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Vetting of Cow Urine Extract of Triphala for Antioxidative and Enzyme Curbing Potentiality: A Scientific Attempt to Evaluate Traditional Base of Herbal Treatment with Cow Urine

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Abstract: Since bygone times natural remedies remain a primal source of medicine for public health. Triphala and cow urine are the inherent natural remedies recommended in the time-honored medicinal system of Ayurveda. Traditionally cow urine is practiced with multifarious plant species for differential medicinal values but still unnoticed by the scientific world for the investigation of its traditional assert. The present appraisal deals with the evaluation of antioxidative and enzyme inhibitory strength of cow urine extract of Triphala by *in vitro* methods. The antioxidative capability of the cow urine extract was noteworthy in DPPH (1,1-diphenyl-2-picrylhydrazyl) and ABTS [2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)] radical curbing mode, successively delineated the IC₅₀ statistics of 95.47 µg/ml and 138.92 µg/ml, respectively. The antidiabetic competence was examined through the approach of inhibitory action averse to α-amylase and α-glucosidase serially rendered the IC₅₀ statistics of 172.97 µg/ml and 199.56 µg/ml while the accomplishment of anti-inflammatory prospects against lipoxygenase was beheld with the IC₅₀ statistics of 123.57 µg/ml. The cow urine extract expressed a substandard rendition of anti-proteinase scrutiny. In the course of experimentation, traditional claims of herbal treatment in combination with cow urine may become instrumental to curb the cultivation of distinct diseases and beneficial outcomes of cow urine extract of Triphala are presumably linked to the essential bioactive chemical substances in the extract.

Keywords: Triphala, Cow urine, Antioxidative, Antidiabetic, Anti-inflammatory

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Introduction

Oxidative stress, diabetes, and inflammatory conditions are globally noticed as intertwined

health issues. Oxidative stress and inflammatory conditions were researched as crucial factors to

flourish hyperglycemia by affecting the operation of pancreatic beta cells and curtailing insulin acuteness. On other hand, a continued hyperglycemic condition in the body may affect the fundamental operative mechanism of mitochondria to cause a generation of expansive oxidative stress and it may produce systemic and local inflammation by raising the levels of inflammatory mediators (Oguntibeju, 2019; Zhang *et al.*, 2020). Comanagement of diabetes and its complications becomes a puzzle for health experts because synthetic agents endorsed in the treatment of diabetes are interrelated with varied side effects like hypoglycemia, gastrointestinal disturbances, allergic conditions, cardiovascular toxicity, etc. These medications are not versatile to restrain the nonidentical complications observed in diabetic patients and hence, such patients are always directed for a lifetime with multi-drug therapy (Mohiuddin *et al.*, 2019; Padhi *et al.*, 2020).

In the world, a plant-based medicinal system generates its importance due to its minute or no side effects, limited cost, and broader health benefits. Numerous research works already substantiated the therapeutic potency of medicinal plants in diverse illness states (Abu-Odeh and Talib, 2021). Ayurveda is the ancestral medicinal system in India, stands on the foundation of natural products, and presently received valuable attention from the modern pharma sector (Parveen *et al.*, 2018). Polyherbal medication is the most popular concept in Ayurveda as it can act with multi-targeted dimensions. The presence of multi-bioactive molecules can act more efficiently by their differential mechanism and contribute exhaustive therapy against illness conditions (Karole *et al.*, 2019). Triphala is a notable ayurvedic blend, formulated by admixing the alike quantity of powdered fruits of Indian Gooseberry (*Emblica officinalis*), Chebulic Myrobalan (*Terminalia chebula*), and Beleric Myrobalan (*Terminalia bellerica*). Ayurvedic practitioners recommended the Triphala as an immunity booster, flatus-reliever, digestive, bowel evacuant, spasmolytic, mucoactive agent, and

bronchodilator. The scientific world researched and vindicated the therapeutic potentiality of Triphala for oxidant inhibitory, febrifuge, immune boosting, antiobesity, bactericidal, pain relieving, antitumor, antihepatotoxic, gastroprotective, radio-shelter, and hypoglycemic effects. The enriched chemical nature of Triphala with phenolic compounds and other bioactive molecules supports its therapeutic aptitude (Peterson *et al.*, 2017). Cow urine is another important medicinal substance described in Ayurvedic documents for the management of different illnesses of the kidney, heart, alimentary system, blood, and skin. Cow urine is an adept substance for the maintenance of regular health as it can withdraw entire imbalances from the human body. Apart from water and urea, cow urine expresses the presence of various components including salts, minerals, amino acids, vitamins, enzymes, and hormonal substances (Bajaj *et al.*, 2022). In many Ayurvedic formulations, cow urine is used as an adjuvant in order to enhance the bio-availability of co-administered drugs and thus improve their therapeutic competence (Singh, 2019). In India, traditional practitioners employed cow urine along with different herbs to treat different illness conditions including pyrexia, convulsion, blood disorders, gastric disorders, and many more. Gayatri Parivar is one of the renowned traditional institutes that formulated herbal products for the treatment of diabetes by using cow urine (Jarald *et al.*, 2008). In order to find out the scientific base behind such traditional therapy, the present antioxidative, and enzyme inhibitory scrutiny was attempted by using the cow urine macerate of Triphala.

Materials and Methods

Collection of Plant Fruits and Cow urine:

Fruits of *Emblica officinalis*, *Terminalia chebula*, and *Terminalia bellerica* were obtained from the medicinal garden of 'Maa Saraswati Herbals', Umari, Dist. Bhandara, MS, India (Fig. 1). The taxonomic characterization of each fruit material was affirmed by the Department of Botany, MB Patel College, Sakoli, Maharashtra, India. Cow

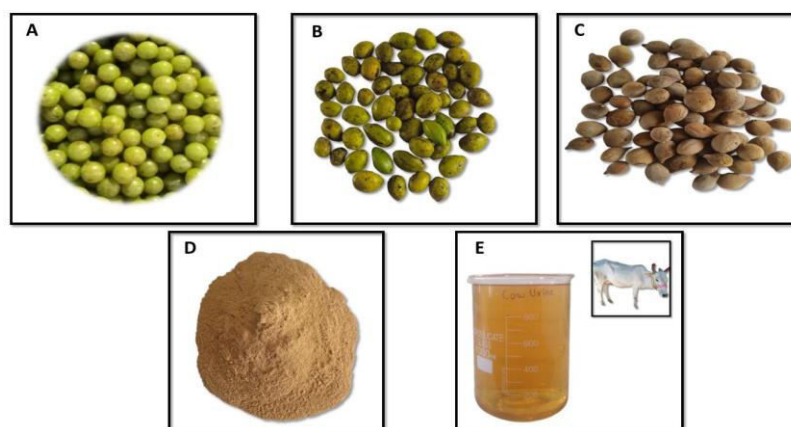


Fig. 1: A: *Emblica officinalis*, B: *Terminalia chebula*, C: *Terminalia bellerica*, D: Triphala, E: Cow urine and source.

urine was collected at dawn time from the local 'Gaolao' breed of cow, filtered in the laboratory, and stored in a glass container for further experimentation. The identification of the cow breed was confirmed by a local veterinarian, Dr. B. S. Girhepunje (MVSc).

Formulation and Maceration of Triphala blend:

The collected fruits of each plant were cleansed, unmoistened, and crumbled separately. Each crumbled fruit material was sieved (sieve number 60) and blended in alike proportions. A quantified blend of formulated Triphala (100 g) was separately macerated with cow urine and distilled water for 5 days with intermittent stirring. On the sixth day, the macerate was filtered and concentrated in an electric water bath (Venkateswarlu *et al.*, 2019).

Phytochemical Scrutiny:

Different preliminary tests were conducted as per standard procedures for the detection of specific bioactive phytochemicals in the cow urine extract and aqueous extract of Triphala (Shaikh and Patil, 2020).

In vitro Antioxidant Activity:

DPPH Radical Scavenging Assay:

The discoloration that emerges in the purple appearance of the DPPH solution is the basis for the determination of antioxidant potential. Experimentally, 1000 μ l of DPPH reagent (0.2 mM;

prepped in methanol) was assorted in 1000 μ l of multifarious masses (50-250 μ g/ml) of the extracts and set aside for half an hour at ambient temperature. The standard comparability was studied against the equivalent concentrations of ascorbic acid by observing the absorbance at 516 nm (Alimi and Ashafa, 2017).

ABTS Radical Scavenging Assay:

The neutralization of radicals of azo compound on reaction with the antioxidant compound is the assessment criteria of performed experimental mode. Equitably quantified solutions of 20 mM ABTS diammonium salt and 70 mM potassium peroxydisulfate were intermingled and incubated in a black paper box for 24 consecutive hours to consummate the augmentation of ABTS radicals. A definite portion of 600 μ l of each test specimen (50-250 μ g/ml) was separately united with 450 μ l of incubated solution and promptly with the pause of 10 min absorbance value was ascertained at 734 nm. Aftereffects were quoted against the parallel concentrations of ascorbic acid (Bhuvana *et al.*, 2017).

In vitro Antidiabetic Activity:

α -Amylase Inhibitory Assay:

A sample tube containing 250 μ l of the trial sample was diluted with an equivalent quantity of phosphate buffer (0.02 M; pH 6.9) carrying α -amylase (500 μ g/ml) and kept aside at standard

ambient temperature. After 10 min, 250 µl of 1 % starch solution (prepped in the same buffer) was supplemented in a sample tube and kept for additional 10 min of incubation at the same standard ambient temperature. Further 500 µl of dinitrosalicylic acid (DNS) reagent was assorted and left to boil in the thermostatic bath for 10 min. Finally, 5000 µl of fresh refined water was incorporated, and the finding of the absorbance was completed spectrophotometrically at 540 nm. The outcomes of trial scrutiny were recognized against the equivalent concentrations of acarbose (Kazeem *et al.*, 2013; Ramana Murty Kadali *et al.*, 2017).

α-Glucosidase Inhibitory Assay:

The estimation of yellow-colored p-nitrophenol liberated from p-nitrophenyl glucopyranoside (p-NPG) is the basis of the present antidiabetic model. In the present assay, the individual concentrations of the test samples (50-250 µg/ml) were allowed to incubate with 100 µl of α-glucosidase (1 unit/ 1000 µl) at ambient temperature for a quarter-hour. In the next step, 50 µl of 3.0 mM p-NPG diluted in 20 mM phosphate buffer (pH 6.9) was comingled for the progression of the chemical reaction and kept at room temperature. After a time interval of 20 min, 2000 µl of 0.1 M soda ash was supplemented into entire trial samples and ascertainment of absorbance was accomplished with the wavelength of 405 nm. The use of a standard drug was followed as like mentioned in the assay of alpha-amylase inhibitory activity (Kazeem *et al.*, 2013; Tamilselvi *et al.*, 2018).

In vitro Anti-inflammatory Activity:

Proteinase Inhibitory Assay:

The deterrence of protein lysis by inhibiting the proteolytic enzyme is the assessment basis of the implemented anti-inflammatory study. Precisely measured 60 µg trypsin, 1000 µl tris-hydrochloride buffer (pH 7.4), and 1000 µl of varied concentration (50-250 µg/ml) of trial samples were fused together and admitted for incubation at 37°C to attain the reaction. After 5

min, 1000 µl of protein solution (0.3% w/v casein) was introduced and retained at the same temperature for extra 20 min. The reaction ended with the incorporation of 2000 µl of hyperchloric acid (70%) and a subsequent spinning process at 3000 rpm in a centrifuge machine. The upper layer of each centrifuged solution was studied for absorbance data at 210 nm. The comparative trials were executed with the analogous concentrations of diclofenac (Agarwal and Shanmugam, 2019).

Lipoxygenase Inhibitory Assay:

The attempted anti-inflammatory trial is based on spectrophotometric scrutiny of the oxidation of linoleic acid to hydroperoxyl derivatives. All trial samples (50-250 µg/ml) were blended with 250 µl of 2M sodium borate buffer (pH 9) followed by a summation of 250 µl enzymatic solution of lipoxygenase (20,000 U/ml) and kept undisturbed at 25°C. After 300 seconds, 1000 µl of linoleic acid solution (0.6 mM) was appended to each sample, and a wavelength of 234 nm was employed to perceive the absorbance. The trials of the standard were operated with diclofenac by using its analogous concentrations (Mohan *et al.*, 2013; Shukla and Choudhary, 2018).

Calculation of % inhibition and IC₅₀ value:

In each experiment, the % inhibition was figured out as per Equation 1 and the IC₅₀ numerals were determined from a regression graph obtained from concentration (x-axis) and % inhibition (y-axis).

$$\text{Equation 1: \% inhibition} = [(Ab_{\text{con}} - Ab_{\text{sam}})/Ab_{\text{con}}] \times 100$$

(Ab_{con}: Absorbance of the control; Ab_{sam}: Absorbance of the sample)

Results

Phytochemical Scrutiny:

The specific qualitative tests employed for the preliminary analysis of individual phytochemicals in aqueous and cow urine extract hinted toward the existence of divergent bio-compounds shown in Table 1 including therapeutically active nitrogenous and phenolic compounds like

Table 1: Phytochemical scrutiny of cow urine extract and aqueous extract of Triphala

Phytochemicals	Test	Cow urine extract	Aqueous extract
Alkaloids	Mayer's Test	+	+
	Dragendroff's Test	+	-
Cardiac Glycosides	Keller-Killiani Test	+	-
Phenolics and Tannins	Ferric Chloride test	+	+
	Lead acetate Test	+	+
Phlobatannins	HCl Test	-	-
Flavonoids	Shinoda Test	-	-
	Alkaline reagent Test	+	+
Terpenoids	Salkowski's Test	+	+
Steroids	Liebermann-Burchard Test	+	-
Anthraquinones	Borntrager's test	+	+
Saponins	Foam Test	+	+
Coumarins	NaOH Test	-	-
Fixed oil and Fat	Spot Test	-	-

alkaloids, flavonoids, tannins, saponins, anthraquinones, and terpenoids. The cow urine extract also displayed positive reports for the existence of cardiac glycosides and steroidal compounds.

Antioxidant Activity:

During antioxidative perusal against DPPH radicals, extract and standard (ascorbic acid) were evinced with concentration-dependently advancement in % inhibition indicating their potential to scavenge DPPH radicals. The competence of cow urine extract for the inhibition of DPPH radicals was observed to be more potent than aqueous extract. The IC_{50} values determined by plotting the regression graph (Fig. 2) were established as 14.31, 95.47, and 157.76 $\mu\text{g/ml}$ for standard (ascorbic acid), cow urine extract, and aqueous extract, respectively. The % inhibitions expressed by cow urine extract from the concentration of 50 to 250 μg were noticed as more dynamic than equivalent concentrations of ascorbic acid. In the ABTS assay, each extract and ascorbic acid showed a concentration-dependent increment in % inhibition, indicating their potentiality to scavenge ABTS⁺ radical cations. The upgraded performance of cow urine extract noticed through the regression graph (Fig. 3)

ascertained the numeral of 138.92 $\mu\text{g/ml}$ as IC_{50} value. The same study highlighted the IC_{50} value of 220.18, and 93.15 $\mu\text{g/ml}$ for aqueous extract, and ascorbic acid, respectively.

Antidiabetic Activity:

As hydrolases are preeminent enzymes recognized in the hydrolytic lysis of saccharides, the antidiabetic ability of trial samples was appraised by their pressive effect against the mentioned enzymes. The attainment of the α -amylase inhibitory assay illustrated in Figure 4 indicates the compelling result for cow urine extract with the numeral of 172.97 $\mu\text{g/ml}$ as IC_{50} value. Despite the IC_{50} value of acarbose being 93.63 $\mu\text{g/ml}$, cow urine extract expresses improved inhibition in concentration dependant aspects. The regression analysis set out an IC_{50} numeral of 251.23 $\mu\text{g/ml}$ for aqueous extract was noticed as weak than the analogous concentrations of cow urine extract. The sequel of α -glucosidase inhibitory experimentation of trial samples and the standard compound is illustrated in Figure 5. At the maximum experimented concentration of 250 $\mu\text{g/ml}$, acarbose expressed doubled % inhibition than aqueous extract while cow urine extract showed moderate performance with 59.35% inhibition. The IC_{50} value of cow urine extract

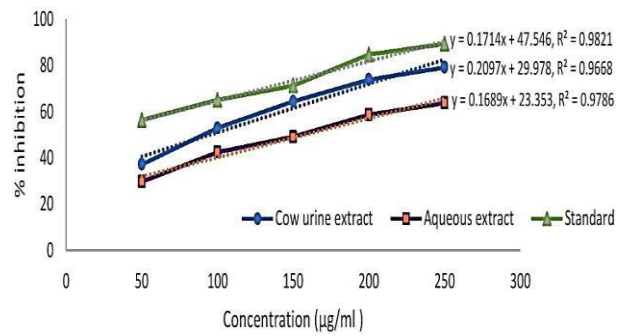


Fig. 2: DPPH radical scavenging assay.

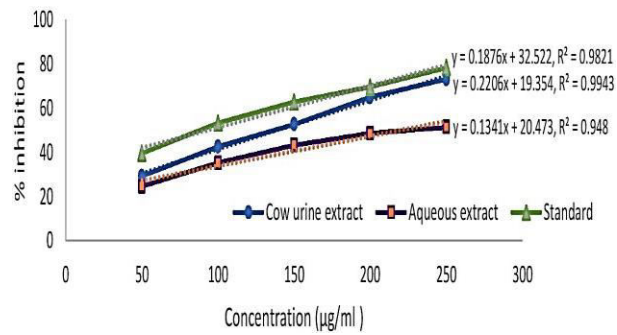


Fig. 3: ABTS radical scavenging assay.

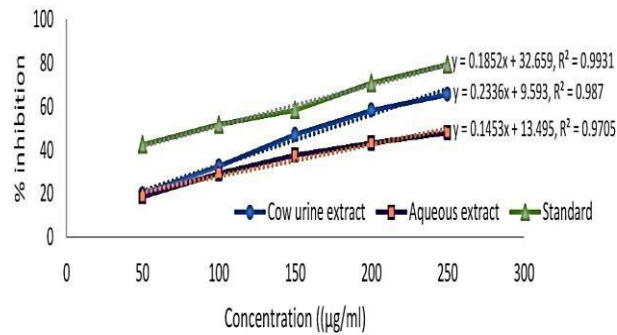


Fig. 4: α-Amylase inhibitory assay.

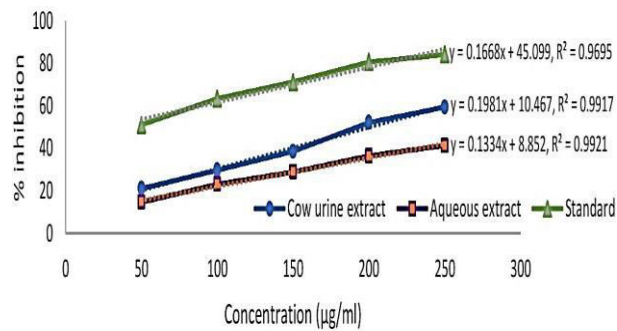


Fig. 5: α-Glucosidase inhibitory assay.

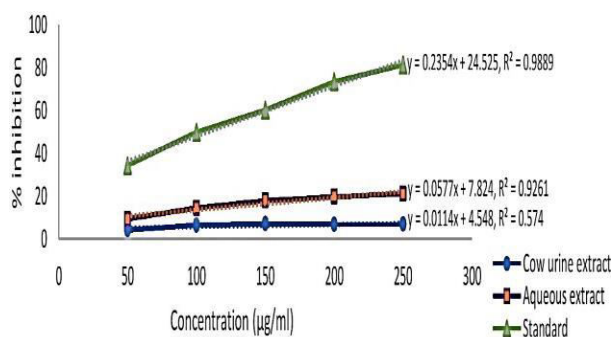


Fig. 6: Proteinase inhibitory assay.

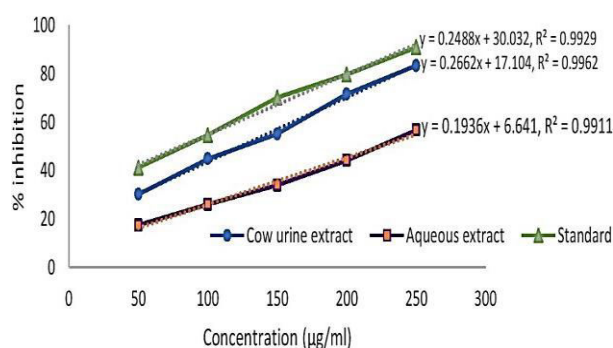


Fig. 7: Lipoxygenase inhibitory assay.

(199.56 µg/ml) was comparatively weaker than the IC_{50} value of acarbose (29.38 µg/ml) but expresses an improvement over the inhibitory performance of an aqueous extract (308.45 µg/ml).

Anti-inflammatory Activity:

The competency experimented in anti-proteinase and anti-lipoxygenase assays represented the anti-inflammatory potentiality. The after-effects of proteinase inhibitory vetting are cited in Figure 6. At the concentration of 250 µg/ml, diclofenac evinced 81.28% inhibition while cow urine extract and aqueous extract were sequentially detected with the results of 6.84% and 21.02% only. The outturn from proteinase inhibitory perusal discerned the very poor value of IC_{50} for cow urine extract, specifying its negligible potentiality than aqueous extract (IC_{50} value: 730.95 µg/ml) and diclofenac (IC_{50} value: 108.22 µg/ml). During experimental probing of anti-lipoxygenase

prospectivity (Fig. 7), the order of potentiality was noticed as diclofenac > cow urine extract > aqueous extract. Within the range of 50 to 250 µg/ml of contents, the statistic of % inhibition for cow urine extract was obtained in the range of 30.2 to 83.36% along with the IC_{50} numeral of 123.57 µg/ml. Aqueous extract displayed minimal inhibition with an IC_{50} statistic of 223.96 µg/ml while diclofenac offered the strongest % inhibition with the numeral of 80.25 µg/ml as the IC_{50} value.

Discussion

In the present study, cow urine extract of Triphala examined for antioxidative and enzyme inhibitory potentiality was ascertained with ameliorated performance in all experimental models except anti-proteinase assay.

Numerous scientific methods are available to scrutinize antioxidant competence but the DPPH assay is an acclaimed trustworthy approach to

disclose its antioxidative efficacy. In presence of antioxidant molecules, nitrogenous radicals of DPPH switch their violet tinge to a yellow one through a reduction mechanism. In the present delve, the reduction process expressed by cow urine extract may be due to the proton donating ability of its antiradical molecules being observed in an enhanced manner with boosting order of concentration. In the ABTS assay, the neutralization effect noticed through the discoloration of blue-colored radical cation may be due to the antioxidant components in both extracts but cow urine extract expressed accelerated performance may be due to the bioactive components of cow urine (Jain *et al.*, 2018). In both experimented models, antioxidant efficaciousness executed by Triphala may be associated with its phenolic components and ascorbic acid but improvement of potentiality in cow urine extract expresses the probability of involvement of bioactive components of cow urine (Peterson *et al.*, 2017; Karole *et al.*, 2019).

The refrainment of the enzymes like hydrolases involved in starch hydrolysis is the most favored approach to examine the antidiabetic consequences. Starch available in the body from different foodstuffs undergoes a breakdown by α -amylase to produce malt sugar and dextrin followed by further cleavage by α -glucosidase into glucose and hence causes an elevation of post-meal blood sugar level (Anh *et al.*, 2021). The experimented antidiabetic scrutiny demonstrated that cow urine extract exerted an inhibitory performance against α -amylase and α -glucosidase as per dose-reliant aspects shown in Figures 5 and 6, respectively. In the present study, the preliminary investigation for the identification of the chemical nature of the extract highlighted the presence of different therapeutically active biocomponents including phenolic components, and priorly published scientific data claim that phenolic components in the extract roll out their inhibitory action against such digestive enzymes. Phenolic compounds express hydrogen bonding with the active site of these enzymes and thus hindered their action to hydrolyze the

carbohydrates. With the consideration of the chemical structure of phenolic compounds, the count and position of hydroxyl groups in molecular structure impart a decisive role while inhibitory action. The interdictory action of each extract against α -amylase in addition to α -glucosidase can become a competent strategy to control the sugar level in diabetic patients (Thilagam *et al.*, 2013). Hyperglycemia endorses the spontaneous oxidation of glucose to generate harmful radicals which are beyond the scavenging strength of autogenous antiradical defenses resulting the different complications in the body. In this case, the antioxidant efficacy of plant material is always considered an adjuvant therapy for the antidiabetic potential to sidestep diabetic complications (Bajaj and Khan, 2012). The improved antioxidant performance of Triphala in presence of cow urine may be responsible to impart improved antihyperglycemic efficacy.

Enzyme-curbing action always remains a prime focus for the inquisition of anti-inflammatory efficaciousness. Ample concentrations of proteolytic enzymes in neutrophils are often known to interconnect with inflammatory circumstances. These enzymes are predominantly involved in arthropathy to engender chronic inflammation; the interdiction of such proteolytic enzymes may sponsor anti-inflammatory possibilities (Patel and Zaveri, 2014). During the anti-proteinase inquest, cow urine extract detected with ineffectuality might be associated with the proteolytic urokinases reported in cow urine (Badhe *et al.*, 2013). Corollaries of the scrutinized model suggested that anti-proteinase action may not become a mode for the anti-inflammatory action of cow urine extract of Triphala. Another model of the anti-lipoxygenase study reflected that it may be the mode for anti-inflammatory adeptness of cow urine extract of Triphala. Lipoxygenase is the prominent enzyme related with the biosynthesis of disparate inflammatory mediators from polyunsaturated fatty acids. These enzymes are promptly aroused under oxidative stress and express the upregulated inflammatory response in

numerous illness conditions like hyperglycemia, allergic reactions, and respiratory diseases (Kulkarni *et al.*, 2021). During anti-lipoxygenase experimentation, the emphatic rendition of cow urine extract may be due to its antioxidant strength. Pre-existing scientific perusal reported that endogenous peptides in cow urine have the propensity to bind with lipoxygenase and terminate their role in inflammatory conditions (Kumar *et al.*, 2021).

Conclusion

It is concluded that the traditional claim of herbal treatment in combination with Cow urine might be instrumental to curb the cultivation of distinct diseases arising due to oxidative stress. Furthermore, the existence of vital bioactive chemical substances may be reasonable for the exerted potentiality by the cow urine extract of Triphala are needed to identify and correlate with the accomplished beneficial synergic outcome at the molecular level.

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