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Original Article

Phytochemical Approach Including Total Phenolic and Flavonoid Contents and Evaluation of *in vitro* ABTS Antioxidant Capacity and Lipoxygenase Inhibition of *Anisosciadium lanatum*

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ABSTRACT

Anisosciadium lanatum Boiss is commonly known in Bedouins as besbas. Traditional, it is palatable plant and used in medicine of livestock to treat skin conditions . The main objective of current approach was to screen the presence of different kind of metabolites applying standard procedures followed by assessment of total phenolic (TPC) and flavonoids (TFC) contents. In addition, the *in vitro* ABTS antioxidant and lipoxygenase activities were evaluated. Different organs (leaves, stems and flowers) of *Anisosciadium lanatum* were extracted using 70% methanol to yield total methanol extracts of leaves (TML), stems (TMS) and flowers (TMF). Results demonstrated that TML, TMS and TMF are characterized by the content of different constituents such as flavonoids, phenolics/tannins, steroids, saponins, and carbohydrates at different levels. Ethyl acetate (EA) and butanol (BT) fractions of TML and TMS demonstrated the highest percentage of TPC and TFC. The results demonstrated the competence of EA and BT as free radical scavenger fractions compared to other fractions and its opportunity to contain bioactive antioxidant metabolites . TML, TMS and TMF exhibited Lipoxygenase inhibitory activity with IC_{50} values of 4.88, 5.40 and 6.05 µg/mL, respectively when compared to that of the positive control baicalein (IC_{50}: 0.27 µg /mL). In conclusion, present investigation highlighted the potential of *Anisosciadium lanatum* to be promising candidate with activity against wide range of inflammatory-related diseases.

Key words: *Anisosciadium lanatum* , Total phenolic, Total flavonoid, ABTS, Lipoxygenase .

INTRODUCTION

Traditionally, medicinal plants are considered to play pivotal roles in treatment of various disorders.[1] Family umbelliferae consists of around 3780 species distributed in in 434 genera. Members of this family spread all over the world.[2,3] Evidenced reports convinced its contents of various phytochemicals including; terpenoids, saponins, flavonoids, coumarins, and poly-acetylenes . Many members of the family umbelliferae demonstrated various biological activities, such as anti-bacterial, hepatoprotective, antiinflammatory, and anticancer activities.[2] Huge numbers of members of this family is reputed to be safe and edible. The genus *Anisosciadium* (member of family umbelliferae) includes three main species; *Anisosciadium isosciadium* Bornm., *Anisosciadium orientale* DC., and *Anisosciadium lanatum* Boiss. This genus is endemic to Southwest Asia.[4] Previous investigations have reported the identification of volatile oil and their cytotoxicity and antioxidant properties of *Anisosciadium orientale*.[4,5] In this context, *Anisosciadium lanatum* Boiss (A. lanatum) is native and spread wildly in the Saudi Arabia.[6,7] *A. lanatum* is perennial herb. Morphologically, the leaves are characterized by a bipinnately parted incision with compound umbel type inflorescences. The flowers are minute with whitish pink colored appearance.[8,9] Traditionally, Bedouins use *A. lanatum* as local medicinal herb for skin sores and boils.[10] The young green leaves are refreshing edible herb[24,25] Additionally, *A. lanatum* is used in livestock treatment for skin conditions in goats and sheep. [6] Recent studies reported the isolation of guaiane sesquiterpene which demonstrated anti-proliferative activity against liver, colon, and lung cancer cells.[11,12] Volatile oil isolated from *A. lanatum* controlled hepatoma HepG2 liver cancer cells through the reciprocal regulation of apoptotic markers .[13] In these contexts, the goal of the current investigation is to highlight the phytochemical contents including; determination of total phenolic and flavonoid contents and antioxidant capacity of various fractions of different polarities of leaves, stems and flowers of *A. lanatum* and further evaluation of *in vitro* and lipoxygenase inhibition activity.

MATERIALS AND METHODS

General

Methanol, petroleum ether (PE), dichloromethane (DC), ethyl acetate (EA) and n-butanol (BT) were of analytical grades. Baicalein, rutin, gallic acid and ascorbic acid were purchased from Sigma Aldrich.

Plant material

lanatum Boiss was collected from Riyadh, Saudi Arabia (April 2014). A voucher specimen (14-Apr-AL) was deposited at the Herbarium museum of the College of Clinical Pharmacy, King Faisal University, Saudi Arabia.

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Extraction

The air dried powders of carefully separated plant organs [leaves, stems and flowers of *A. lanatum* Boiss, 300, 250 and 100 g respectively] were thoroughly extracted at room temperature (1week) *via* cold maceration using five liters of 70% methanol.[14] The compiled extract was concentrated to yield the total methanol extracts of leaves (TML), stems (TMS) and flowers (TMF), 25, 15 and 11 g respectively, which were then stored in freezer for the next steps. The various extracts were suspended in distilled water (100 ml) using a separating funnel and partitioned with PE (6×100 ml). The PE fractions were combined and concentrated to yield 4, 1 and 1.5 g respectively then were stored in well- closed container. The remaining aqueous fractions (AQ) were subjected to fractionation with DC (4×250 ml). DC fractions were also combined and concentrated to yield 3, 2 and 1 g, respectively. Likewise, EA as well BT extracts were also obtained to yield 4, 2 and 2 g respectively for EA fraction and to give 3, 2 and 1 g respectively for BT fraction. The remaining AQ was also freeze-dried to powder to give 4, 3 and 0.5 g respectively. The various extracts of leaves, stems and flowers were subjected to the phytochemical screening tests using referenced procedures.

Phytochemical screening

Qualitative phytochemical screening of available metabolites was performed applying the described and mentioned methods.[15,16]

Flavonoids

Two milliliters of each corresponding extract, was mixed with one mL of 2 % NaOH. The availability of flavonoids is recorded once; an intense yellow color is produced and disappeared by addition of few drops of any diluted acid.

Saponins

One mL of each corresponding extract was suspended in twenty mL of distilled water and then the mixture was vigorously shaken. The presence of saponins is confirmed by the formation of persistence froth for at least 15 min.

Steroids

Half mL of each corresponding extract was dissolved in five mL of chloroform and few drops of acetic anhydride. Then, few drops of concentrated sulfuric acid were added from the side of the test tube. The coloration of the upper yellow layer with green/blue color indicated the presence of steroids.

Alkaloids

Dragendorff's reagent was used to detect the presence of alkaloids *via* the development of orange or orange-red precipitate. Part of corresponding extract (0.5.ml) was mixed with dilute hydrochloric acid (1.5 ml) and followed by filtration. Few drops of Dragendorff's reagent was added to the filtrate and monitored.

Anthraquinones

Half mL of each corresponding extract was heated with dilute hydrochloric acid then filtered and cooled. The filtrate was extracted with benzene and diluted ammonia solution was added to the reaction media. The aqueous ammonia layer became pink to red due to the presence of derivatives of anthraquinones.

Tannins/Phenolics

Half mL of each corresponding extract was mixed with few drops of 5% ferric chloride solution. The production of intense blue-greenish indicates the presence of tannins/phenolics.

Cardiac glycosides

Part of corresponding extract (10 mL) was mixed with (4 mL) of solution of glacial acetic acid and 1 drop of 2% ferric chloride followed by 1 mL of concentrated sulphuric acid. A brown ring formed between the layers indicted the presence of cardiac glycosides.

Carbohydrates

Two mL of each corresponding extract was mixed with a 10 mL Molisch reagent. Then, 2 mL of concentrated sulphuric acid was added from the side of the test tube. The formation of a violet ring at the intersection of the two layers indicated the presence of carbohydrates.

Calculation of total phenolic contents (CTPC)

The estimation of TPC was performed in accordance to the previously described method using Folin-Ciocalteu reagent.[17] Stock solutions with a concentration of 1 mg/ml were prepared in methanol, were freshly prepared for each of different fractions . Half milliliter of Folin-Ciocalteu reagent together with six ml of distilled water was added to 0.1 ml of solution of each fraction of different polarities followed by addition of 1.5 ml of 20% $NaCO_3$ solution to get a final volume of 10. The reactants were set aside at room temperature for two hours. Lastly for all tests, the absorbance was measured at 760 nm. Calibration curve of the standard drug (gallic acid) was prepared using sequential dilution (0.5, 0.4, 0.3, 0.2 and 0.1 mg/ml in distilled water).

Calculation of total flavonoid contents (CTFC)

According to Khalil *et al* method[18] with minor modifications, the assessment of the content of total flavonoids was performed. Equal proportion s of distilled water and acetone (50 ml from each) were co-mixed and followed by addition of 10 mg from each fraction involved in the study . Volume of 0.25 ml of serially diluted samples, were mixed with 0.75 µl of a $NaNO_2$ (5% w/v) solution, then, 0.15 ml of a freshly prepared $AlCl_3$ (10% w/v) solution. The prepared reaction media were mixed with 0.5 ml of 1 M NaOH solution. Then, the volume of prepared mixture was adjusted with distilled water to a volume of 10 ml . The prepared reaction media were kept for 5 min. by the end of experiment, the absorbance of each prepared mixture was measured at 510 nm against the same reactants but without the presence of tested fractions. Calibration curve was developed for the reference compound (rutin, using the following concentrations, 0.0, 0.1, 0.5, 1, 5 and 10 µg/ml).

Radical scavenger activity using ABTS method

To estimate the antioxidant power, (2, 2'-azino-bis-(3-ethyl-benzothiazoline-6-sulfonic acid) method was implemented, where, the generation of the free radical of ABTS was developed *via* the mixing of 7 mM ABTS together with 2.45 mM potassium persulfate according to Dalya *et al* method.[19] The prepared mixtures were kept in dark at room temperature and the reaction flasks were covered with aluminum foil to keep in dark for 16 hours before its usage. The generated ABTS•1 free radical solution was diluted with ethanol until an absorbance of 0.70 at 734 nm was attained. Stock solutions were prepared for all reactants, standard, and tested fractions. At the concentration of 1 mg/ml standard (Sodium ascorbate) was prepared. The corresponding extracts were made in distilled water and serial dilutions of 1, 5 and 10 µg/mL. A volume of 0.5 ml of each dilution was added to 2 ml ABTS free radical solution then kept to allow reaction for half an hour. Then, the absorbance was measured at 734 nm. A blank experiment was developed using 0.5 ml methanol. The percentage inhibition of free radical was calculated through the formula:

$$\%\ of\ inhibition\ = \frac{Ab.B - Ab.S}{Ab.B}\ X\ 100$$

Where; Ab. B and Ab. S are the absorbance of blank and sample or standard, respectively. The experiments were performed in triplicate and

standard error of mean were calculated and used in the establishment of calibration curves. Form these curves, linear regression was performed and 50% inhibitory concentration (IC_{50}) was calculated for samples and standard and used in comparison; the lower the IC_{50}, where the greater the antioxidant power.

Lipoxygenase inhibitory activity assay

Lipoxygenase inhibitory activity was calculated using the method described by Alshawush *et al.*[16] Ten µL of the serial concentrations of tested sample solutions were mixed with160 µL of 0.1 mM sodium phosphate buffer with pH (7) and 20 µL of lipoxygenase solution and kept for 5 min at room temperature. Solution of linoleic acid (10 µL) as substrate, was added to initiate the interaction and wait for 10 min. The test sample and the control were dissolved in 50% ethanol. The percentage of inhibition of lipoxygenase was calculated according to the following equation

$$\%\ of\ inhibition\ of\ lipoxygenase\ = \frac{R-S}{R}\ X\ 100$$

Where; R ($Abs_{control}$ – $Abs_{background}$) is the activity of the enzyme without sample and S (Abs_{sample} – $Abs_{background}$) is the activity of enzyme with sample. Where $Abs_{control}$ = absorbance of control, $Abs_{background}$ = absorbance of background and Abs_{sample} = absorbance of sample. IC_{50} values were determined by the equation of nonlinear regression with the help of Excel Microsoft Office. All the reactions were performed in triplicate.

RESULTS AND DISCUSSIONS

Phytochemical screening of different fractions of different organs of A. *lanatum*

The preliminary screening for various chemical constituents in different fractions of TML, TMS and TMF showed the presence of different metabolites such as flavonoids, saponins, steroids, tannins/phenol and carbohydrates at different levels in different fractions of the flower extract and the absence of anthraquinones, alkaloids and cardiac glycosides, as shown in Table 1.

CTPC

TPC in different fractions of TML, TMS and TMF, was calculated using the regression equation: (y = 0.1792x - 0.0074, R^2=0.9518). The quantity of phenolic constituents was expressed as the equivalence of milligrams of standard Gallic acid per gram of dried plant extract (mg GAE/g) (Figure 2A). Results demonstrated that the quantity of TPC ranged from (0.5±1.10) mg GAE/g of dry extract for the DC of TMF to (44.25±0.91) mg GAE/g of dry extract for the EA of TML. Hence, the EA and BT fractions of TML and TMS demonstrated the highest percentage of TPC followed by AQ fractions of TML and TMS. While, PE fraction showed the least phenolic constituents for all kind of fractions (Figure 2B).

CTFC

TFC in different fractions of TML, TMS and TMF was estimated using the regression equation: (y= 0.0366x + 0.0075, R^2 = 0.9752) (Figure 3A). The results were expressed as the equivalence of milligrams of rutin per gram of dried plant extract (mg RE/g). Results depicted that the amount of TFC ranged from (0.078±0.10) to (4.25±2.2) mg QE/g of dry extract. Similarly, to those of TPC, EA and BT were richest fractions in flavonoids contents then AQ fraction. On the other hand, PE fractions were hardly found to contain flavonoid constituents for all extracts (Figure 3B). From results of both TPC and TFC, ET and BT fractions are worthy of for future consideration to identify their constituents.

Antioxidant capacity (ABTS)

The ABTS assessment method is considered one of the most important protocols to assess the antioxidant capability of plant extracts. Remarkably, the decrease of the absorbance of tested experiment

Figure 1: Photograph of *A. lanatum* Boiss during the flowering stage.

Table 1: Preliminary phytochemical screening of various fractions of various organs of *Anisosciadium lanatum*.

Chemical test/Plant organ	Leaves					Stems					Flowers				
	PE	DC	EA	BT	AQ	PE	DC	EA	BT	AQ	PE	DC	EA	BT	AQ
-Flavonoids Alkaline solution test	-	-	+	+	+	-	-	+	+	+	-	-	+	+	+
-Saponins Foam test	-	-	+	+	+	-	+	+	+	+	-	-	±	±	-
-Triterpenoids/steroids Liebermann-Burchard test	+	+	+	+	-	-	-	+	+	-	+	+	+	+	-
-Alkaloids Dragendorff's reagent	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
-Anthraquinones Borntrager's test	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
-Tannins 10% FeCl	-	-	+	+	-	-	-	+	+	-	-	-	±	±	-
-Cardiac glycosides[3] Keller Killiani test	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
-Carbohydrates Molisch's test	-	-	+	+	+	-	-	+	+	+	-	-	+	+	+

PE, Petroleum ether fraction; DC, Dichloromethane fraction; EA, Ethyl acetate fraction; BT, Butanol fraction; AQ, Aqueous fraction. + (present); - (absent); ± (traces)

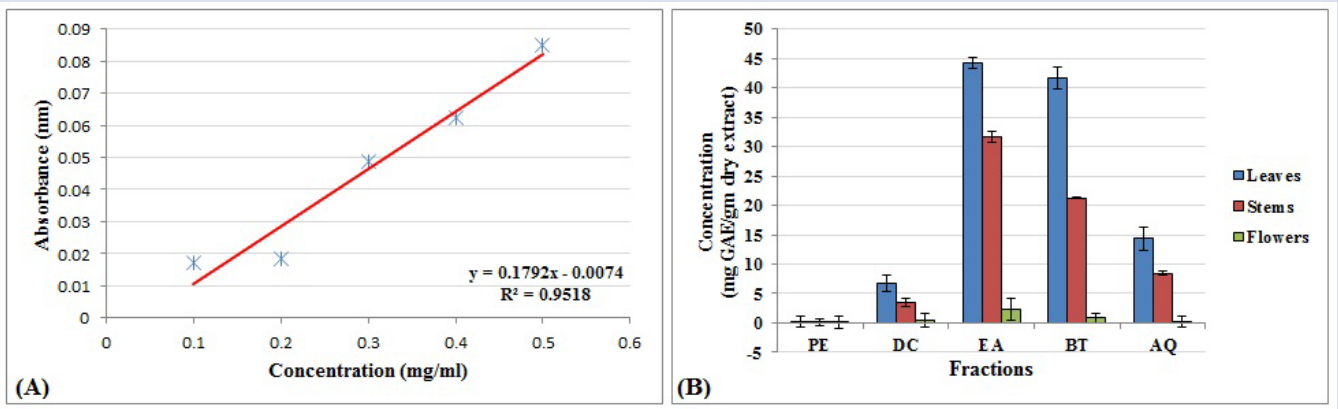

Figure 2: Calibration curve for gallic acid (A) and total phenolic contents (B) of various fractions of *A. lanatum*. PE; petroleum ether fraction, DC; Dichloromethane fraction, EA; ethyl acetate fraction, BT; n-butanol, AQ; remaining aqueous fraction, GAE/g; Gallic acid equivalence per gram of dried plant extract.

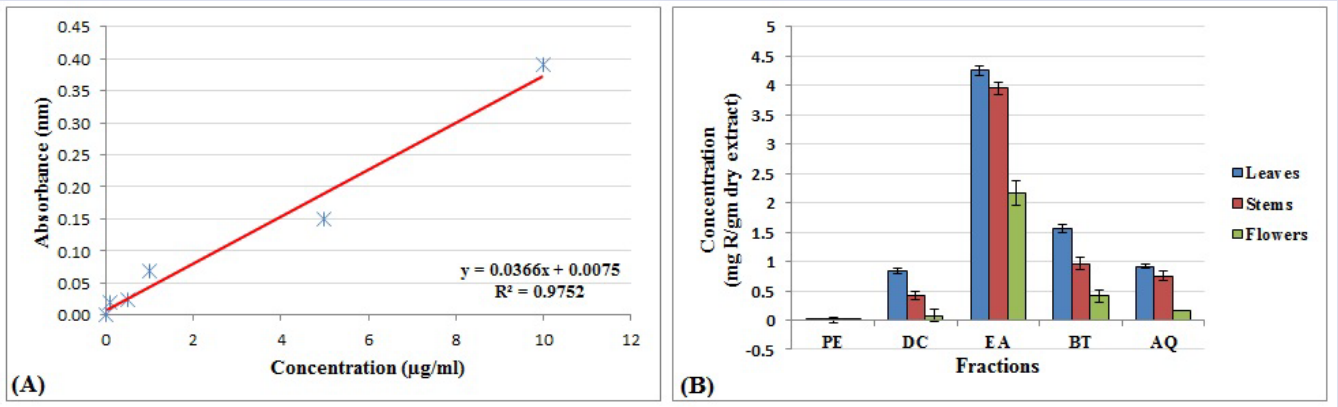

Figure 3: Calibration curve for rutin (A) and total flavonoid contents (B) of various fractions of *A. lanatum*. PE; petroleum ether fraction, DC; Dichloromethane fraction, EA; ethyl acetate fraction, BT; n-butanol, AQ; remaining aqueous fraction, RE/g; rutin equivalence per gram of dried plant extract.

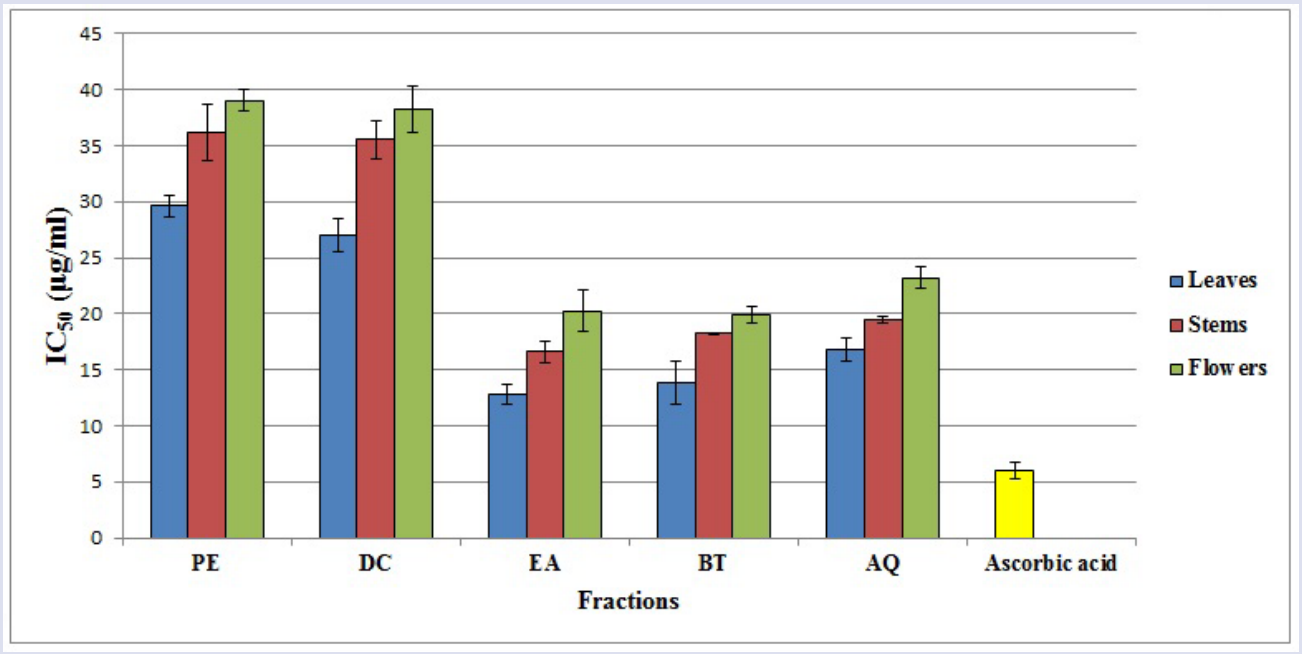

Figure 4: Antioxidant capacity (ABTS) of various fractions of *A. lanatum*. PE; petroleum ether fraction, DC; dichloromethane fraction, EA; ethyl acetate fraction, BT; n-butanol, AQ; remaining aqueous fraction.

indicts the more antioxidant capacity of the tested samples.[19] basically; the reduction in absorbance is predictable due to the interaction between the antioxidant constituents and the produced free radical. The 50% inhibition concentration (IC_{50}) was calculated and used as a mean of assessment and comparison between those of antioxidant material with a well-established standard (ascorbic acid). The results showed moderate ABTS scavenging activity of all extracts ranged from IC_{50} of (13.83±1.9) µg/ml to (39.03±1.0) µg/ml. Results were compared with IC_{50} of the positive standard 5.97±0.7 µg/ml (Figure 4). The study demonstrated the efficiency of EA and BT as free radical scavenger fractions compared to other fractions and its opportunity to hold bioactive antioxidant metabolites.

Lipoxygenase inhibitory activity assay

Lipoxygenases enzymes are responsible for the conversion of polyunsaturated fatty acid into biologically active markers associated with the inflammatory and other immune responses. Lipoxygenase inhibitors are considered as promising therapeutic candidates against wide range of inflammatory-related diseases.[19] IC_{50} values of lipoxygenase inhibition activity was calculated using the regression equations: ($y = 0.1265x - 1.4811$, $R^2 = 0.8685$) for TML, regression equation: ($y = 0.1351x - 0.6791$, $R^2 = 0.9741$) for TMS, regression equation: ($y = 0.2325x - 5.5738$, $R^2 = 0.921$) for TMF and regression equation: ($y = 0.0063x - 0.0415$, $R^2 = 0.955$) for baicalein (the positive control). Based on obtained results, the IC_{50} values were 4.88, 5.40 and 6.05 µg/mL for TML, TMS and TMF respectively. Results were compared with the positive control baicalein (IC_{50}: 0.27 µg /mL).

CONCLUSION

Flavonoids, saponins, steroids, tannins/phenol, and carbohydrates were detected in different fractions of different organs of *A. lanatum*. Anthraquinones, alkaloids and cardiac glycosides were absent in all fractions . The approach highlighted the richness of *Anisosciadium lanatum* in phenolic and flavonoid contents. Substantial *in vitro* antioxidant and lipoxygenase inhibitory activities were revealed . According to these findings, more in-depth research is needed for future development of *in vivo* studies to evaluate the potential anti-inflammatory and other related activities.

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