Biological Evaluation of Benzothiazole-Thiourea Derivatives, Plant Extracts and their Combinations

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Abstract- The synthesis of benzothiazole derivatives and investigation of their chemical and biological behavior have gained more importance in recent decades. The structural formula of all derivatives was confirmed and characterized by elemental analysis and spectral studies. A methanol extract of *Pongamia pinnata*, *Syzigium cumini*, *Delonix regia*, *Adathoda zeylanica* was done by Soxhlet. The combinations of the plant extracts with the organic compounds were evaluated for anti-inflammatory, anti-oxidant activity and checked for their cytotoxicity. Our findings indicate that the plant extract and organic compounds have a significant effect on biological evaluations.

Index Terms- Benzothiazole-thiourea, Pongamia pinnata, Syzigium cumini, Delonix regia, Adathoda zeylanica, anti-inflammatory, anti-oxidant

I. Introduction

Benzothiazole is a privileged bicyclic ring system with multiple applications. A large number of therapeutic agents are synthesized with the help of benzothiazole nucleus. Being a heterocyclic compound, benzothiazole finds use in research as a starting material for the synthesis of larger, usually bioactive structures. Its aromaticity makes it relatively stable; although, as a heterocycle, it has reactive sites, which allow for functionalization. During recent years there have been some interesting developments in the biological activities of benzothiazole derivatives. These compounds have special significance in the field of medicinal chemistry due to their remarkable pharmacological potentialities [1].

The inflammatory response is a natural defense mechanism that is triggered whenever body tissues are damaged in any way. Most of the body defense elements are located in the blood and inflammation is the means by which body defense cells and defense chemicals leave the blood and enter the tissue around the injured or infected site. Inflammation occurs in response to physical trauma, intense heat and irritating chemicals, as well as to infection by viruses and bacteria. [1] The four cardinal signs of inflammation are redness, heat, swelling and pain. Many experts consider impairment of function to be the fifth cardinal sign of inflammation. Inflammation is often associated with the inflammatory response to tissue injury or trauma, but inflammation is at work in the body at a bio-molecular level on a constant, basis without any symptomology. Inflammation helps to maintain homeostasis in the body by coordinating immune function, including T cell mediation to identify and eliminate cancer cells.[2]

Many medicinal plants are used in developing countries for the management of inflammatory conditions. The validation of the folkloric claims of these medicinal plants will provide scientific basis for the conservation of tropical medicinal resources, the deployment of beneficial ones as phytomedicine in the primary healthcare and the development of potential bioactive constituents. These could provide novel compound or precursors in drug development, and utilization of isolated compounds as investigative, evaluative and other research tools in drug development and testing processes.

Research continues to demonstrate that the inflammation is a normal, natural and beneficial process to the human body. Treating inflammation with chemicals results in the halting of this natural process and often leads to chronic conditions. No wonder that so many health conditions end in the word *"itis"*.[2] Reactive Oxygen species (ROS) generated endogenously or exogenously are associated with the pathogenesis of various diseases such as atherosclerosis, diabetes, cancer, arthritis and aging process. [3] Inflammation is a complex process and ROS play an important role in the pathogenesis of inflammatory diseases. Thus antioxidants which can scavenge ROS are expected to improve these disorders.

II. Materials and Methods

The organic compounds were obtained from the chemotherapy department of Haffkine institute where they were synthesized. All raw materials used in the synthesis have been obtained from M/s Fluka AG (Bachs-Switzerland) and M/s Sigma Aldrich chemicals and Co. Inc. (Milwoukee, WI, USA). Melting

Points were recorded on a Thermonik Melting point apparatus (Campbell Electronics, Mumbai, India) and are uncorrected. IR spectra were recorded on an IR-Affinity, Shimadzu using DRS system. 1H-NMR spectra have been recorded on a JEOL AL-400 FT-NMR spectrometer (400 MHz-JEOL Ltd. Tokyo, Japan), using TMS as internal standard in solvent DMSO. Elemental analysis has been carried out on a C, H, N Elemental Analyzer (Thermo-Finnigan Flash, EA 1112, Italy). Mass data have been recorded on Agilent GC-MS.

Collection of plants and organic compounds

The leaves of the plants Delonix regia and Adathoda zeylanica were collected in the month of January from the garden of Haffkine institute (Parel) where it was found growing wild. The seeds of *Syzigium cumini* and *Pongamia pinnata* were obtained in bulk from a local ayurvedic vendor in Mumbai. The plant materials were authenticated by Dr. Ganesh Iyer, Department of life Sciences, Ramnarain Ruia College.

Synthesis of Benzothiazole derivatives [4,5]

(E)-1-((1-(6-methoxybenzo[d]thiazol-2-yl)-3-phenyl-1H-pyrazol-4-yl)methylene)-3-phenylthiourea (A)

In continuation of our earlier work an equimolar amount of carbaldehyde derivative of benzothiazole (0.01 mol), phenyl thiourea (0.01 mol) and 1-2 drops of glacial acetic acid, reflux the reaction mixture for 8-10 hrs. Completion of reaction was monitoring by TLC. The reaction mixture was poured into the ice-cold water. Product was filtered wash with water. Recrystallized the product with ethyl alcohol. Other derivatives are prepared in similar manner.

White solid; Yield, 75%; mp = 186 -188^{0}C; IR (KBr) υ/cm^{-1}: 3461 (-NH), 2836(-OCH_3), 1598 (-NH), 1320 (C-N), 1663(C=N); ^{1}H NMR (400 MHz, DMSO, δ ppm): 3.9 (s, 3H, CH_3), 5.6 (S,1H, NH), 7-8 (m, 14H, Ar-H), 8.6 (s, 1H, NH); ^{13}C NMR (400 MHz, DMSO, δ ppm): 55.9, 156.6, 114.8, 122.5, 144.2, 136.4, 104.9, 160.8, 137.5, 110.8, 143.5, 133.0, 127.4, 127.6, 129.4, 128.7, 163.7, 185.9, 137.4, 126.2, 129.0, 128.4. MS (EI) m/z 469 [M^+] ($C_{25}H_{19}N_5OS_2$). Elemental analysis: C, 63.94; H, 4.08; N, 14.42 found: C, 64.11; H, 3.89; N, 14.78

(E)-1-(4-hydroxyphenyl)-3-((1-(6-methoxybenzo[d]thiazol-2-yl)-3-phenyl-1H-pyrazol-4-yl) methylene)-3-phenylthiourea (B)

Brown solid; Yield, 68%; mp = 203-206^{0}C; IR (KBr) υ/cm^{-1}: 3544 (-OH), 2819 (-OCH_3), 1607 (-NH), 1319 (C-N), 1670 (C=N); ^{1}H NMR (400 MHz, DMSO, δ ppm): 4.1 (s, 3H, CH_3), 5.8 (S,1H, NH), 7-8 (m, 13H, Ar-H), 8.7 (s, 1H, NH); ^{13}C NMR (400 MHz, DMSO, δ ppm): 55.6, 156.4, 114.7, 122.3, 144.0, 136.3, 104.7, 160.8, 137.7, 110.9, 143.5, 133.0, 127.4, 127.6, 129.4, 128.7, 163.7, 185.9, 129.5, 127.4, 116.2, 154.5 MS (EI) m/z 486 [M^+] ($C_{25}H_{19}N_5O_2S_2$). Elemental analysis: C, 61.84; H, 3.94; N, 14.91 found: C, 64.18; H, 4.53; N, 14.71

(E)-1-(4-chlorophenyl)-3-((1-(6-methoxybenzo[d]thiazol-2-yl)-3-phenyl-1H-pyrazol-4-yl) methylene)-3-phenylthiourea (C)

Brown solid; Yield, 59%; mp = 129-131^{0}C; IR (KBr) υ/cm^{-1}: 3438 (-NH), 2832 (-OCH_3), 1588 (-NH), 1326 (C-N), 1684 (C=N), 758 (Cl); ^{1}H NMR (400 MHz, DMSO, δ ppm): 3.7 (s, 3H, CH_3), 5.4 (S,1H, NH), 7-8 (m, 13H, Ar-H), 8.5 (s, 1H, NH); ^{13}C NMR (400 MHz, DMSO, δ ppm): 55.8, 156.7, 114.7, 122.6, 144.1, 136.3, 104.9, 160.9, 137.5, 110.8, 143.0, 133.4, 127.4, 129.3, 128.7, 163.6, 185.4, 135.2, 131.4, 129.2, 133.8 MS (EI) m/z 504 [M^+] ($C_{25}H_{18}ClN_5OS_2$). Elemental analysis: C, 59.57; H, 3.60; N, 13.89 found: C, 60.04; H, 3.76; N, 14.03

(E)-1-(4-Fluorophenyl)-3-((1-(6-methoxybenzo[d]thiazol-2-yl)-3-phenyl-1H-pyrazol-4-yl) methylene)-3-phenylthiourea (D)

Grey solid; Yield, 63%; mp = 147-149^{0}C; IR (KBr) υ/cm^{-1}: 3395 (-NH), 2814 (-OCH_3), 1563 (-NH), 1312 (C-N), 1694 (C=N), 1140 (F); ^{1}H NMR (400 MHz, DMSO, δ ppm): 4.2 (s, 3H, CH_3), 5.4 (S,1H, NH), 7-8 (m, 13H, Ar-H), 8.6 (s, 1H, NH); ^{13}C NMR (400 MHz, DMSO, δ ppm): 55.8, 156.7, 114.6, 122.6, 144.0, 136.2, 105.0, 161.2, 137.3, 110.4, 143.0, 133.4, 127.6, 129.2, 128.8, 163.7, 185.7, 132.8, 131.0, 115.8, 163.3 MS (EI) m/z 488 [M^+] ($C_{25}H_{18}FN_5OS_2$). Elemental analysis: C, 61.58; H, 3.72; N, 13.36 found: C, 61.85; H, 3.79; N, 13.87

Processing of plant material

The leaves and seeds of plants were chopped to small pieces and dried under the shade. It was then powdered with a mechanical grinder to obtain a coarse powder. The plant extracts were then obtained by means of a Soxhlet extractor using methanol as a solvent.

Phytochemical screening [6,7]

Phytochemical screening of the different extracts was performed using standard procedures.

1) Test for Phenolic compounds:

2ml of 3% extract were taken and add 2ml of 1% $FeCl_3$ (ferric chloride) in 0.5 N HCl (hydrochloric acid). The deep blue coloration of the solution is an indication of presence of phenolic compounds.

2) Test for Terpenoids

To 0.5g each of extract was added 2 ml of chloroform. Concentrated H_2SO_4 (sulphuric acid). 3ml was carefully added to form a layer. A reddish brown coloration of the interface indicates the presence of terpenoids.

3) Test for Organic acids

To 1 ml of extract add 1% of $NaHCO_3$ (sodium bicarbonate) slowly and presence of effervescence indicates positive results.

4) Test for Flavonoids

Dilute ammonia (5 ml) was added to a portion of an aqueous filtrate of the extract. Concentrated sulphuric acid (1 ml) was added. A yellow coloration that disappears on standing indicates the presence of Flavonoids.

5) Test for Saponins

To 0.5g of extract was added 5 ml of distilled water in a test tube. The solution was shaken vigorously and observed for stable persistent froth. The frothing was mixed with 3 drops of olive oil and shaken vigorously after which it was observed for the formation of emulsion.

6) Test for Tannins

About 0.5 gm of the extract was boiled in 10ml of water in a test tube and then filtered. A few drops of 0.1% ferric chloride was added and observed for brownish green or blue black coloration.

7) Test for Alkaloids

To 1 ml of extract in a test tube, add picric acid solution till the formation of orange color which indicates positive results.

MTT cell viability assay [8]

BHK21 cells were used for checking the cytotoxicity of the plant extracts and organic compounds. Seeding of cells were done in a 96 well plate using DMEM. The plate was incubated overnight at 37°C in humidified incubator, 5% CO_2. The cells were observed for monolayer under the microscope and then test compounds were added to the plate including negative control (DMEM), positive control (DMSO) and vehicle control with the final volume of 100µl per well. The plate was incubated overnight at 37°C in humidified incubator, 5% CO_2. Next day, 5mg/ml of MTT reagent was prepared and added in 9 ml of MEM to make a volume of 10 ml of MTT reagent. The previous media was discarded and 100µl of MTT in each well of 96 well plates was added. The plate was incubated at 37°C for 3 hours. 100µl of stop mix solution (1 volume) was added in each well of the plate to dissolve the formazane precipitate which gives purple color over incubation period. The plate was read on a plate reader at 550 nm and the results were calculated as % viability.

Assessment of Antioxidant activity by DPPH radical scavenging assay: [9-12]

The stock solutions of the methanolic plant extracts and the organic compounds was prepared in methanol from the working concentrations were prepared with a total volume of 1ml using methanol. DPPH (0.0002% w/v) was prepared in methanol. To the diluted samples, 1 ml of the DPPH solution was added and the reaction was incubated in dark for 30 minutes. After 30 minutes, the absorbance was taken at 517 nm using methanol as blank and DPPH as control.BHT (Butylated hydroxyl toluene) was used as a standard against which the graph of the sample was plotted. The percentage inhibition of DPPH activity was calculated using the formula

Percentage inhibition = [O.D of control – O.D of test]/ O.D of control x 100

HRBC membrane stabilization assay: [13-16]

Blood was collected from healthy volunteer who was not taken any NSAIDS for two weeks prior to the experiment. The collected blood was mixed with equal volume of sterilized Alsever solution (2% dextrose, 0.8% sodium citrate, 0.5% citric acid and 0.42% sodium chloride in water). The blood was centrifuged at 3000 rpm and packed cell were washed with isosaline (0.85%.pH 7.2) and a 10 % (v/v) suspension was made with isosaline. The assay mixture contained the drug, 1 ml of phosphate buffer (0.15M, pH 7.4), 2 ml of hypo saline (0.36%) and 0.5ml of HRBC suspension. Diclofenac was used as reference drug. Instead of hypo saline 2ml of distilled water was used in the control. All the assay mixture were incubated at 37°C for 30 min and centrifuged. The hemoglobin content in the supernatant solution was estimated using spectrophotometer at 560 nm. The percentage protection was calculated by following equation

% protection= [O.D of control – O.D of test/ O.D of control x 100

Phytochemical screening:

The Phytochemical screening using standard procedures revealed the presence of Phenolic compounds, Terpenoids, Flavonoids, and Tannins in all the extracts. Alkaloids were found only in Syzigium cumini and the plant extracts exhibited negative results for Organic acids and Saponins.

Figure 1: Schematic representation of Benzothiazol

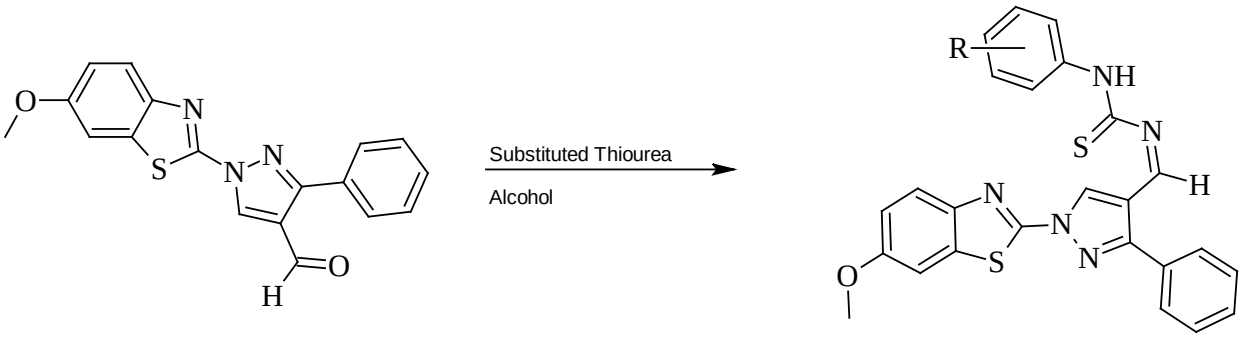

Figure 2: Plant images with scientific name

Pongamia pinnata

Syzygium cumini

Delonix regia

Adathoda zeylanica

Table no.1: Physical Characterization of synthesized compounds

Sr. No.	Comp.	R	Color	Melting Point	Mol Wt.	Empirical Formula	Yield %
1	A	H	White	186-188	469	$C_{25}H_{19}N_5OS_2$	75
2	B	OH	Brown	203-206	486	$C_{25}H_{19}N_5O_2S_2$	68
3	C	Cl	Brown	129-131	504	$C_{25}H_{18}ClN_5OS_2$	59
4	D	F	grey	147-149	488	$C_{25}H_{18}FN_5OS_2$	63

Table no.2 – Preliminary Phytochemical screening

Test for compound	***Pongamia pinnata***	***Syzigium cumini***	***Delonix regia***	***Adathoda zeylanica***
Phenolic compounds	+	+	+	+
Terpenoids	+	+	+	+
Organic acids	-	-	-	-
Flavonoids	+	+	+	+
Saponins	-	-	-	-
Tannins	+	+	+	+
Alkaloids	-	+	-	-

Table no.3: Biological Evaluation of Plant Extract and Synthesized compounds

Name		**Anti-oxidant assay**	**Anti-inflammatory assay**
	Concentration (mg/ml)	**Concentration (µg/ml) IC_{50}**	**% protection at 50 µg/ml**
Standard	-	67.98	97.78
Pongamia pinnata	76.99	202.97	95.78
Syzigium cumini	64.51	67.50	97.85
Delonix regia	61.66	116.55	93.18
Adathoda zeylanica	60.60	174.21	98.70
Comp A	33.70	125.00	98.03
Comp B	32.59	305.81	98.36
Comp C	33.06	249.27	92.91
Comp D	28.95	162.03	98.29
Pongamia pinnata + Comp A	-	129.53	91.02
Pongamia pinnata + Comp B	-	214.74	92.04
Pongamia pinnata + Comp C	-	175.48	93.14
Pongamia pinnata + Comp D	-	159.27	89.66
Syzigium cumini + Comp A	-	60.00	97.72
Syzigium cumini + Comp B	-	78.00	96.70
Syzigium cumini + Comp C	-	70.00	94.02
Syzigium cumini + Comp D	-	58.00	96.16
Delonix regia + Comp A	-	120.00	92.06
Delonix regia + Comp B	-	188.44	92.25
Delonix regia + Comp C	-	196.27	80.12
Delonix regia + Comp D	-	142.75	87.25
Adathoda zeylanica + Comp A	-	120.00	97.56
Adathoda zeylanica + Comp B	-	193.86	96.23
Adathoda zeylanica + Comp C	-	157.52	87.33
Adathoda zeylanica + Comp D	-	143.43	88.52

III. Result and Discussion:

We have described the synthesis of Benzothiazole-thiourea derivatives. New compounds (A-D) have been synthesized by the reaction of Carbaldehyde benzothiazole derivatives with various substituted thiourea in good yield. The structures of compounds are confirmed by IR, NMR and Mass spectral data and are further supported by correct elemental analysis

The plant extracts and organic compounds were tested by MTT assay for their cytotoxicity. The median cytotoxicity concentration was found to be 76.99 mg/ml, 64.51 mg/ml, 61.66 mg/ml, 60.60 mg/ml for *Pongamia pinnata, Syzigium cumini, Delonix regia and Adathoda zeylanica* respectively & 33.70 mg/ml, 32.59 mg/ml, 33.06 mg/ml, 28.95 mg/ml for Compounds (A,B,C,D) respectively. The compounds were found to be more cytotoxic than the plants since they had lower CC_{50} values. Values lower than the CC_{50} detected were used for carrying out the anti-oxidant and anti-inflammatory assays.

S.cumini was found to be a strong anti-oxidant since its value was found to be almost equal to the standard. The IC_{50} values of Compounds (A, B, C, D) were 125µg/ml, 305.81µg/ml, 249.27µg/ml, and 162.03µg/ml respectively. *Pongamia pinnata* + Compounds (A, B, C, D) showed IC50 values 129.53 µg/ml, 214.74µg/ml, 175.48µg/ml, 159.27µg/ml respectively. *P. pinnata* with the compound C and D showed better anti-oxidant activity than individual components. *Syzigium cumini* + Compounds (A, B, C, D) showed IC_{50} values 60µg/ml, 78µg/ml, 70µg/ml, 58µg/ml respectively. The combinations *S.cumini* with compound A and D in this case have produced an even better activity than the standard. *Delonix regia* + Compounds (A, B, C, D) showed IC50 values 120µg/ml, 188.44µg/ml, 196.27µg/ml, 142.75 µg/ml respectively. *Adathoda zeylanica* + Compounds (A, B, C, D) showed IC_{50} values 120 µg/ml, 193.86µg/ml, 157.52µg/ml, 143.43µg/ml respectively. *A. zeylanica* produced a superior activity with Compound A, C, D when compared with the individuals. The compound B was therefore not found to contribute to increasing the anti-oxidant potential with any of the plant extract.

The percentage protection at 50µg/ml of *Pongamia pinnata, Syzigium cumini, Delonix regia and Adathoda zeylanica* was found to be 95.78%, 97.85%, 93.18% and 98.7% respectively. The percentage protection values of Compounds (A, B, C, D) were 98.03%, 98.36%, 92.91% and 98.29% respectively. The plants and the organic compounds thus displayed a very strong anti-inflammatory activity individually and allowed very few cells to undergo bursting. *Pongamia pinnata* + Compounds (A, B, C, D) showed percentage protection values 91.02%, 92.04%, 93.14, 89.66% respectively. *Syzigium cumini* + Compounds (A, B, C, D) and 1-(5-methoxy-1, 3-benzothiazol-2-yl)) showed percentage protection values 97.72%, 96.7%, 94.02%, 96.16%. *Delonix regia* + Compounds (A,B,C,D) showed percentage protection values 92.06%, 92.25%, 80.12%, 87.25% respectively. *Adathoda zeylanica* +Compounds (A,B,C,D) showed percentage protection values 97.56%, 96.23%, 87.33% and 87.25% respectively. These were compared with the percentage protection of the Standard Diclofenac which is 97.78%. The combinations carried out in this case did not provide a superior activity than the individual extracts or organic compounds. A depreciation of the activity as compared to the activity of individual components was found in most cases except *P.pinnata*with Compound C and S.cumini with Compounds A and C wherein a middle ground was established. Nevertheless, the tested plants and organic compounds exhibited excellent anti-inflammatory activities since the percentage protection observed in most of the cases was found to be around 90%. These compounds and plants need to be further tried and tested for their *in-vivo* activity and combinations within plant extracts and within organic compounds may possibly bring about a 100% protection. The plants and organic compounds were therefore found to possess very good membrane stabilizing property which is one of the preliminary steps involved in the screening of anti-inflammatory property. Anti-oxidants are very important in the biology of living cells and are an important part of inflammatory process. Combining anti-oxidants has been found to increase the activity in a number of cases and could serve to eliminate the oxidative stress during inflammation. However, combinations did not serve to produce superior activity in case of HRBC membrane stabilization although the plants and organic compounds themselves exhibited excellent anti-inflammatory properties. But further in vivo studies have to be carried out to confirm the claim.

IV. Conclusion:

Plants were found to be better anti-oxidants than the organic compounds. Combining the agents brought about a superior activity than the activity of individual components in a number of cases. The combination of *S.cumini* with comp D was the most anti-oxidant with an IC50 of 58µg/ml which was even superior to the standard BHT. It also went on to show excellent anti-inflammatory activity hence, further studies on this can help to produce a strong anti-inflammatory agent with excellent anti-oxidant property. The plants and the organic compounds were found to have significant anti-inflammatory activity since they exhibited percentage protection of around 90% individually as well as in combinations. However, the combinations did not bring about any significant increase when compared to the individual values. *Adathoda zeylanica* and Compounds A, B and D were found to have the most anti-inflammatory activity among the tested extracts and compounds.

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