herb and a food plant all over the world, including India, Pakistan, Nepal and Japan. Ethnomedicinally, *A. indica* has been employed by local people to alleviate chronic fever, dyspepsia, hepatobiliary ailments, as well as an anthelmintic, antiseptic, anti-spasmodic, emmenagogue, expectorant and stomachic (Rather *et al.* 2014, Ruwali *et al.* 2015). Regarding phytochemistry of *A. indica*, literature survey revealed that some efforts have been directed towards chemical analysis of essential oils (Chanphen *et al.*1998, Shah and Rawat 2008, Rather *et al.* 2014).

Although, there is a single report on antioxidant activity on the essential oil of *A. indica* (Rashid *et al*. 2013), in spite of sincere efforts, authors could not find any reports on antioxidative activity of *A. indica* extracts and to the best of the knowledge the present communication is the first report of the antioxidative potential of *Artemisia indica* Willd. extracts.

MATERIALS AND METHODS

Plant material: Fresh aerial parts of *Artemisia indica* Willd. (family: Asteraceae) specimens were collected at an altitude of 1560 m, strictly abiding by the standard precautions in June 2012 from the Kumaun hills of Okhalkanda block (latitude 29º39' N and longitude 79º67' E), near Bhimtal, district Nainital, Uttarakhand (India). The plant specimens were authenticated in the Botanical Survey of India (BSI), Northern Circle, Dehradun (Uttarakhand). A voucher specimen (Acc. no. 114879) was deposited at the herbarium of BSI.

Preparation of plant extracts: The extraction of dried and finely powdered plant material was done by Soxhlet extraction (1:10 w/v of extract:solvent) with 3 different solvents, *viz.* methanol, ethanol, and 50% hydromethanol (to compare the extraction efficacies of these solvents) to yield respective crude extracts of *Artemisia indica* (AEs) *viz. Artemisia indica* methanol extract (AME), *Artemisia indica* ethanol extract (AEE) and *Artemisia indica* hydromethanol extract (AHME). Extract preparation in detail is described elsewhere (Ruwali *et al.* 2015).

DPPH free radical scavenging activity: DPPH radical scavenging activity assay of AEs was determined according to the methods of Blois (1958) and Mensor *et al.* (2001). Briefly, 1 ml each of the AEs at variable concentrations (25, 50, 100, 200, 400 µg/ml) was added to 1 ml of DPPH (2, 2-diphenyl-1-picrylhydrazyl) solution (0.2 mM in methanol; as the stable free radical source). The mixture was shaken and kept for 30 min at room temperature. The decrease in absorbance due to each extract was determined at 517 nm. Quercetin was used as the standard. The DPPH scavenging activity was calculated using the fallowing formula:

$$\text{DPPH scavenging activity (\%)} = [(A_c - A_s / A_c) \times 100]$$

Where A_c is the absorbance of the control (DPPH + methanol), and A_s is the absorbance of AEs or standard.

Hydrogen peroxide (H_2O_2) scavenging activity: The ability of the AEs to scavenge H_2O_2 was determined as per Ruch *et al.* (1989) with slight modification. Briefly, a solution of H_2O_2 (40 mM) was prepared in phosphate buffer (50 mM; pH 7.4). Various concentrations (25, 50, 100, 200 µg/ml) of AEs and standard (Quercetin) were prepared in triple glass distilled water (Dw). To 1 ml of each of these extracts/ standard, 0.6 ml H_2O_2 solution was then added, and total volume was made up to 4 ml with Dw. Absorbance at 230 nm was determined 10 min later against a blank solution (phosphate buffer without H_2O_2). The percentage of H_2O_2 scavenging of both AEs and standard were calculated as:

$$\text{\% Scavenged } H_2O_2 = (A_c - A_s)/A_c \times 100$$

Where A_c is the absorbance of the control (Dw + H_2O_2) and A_s is the absorbance in the presence of AEs or standard.

Nitric oxide scavenging activity: Nitric oxide scavenging activity assay was performed as per Marcocci *et al.* (1994). Sodium nitroprusside (5 mM) was prepared in phosphate buffer saline (pH 7.4). To 1 ml of various concentrations (25, 50, 100, 200, 400 µg/ml) of AEs, 0.3 ml of sodium nitroprusside (as above) was added. The test tubes (having the above cocktail) were then incubated at 25ºC for 5 h, after which 0.5 ml of Griess reagent was added. The absorbance of the chromophore was read at 546 nm. Quercetin was used as the standard. The percent NO scavenging activity was calculated as:

$$\text{\% NO scavenging activity} = A_c - A_s / A_c \times 100$$

where, A_c is the absorbance of the control (without extract or standard), and A_s is the absorbance of extract or standard.

Total antioxidant capacity: The total antioxidant capacity (TAC) of the AEs was evaluated by the phosphomolybdenum method as described by Prieto *et al.* (1999) and Aliyu *et al.* (2013). AEs (0.3 ml) was combined

with 3 ml of the reagent solution (0.6 M sulphuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate). The tubes containing the above reaction cocktail were incubated at 95ºC for 90 min. The tubes then were allowed to cool down to the room temperature and the absorbance measured at 695 nm against blank (methanol (0.3 ml) + reagent solution (3 ml). The TAC is expressed as mg of ascorbic acid equivalents (AAE) per gm of dried extract. The calibration curve was prepared by mixing various reducing concentrations of ascorbic acid (1000, 500, 250, 125, 62.5 and 31.25 µg/ml) with methanol.

Statistical analysis: All experiments (for each sample) were performed in triplicate and data were reported as mean ± SD. The data were statistically analyzed by CRD (completely randomized block design) using ANOVA (single factor or two factor) as per the data (Snedecor and Cochran 1989). The critical difference at 5% ($P \leq 0.05$) level of significance for each character was worked out for comparing the significance among the treatment means. The IC_{50} value was obtained by linear regression analysis of dose-response curve plotting between percent inhibition and concentrations of respective extracts.

RESULTS AND DISCUSSION

The antioxidant activity of putative antioxidants has been attributed to various mechanisms, notable among which are prevention of chain initiation, binding of transition metal ion catalysts, decomposition of peroxides, prevention of continued hydrogen abstraction, reductive capacity and radical scavenging (Gulcin *et al.* 2004). A number of techniques and modifications have been proposed to evaluate antioxidant activity and to explain how antioxidants function. Of these, DPPH assay, total antioxidant activity, active oxygen species such as H_2O_2, O_2^- and OH quenching assays are one of the most commonly used for the evaluation of antioxidant activities of extracts (Chang *et al.* 2002, Gulcin *et al.* 2004). Due to the complex reactive repertoire of phytochemicals in general and extracts (owing to the chemical and biophysical nature of solvent system) in particular, it has been recommended that the antioxidant activities of plant extracts and essential oils must be tested by at least two systems to establish authenticity (Rashid *et al.* 2013), and this is more so needed in the instance of reporting initial studies. For this reason, the *in vitro* antioxidant activity of AEs was evaluated by four different spectrophotometric methods *viz.* DPPH free radical scavenging, H_2O_2 scavenging, NO scavenging and total antioxidant capacity assays.

DPPH radical scavenging activity: DPPH assay is considered a valid, accurate, easy and economic method to evaluate radical scavenging activity of antioxidants, since the radical compound is stable and need not be generated (Molyneux 2004). The advantage of this method is that DPPH is allowed to react with the whole sample and sufficient time given in the method allows DPPH to react slowly even with weak antioxidants. DPPH method may be utilized in aqueous and nonpolar organic solvents and can be used to examine both hydrophilic and lipophilic antioxidants (Prior *et al.* 2005).

DPPH free radical scavenging activities of the various AEs are shown in Fig. 1. For each extract, 5 concentrations in triplicate, were tested. All extracts exhibited a promising DPPH scavenging effect in a concentration-dependent manner. AME exhibited considerably higher DPPH radical scavenging activity ($P \leq 0.05$) than other extracts, and the lowest DPPH scavenging activity was found in AHME. The free radical scavenging activities of extracts decreased in the order of AME>AEE>AHME. This trend was in agreement to that observed in the total phenolic content (TPC) and total flavonoid content (TFC) of these extracts reported elsewhere by us (Ruwali *et al.* 2015). The IC_{50} values of these AEs (Table 1) were also calculated to further evaluate the antioxidant activity. Here, IC_{50} means the concentration of antioxidant required for 50% scavenging of DPPH radicals in the specified time. The lower the IC_{50} value, the greater is the free radical scavenging activity. The highest DPPH radical scavenging effect was obtained in AME with the significantly ($P \leq 0.05$) lowest IC_{50} of 83.8±1.2, followed by AEE (363.3±4.61) and AHME (484.9±11.24). However, when compared with the extracts, Quercetin showed higher radical scavenging ability with IC_{50} of 57.14±3.18.

Hydrogen peroxide (H_2O_2) scavenging activity: Hydrogen peroxide is widely regarded as a cytotoxic agent whose levels must be minimized by the action of antioxidant defence enzymes (Halliwell *et al.* 2000). In chemical terms, H_2O_2 is poorly reactive, none the less the danger of H_2O_2 largely comes from its ready conversion to the indiscriminately reactive hydroxyl radical (OH^-), either by exposure to ultraviolet light or by interaction with a range of transition metal ions, of which the most important *in vivo* is probably iron (Ueda *et al.* 1996).

The potential of AEs to scavenge H_2O_2 was determined according to the method of Ruch *et al.* (1989). The scavenging ability of AEs on H_2O_2 is shown in Fig. 2 along with Quercetin as the standard. All AEs were capable of scavenging H_2O_2 in a dose-dependent manner. AME

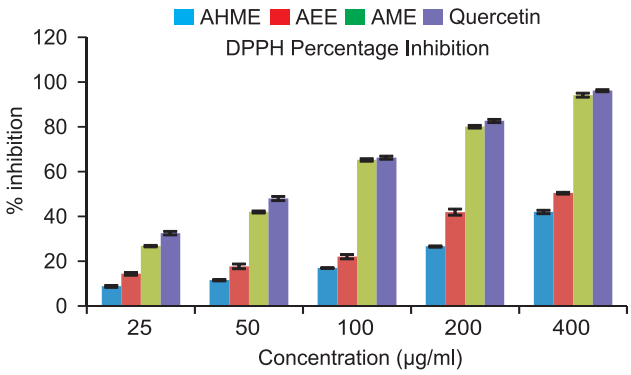

Fig. 1. DPPH scavenging activity of *Artemisia indica* extracts. Values expressed are mean ± standard deviation (n, 3). Percentage inhibition is expressed in µg/ml of the extract. AME, *A. indica* methanol extract; AEE, *A. indica* ethanol extract; AHME, *A. indica* hydro-methanol extract.

exhibited a maximum activity of 68.3% inhibition (P≤0.05), comparable to that of Quercetin with an activity of 77.7% at the same concentration of 200 µg/ml. While for the same concentration (200 µg/ml) AEE and AHME showed a much lesser inhibition of 39.8% and 25%, respectively. Various lower concentrations also had the similar pattern. The overall trend for the H_2O_2 scavenging activities of various AEs was AME>AEE>AHME. In terms of the IC_{50} value (Table 1), the highest H_2O_2 scavenging effect (P≤0.05) was obtained in AME with the lowest IC_{50} of 118.4±0.85 in comparison to Quercetin (61.7±1.45).

The H_2O_2 scavenging capacity of an extract may be attributed to the structural features of their active components, which determine their electron donating abilities. Scavenging of H_2O_2 by various AEs may be

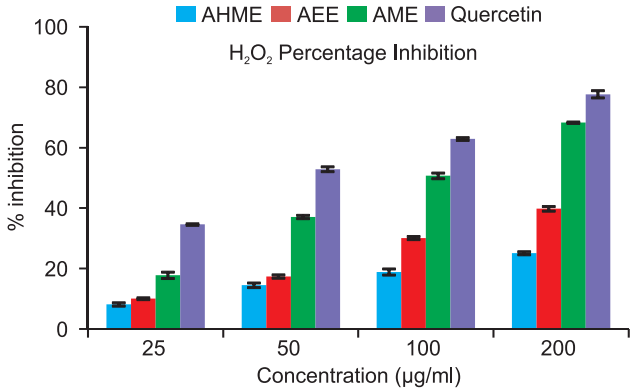

Fig. 2. Hydrogen peroxide scavenging effects of *Artemisia indica* extracts. Values expressed are mean ± standard deviation (n, 3). Percentage inhibition is expressed in µg/ml of the extract. AME, *A. indica* methanol extract; AEE, *A. indica* ethanol extract, AHME; *A. indica* hydro-methanol extract.

attributed to their phenolics, which could donate electrons to H_2O_2, thus neutralizing it to water.

Nitric oxide scavenging activity: Nitric oxide has earned the reputation of being a signaling mediator with many diverse and often opposing biological activities. The diversity in response to this simple diatomic molecule comes from the enormous variety of chemical reactions and biological properties associated with it. In general, lower NO concentrations promote cell survival and proliferation, whereas higher levels favour cell cycle arrest, apoptosis, and senescence (Thomas *et al.* 2008). Excessive production of NO contributes to cell death and damages in a range of ways, but the most important is by the production of peroxynitrite and various oxides of nitrogen upon reacting with superoxide and oxygen (Murphy 1999).

NO scavenging activity of AEs was investigated using sodium nitroprusside, which spontaneously produces NO when dissolved in aqueous solution at physiological pH. NO interacts with oxygen to produce stable products, nitrite and nitrate. Scavengers of NO compete with oxygen, leading to a reduced production of nitrite. The concentration of nitrite in aqueous solution can be assayed spectrophotometrically by using the Greiss reagent, with

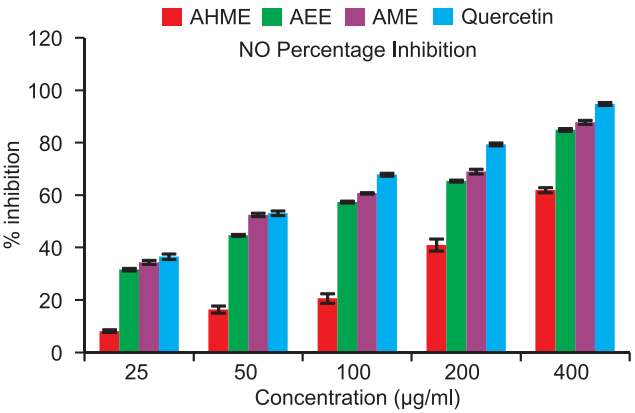

Fig. 3. Nitric oxide scavenging effects of *Artemisia indica* extracts. Values expressed are mean ± standard deviation (n, 3). Percentage inhibition is expressed in µg/ml of the extract. AME, *A. indica* methanol extract; AEE, *A. indica* ethanol extract; AHME, *A. indica* hydro-methanol extract.

which nitrite reacts to give a stable product. Fig. 3 illustrates a significant NO scavenging potential of AEs along with the Quercetin (standard). The scavenging of NO by various AEs exhibited an increment in a concentration dependent manner. AME and AEE exhibited a comparable NO scavenging (P≤0.05) with the standard Quercetin at various concentrations used, while AHME comparatively had lesser percentage inhibition values. AME and AEE gave a IC_{50} values of 65.1±4.6 and 101.2±0.70 µg/ml, respectively

Table 1. IC_{50} (Inhibitory concentration at 50%) of various scavenging activity assays of *Artemisia indica* extracts.

Scavenging activity assay	AME (IC_{50})	AEE (IC_{50})	AHME (IC_{50})	Quercetin (IC_{50})
DPPH radical scavenging activity	83.8 ±1.21	363.3 ±4.61	484.9 ±11.24	57.14 ±3.18
H_2O_2 scavenging activity	118.4 ±0.85	249.8 ±4.5	472.9 ±19	61.7 ±1.45
NO scavenging activity	65.1 ±4.6	101.2 ±0.70	300.5 ±6.20	35.98 ±0.23

Values expressed are mean±standard deviation (n, 3). AME, *A. indica* methanol extract; AEE, *A. indica* ethanol extract; AHME, *A. indica* hydro-methanol extract

(P≤0.05), as compared to 35.98±0.23 µg/ml of Quercetin (Table 1).

Total antioxidant capacity: The total antioxidant capacity (TAC) of the AEs were determined by phosphomolybdenum method using ascorbic acid as standard and this method is quantitative, since the TAC is expressed as ascorbic acid equivalents (AAE). The assay being simple and independent of other antioxidant measurements can be employed to evaluates both water-soluble and fat-soluble antioxidants (Prieto *et al.* 1999, Aliyu *et al.* 2013). The antioxidant capacity of various AEs was in the order AME>AEE>AHME (Fig. 4). AME exhibited a maximum potential TAC of 201.6±1.44 mg AAE/ gm extract (P≤0.05), followed by AEE (103.4±10.8) and AHME (49.1±6.15)

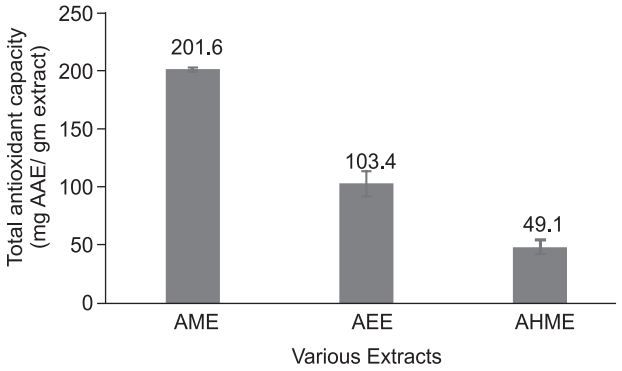

Fig. 4. Total antioxidant capacity of *Artemisia indica* extracts. Values expressed are mean ± standard deviation (n, 3). Total antioxidant capacity is expressed as mg of AAE (ascorbic acid equivalent) per g of extract. AME, *A. indica* methanol extract; AEE, *A. indica* ethanol extract; AHME, *A. indica* hydro-methanol extract.

($P \leq 0.05$), which could be attributed to the presence of phenolic compounds.

The research and characterization of new bioactive substances, for the efficient designing of functional food, nutraceuticals, or drugs, have been intensified. Besides, the quest for newer biomolecules with beneficial biological properties, and especially for the alternative resources for already characterized and desirable phytochemicals becomes more important in curbing overexploitation of a particular species. *Artemisia annua* may be quoted as an example here. Furthermore, a critical consideration, almost never considered in seriousness, is the importance of characterizing the local and geo-climatically endemic plant species as a resource of phytomedicine to the local and ethnic people. These considerations became the basic aim behind the present work of validation of the ethnomedicinal claims about *Artemisia indica*, with special reference to its antioxidative potential.

A fair number of reports are there affirming the antioxidative/ antiradical potential of various species of *Artemisia* (Nikolova *et al.* 2010, Bora and Sharma 2011, Lee 2014) and only one regarding the antioxidative activity of essential oils of *A. indica* (Rashid *et al.* 2013), but probably none regarding crude extracts of aerial parts of *A. indica*. The overall results of the *in vitro* antioxidative activity of *A. indica* extracts being reported here, as evaluated by the DPPH, H_2O_2, NO scavenging and TAC assays, are in good agreement with all available reports.

Phenolic or polyphenolic compounds, including the most prevalent flavonoid group, are plant metabolites widely spread throughout the plant kingdom. Phenolic compounds exhibit considerable free-radical scavenging activity, which is determined by their reactivity as hydrogen- or electron-donating agents, their reactivity with other antioxidants and their metal chelating properties, as well as the stability of the resulting antioxidant-derived radicals (Wojdylo *et al.* 2007).

The *in vitro* antioxidative potential of various AEs as evident by the above-mentioned experimentation may well be attributed to the presence of various polyphenolic compounds, as we have earlier reported that *A. indica* possess a good amount of TPC and TFC (Ruwali *et al.* 2015). Another important outcome of this study was that methanol proved to be better solvent system (as compared to ethanol and 50% hydro-methanol) in context to the antioxidative activity of *A. indica*, as AME gave consistently better results. Therefore, choice of extraction solvent is an important criterion regarding selecting a specific biological activity. This was a confirmation of our earlier investigation of the qualitative and quantitative phytochemical evaluation of *A. indica* (Ruwali *et al.* 2015). It would be worthwhile an attempt to further investigate and to separate the active components and evaluate the antioxidant activity of individual component, so as to confirm the bioactive nature of these compounds. In gist, *A. indica* has a serious potential to be developed/used as a natural source of antioxidant not only as a food supplement for animals and humans, but also as an antioxidant additive in packaged animal feed and human food industry.

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