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Original Article

Study of in-vitro anti-inflammatory and immunomodulatory effect of Ayurvedic plants – Murva

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ABSTRACT

Background and Aims: Toxic chemicals, pathogens and damaged cells trigger the inflammatory process and cause disease in the kidney, heart, brain, liver, lung and reproductive system. The present study evaluates the anti-inflammatory and immunomodulatory effect of Ayurvedic plants under the Murva category.

Methods: *Marsdenia tenacissima* is an acceptable source of Murva. *Sansevieria roxburghiana* is considered as Murva in west Bengal. Methanol extracts of both plants were used to evaluate anti-inflammatory and immunomodulatory effects by certain *in vitro* methods. The *in vitro* anti-inflammatory effects of methanol extract *Marsdenia tenacissima* (MEMT) and methanol extract of *Sansevieria roxburghiana* (MESR) were assessed by heat and hypotonic induced haemolysis on the red blood cells of rats. Nitro blue tetrazolium (NBT) assay and inhibition of TNF-α release in DAL cell lines were conducted to assess the immunomodulatory potential of extracts.

Results: MEMT, MESR and a combination of MEMT&MESR showed significant ($p<0.001$) inhibition of haemolysis on heat and hypotonic induced methods compared with the standard Diclofenac Sodium. In NBT reduction test, MEMT showed a more significant ($p<0.001$) result than MESR. Similarly, the inhibition of TNF-α release was also significantly enhanced by 400 µg/mL of MEMT.

Conclusion: The present research results revealed that Murva is a safe Ayurveda drug for the treatment of cancer and cardiac disease with the protection of systemic immunity and set free from an inflammatory condition.

Keywords: Murva; *Marsdenia tenacissima, Sansevieria roxburghiana, in vitro* anti-inflammatory, immunomodulatory, NBT assay

INTRODUCTION

Inflammation is the response of the immune system when exposed to harmful stimuli like pathogens, toxic compounds, damaged cells or radiation. Inflammation is a defence mechanism and acts by removing harmful stimuli and by initiating a healing process. The inflammatory process is mediated by the migration of leukocytes to the affected site and the release of inflammatory mediators. Chronic inflammation occurs when acute inflammation fails to correct or treat the affected site which leads to the development of cardiovascular diseases, cancer, arthritis, and type 2 diabetes mellitus (Chen L et al., 2018). Inflammation is the major component in tumour progression where most of the cancer arises from infection or chronic irritation and inflammation. The tumour cells are surrounded by inflammatory cells which release inflammatory mediators such as selectin, chemokines and their respective receptors. These mediators initiate cancer invasion, migration and metastasis process. Thus, an anti-inflammatory drug plays an effective role in the early tumour stage and malignant conversion (Huang, Zhang, & Ding, 2018). Inflammation is also

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caused by drug resistance or by inflammatory mediators and causes the therapeutic failure of various first-line anticancer drugs such as doxorubicin, cisplatin, 5-fluorouracil, and paclitaxel (Bag, Devi & Bhaigyabati, 2015). Similarly, inflammation is also linked to cardio stress. The affected myocyte has elevated levels of endothelial adhesion molecules which affect the release and production of inflammatory mediators such as chemokine, cytokine production and release (Chen et al., 2018). However, a single drug which compromises all the above incidents is quite difficult to find. Hence a new strategy was developed in pharmaceutical industries to establish a formulation with extended action of cardio protection from doxorubicin and anticancer activity free from myelosuppression and antianaemic effect. Under this comprehensive concept, medicinal herbs and their formulations have recently received greater attention in regard to the treatments of various life threatening diseases because of their efficacy and rapid curative properties. Among the herbal preparations, Ayurvedic formulations have been placed in the first position for thousands of years due to their lower toxicity and wider acceptability. Ayurveda is a Sanskrit word which means longevity and which is a traditional system of medicine in India and other south Asian countries. Ayurvedic treatment is based on the establishment of equilibrium in different elements of the human body, intellect, mind and soul (Lokhande, Jagdale, & Chabukswar, 2006). These drugs mainly act by stimulating the specific function of the organ, by altering hormones, affecting immunity and neurotransmitter as well as through antioxidant mechanism. It is composed of herbal preparations that are combined with supplements at different levels and are safe, effective and indigenous remedies for the people in India and China (Ven Murthy, Ranjekar, Ramassamy, & Deshpande, 2010; Arya& Guota, 2011). In Ayurveda, various therapeutic approaches are used which are based on years of experience, empiricism, and observation (Gogtay, Bhatt,& Dalvi, 2002) The major problem in Ayurveda is the standardisation and identification of medicinal plants and formulation because some drugs have more than one botanical source which creates difficulty in identification of the sources. One of such Ayurvedic plants is Murva. It is a controversial drug, a combination of 11 medicinal plant roots found in different parts of India. Due to its availability in south India, *Marsdenia tenacissima (Roxb.)* Wight et Arn., (Asclepiadaceae) (MT) is an acceptable source of Murva. However, *Sansevieria roxburghiana* (Schult and Schult.f (SR) (*S. zeylanica* Roxb.) (Asparagaceae) is also considered Murva in west Bengal, hence both plants were selected for this study (Mathew, Asirvatham, & Tomy, 2021) The remaining plants in Murva, their scientific names, and their source locations are *Helicteres isora* L (Sterculiaceae) from Punjab, *Maerua arneria* (Capparaceae) from Bihar, *Chonomorpha fragrans* (Apocynaceae) from Kerala, *Clematis triloba* (Ranunculaceae), *Wattakaka volubilis* (Linn. f.) Stapf (Asclepidaceae) and *Salvadora persica L* (Salvadoracae) from South India, *Argyreia nerova*, (Convolvulaceae), *Maerua oblongifolia* (Capparaceae), and *Dregea volubilis* (Apocynaceae) from other regions of India (Mathew et al., 2021). Traditionally, Murva is used for the treatment of anaemia, diabetes, stomach disorder, typhoid, cough, fever and urinary tract infections. MT is traditionally used as a drug in clinical conditions like abdominal pain caused by worm infections, Vata conditions like constipation, light and interrupted sleep, nervousness, anxiety, tremors, fever and also disorders of heart, skin and neuroprotection (Tiwari, Singh& Tiwari 2018). In Chinese folk medicine, stems are used for the treatment of cancer and asthma. Traditionally the root is used in the treatment of fever by local practitioners (Hatapakki & Hukkeri, 2011) whereas the whole plant extract of SR is traditionally indicated in used as cardiotonic, febrifuge, expectorant, purgative, and tonic, and indicated in the treatment of glandular enlargement, purgative, and rheumatism. Mucilaginous rhizome is used for sustained chronic persistent cough, consumptive complaint, for mitigating common cough, cold and ear pain. The tender shoot juice administration was found to be effective in children for removing viscid phlegm from throats. The root is used for snakebite and haemorrhoids. In Bangladesh, young leaf juice is used for ear infection (Obydulla, 2016). The roasted leaves are traditionally used as emollient but, none of them have been experimentally proven its anti-inflammatory and immunomodulatory effect. The current study aimed to evaluate the anti-inflammatory and immunomodulatory potential by stabilization of cell membrane HRBC assay.

MATERIALS AND METHODS

Plant material collection

The roots of *Marsdenia tenacissima* (Roxb.) Moon (MT) and rhizome and roots of *Sansevieria roxburghiana* Schult. & Schult. F (SR) were collected in October 2018 from Cherthala, Kerala, India. Dr. K. Madhava Chetty identified and authenticated the plant MT, and the herbarium specimen (voucher number 1132) was deposited to the Department of Botany in Sri Venkateswara University Tirupathi, A.P. Similarly, SR also authenticated the herbarium specimen (No: AAM001) which was deposited to Dr. Jose Mathew, Department of Botany, Santana Dharma College, Alappuzha, Kerala. India.

Extraction procedure

The Roots of MT, Rhizome and roots of SR were washed and cleaned, and dried at room temperature (shade dry). About 300g of the coarsely powdered drug was successively extracted using Soxhlet apparatus with increasing polarity of solvents using petroleum ether (50-60^{o} C for 72 hours), chloroform (60-70^{o} C for 48 hours), methanol (70-80^{o} C for 48 hours), and marc from the methanol extract was macerated with chloroform-water (10:90) for 24 hours to obtain the aqueous extract (Hepsibah & Jothi, 2017). Petroleum ether extract of MT (PEMT), SR (PESR), chloroform extract of MT (CEMT), SR (CESR), methanol extract of MT (MEMT), SR (MESR) and aqueous extract of MT (AEMT), SR (AESR) were collected by rotary evaporator and then dried and stored in an airtight container for experimental purposes.

Cancer cell line

The transplantable Dalton ascetic lymphoma cells (DAL) were obtained from Amala cancer research institute, Thrissur, Kerala. The DAL cells were kept in the ascetic form *in vivo* through sequential passages in Swiss albino mice, by intraperitoneal transplantation of $2x10^{6}$ cells /mouse after every 14 days. Eight days after transplantation, ascetic fluid was taken from the DAL bearing mouse.

Preliminary phytochemical analysis

Phytochemicals are naturally occurring chemical compounds obtained from plants. The term commonly refers to those chemicals that might have a role in health, but are not confirmed as essential nutrients (da Silva et al., 2016; Gul, Jan, Faridullah, Sherani, & Jahan, 2017). Test for alkaloids, carbohydrates, glycosides, phytosterols, coumarins, flavonoids, phenolic compounds, tannins, saponins, fixed oil, protein and amino acids were conducted as described by Trease & Evans, 1989.

Tests for alkaloids

- Drageendorff's Test (Potassium bismuth iodide solution)

Two millilitres (2 mL) of acidic solution of plant extract were neutralized with 10% ammonia solution. Dragendorff's reagent was added and turbidity or precipitate was observed as indicative of presence of alkaloids.

- Wagner's Test (Potassium iodide solution)

2 mL of plant extract were boiled with 5 mL of 2% HCl on a steam bath. The mixture was filtered and 1 mL portion of the filtrate was treated with 2 drops Wagner's reagent. A reddish-brown precipitate indicates the presence of alkaloids.

- Mayer's Test (Potassium mercuric iodide solution)

Drops of Mayer's reagent were added to a portion of the acidic solution in a test tube and observed for an opalescence or yellowish precipitate indicative of the presence of alkaloids.

- Hager's Test (Iodine-picric acid)

For this test procedure, few drops of Hager's reagent (saturated picric acid solution) were added to 2 mL of the respective plant extract. Bright yellow precipitate formation indicated the existence of alkaloids.

Test for carbohydrate

- Molisch Test

A few drops of Molisch's solution were added to 2 mL of aqueous solution of the extract, thereafter a small volume of concentrated sulphuric acid was allowed to run down the side of the test tube to form a layer without shaking. The interface was observed for a purple colour as indicative of positive for carbohydrates.

- Seliwanoff's Test

5 mL of Seliwanoff's reagent were added in a test tube containing 1 mL of plant extract, heated using hot water. The colour of the test tube changed to red, indicating keto sugar (Fructose and Sucrose) was present in the solution.

- Benedict's Test

A mixture which contains 2 mL of plant extract and Benedict's solution (approximately 5 mL) was heated in a test tube for around two minutes and was then allowed to cool. Red coloured precipitate indicated the presence of carbohydrate.

Test for Glycosides

- Legal Test

The plant extract was dissolved in 1 mL of water, with a few drops of 10% sodium hydroxide and 1 mL of 0.3% nitroprusside sodium reagent. The mixture turned a dark red colour almost instantly.

- Baljet Test

Dissolved the plant extract in the in 3 mL of methanolic sodium picrate solution. Added 1 mL of N-sodium hydroxide solution to the liquid. The mixture acquired at once a light wine-red colour.

- Borntrager's Test

5 mL of plant extract were added in 5 mL of 5% ferric chloride solution and 5 mL dil. hydrochloric acid, heated for 5 minutes in water bath. Cooled and added 3 mL of benzene or organic solvent. Shook well. Separated organic layer, added equal volume of 10% ammonia solution. The formed rose pink/red at ammonia layer showed the presence of glycosides.

- Keller-Killiani Test

5 mL of the plant extract were added to3 mL of concentrated acetic acid. Added 1 drop of iron (III) chloride test solution to the liquid and carefully transferred it on concentrated sulphuric acid. A reddish-brown ring formed at the interface, the upper acetic acid layer soon turned bluish green.

Test for phytosterol

- Liebermann-Burchard Test

The amount of 0.5 g of the extract was dissolved in 10 mL anhydrous chloroform and filtered. The solution was divided into two equal portions for the following tests. The first portion of the solution above was mixed with 1 mL of acetic anhydride followed by the addition of 1 mL of concentrated sulphuric acid down the side of the test tube to form a layer underneath. The test tube was observed for green colouration as indicative of steroids.

- Salkowski Test

5 mL of plant extract were mixed in 2 mL of chloroform followed by the careful addition of 3 mL concentrated sulphuric acid to form a layer. A layer of the reddish-brown coloration was formed at the interface thus indicating a positive result for the presence of terpenoids.

Test for coumarins

A volume of 1 mL of 10% NaOH solution was added to 1 mL of the plant extract. Yellow colour was formed when it was placed in a water bath. It confirmed the existence of coumarins in the tested samples.

Test for Flavonoids

- Shinoda Test

Pieces of magnesium ribbon and concentrated HCl were mixed with aqueous crude plant extract after few minutes and pink colour showed the presence of flavonoid.

Test for Phenolic compounds

- $FeCl_3$ test

A few drops of $FeCl_3$ solution were added to 1 mL of plant extract samples. Blackish red precipitate revealed the existence of flavonoids in the test samples

Test for Tannin

- Gelatin Test

To a 1% gelatine solution, added a drop of 10% sodium chloride. If a 1% solution of tannin is added to the gelatine solution, tannins cause precipitation of gelatine from solution.

Test for Protein and Amino acid

- Biuret Test

A quantity (2 mL) of the extract was put in a test-tube and 5 drops of 1% hydrated copper sulphate were added. A quantity, 2 mL of 40% sodium hydroxide, was also added and the test tube shaken vigorously to mix the contents. A purple coloration shows the presence of proteins.

- Xanthoprotein Test

1 mL of extracts was treated with 1 mL of concentrated HNO_3. A white precipitate was formed then boiled and cooled. Then 20% of NaOH or NH_3 was subsequently added, which leads to the formation of an orange colour, which revealed the presence of aromatic amino acids.

- Lead Acetate Test

A fraction of the extracts was treated with 1 mL of lead acetate. A white precipitate formed, which indicated the presence of proteins.

Test for Saponins

1 mL of methanol extract was diluted with distilled water to 20 mL and shaken for 15 minutes in a graduated cylinder. The presence of saponin was confirmed by the formation of a layer of foam.

Test for Fixed Oils

- Spot Test

A quantity of 0.1 g of the extract was pressed between filter paper and the paper observed. Translucency of the filter paper indicated the presence of oils.

- Saponification Test

A few drops of 0.5 N alcoholic potassium hydroxide were added to 1 mL of plant extract along with drop of phenolphthalein, the mixture was heated 2 hours. Formation of soap or partial neutralization of alkali indicated the presence of fixed oils and fats.

Cytoprotective assays

Preparation of RBC Suspension

The study protocol (SJCP/IAEC/2018-4/35) was approved by the Institutional Animal Ethics Committee (IAEC), St. Joseph's College of Pharmacy Cherthala, Kerala, India. About 3ml of fresh blood were collected from the male rat and transferred to heparinised tube. The tube was centrifuged for 10 min at 3000 rpm. 40%w/v suspension was prepared by dissolving red blood pellets with normal saline. Then the solution was reconstituted with isotonic buffer (10mM sodium phosphate buffer, pH 7.4) and it was used for the experiment's purposes (Raju, Seeja, Deshami, Sinchu, & Malu, 2015). .

Human Red Blood Cell (HRBC) Membrane Stabilization test

By using normal saline, a 10 % blood suspension was prepared. The reaction mixture contained 1 mL of 10% RBC suspension and 1mL of different concentrations of MEMT, MESR and a combination of MEMT&MESR. Instead of 1 mL of test drug, normal saline was added in control test tube. Standard drug Diclofenac sodium (200 µg/mL) concentration was used. All the reaction mixtures were incubated for 30 minutes at 56°C in the water bath. After incubation, the test tube was cooled and centrifuged for 5 min at 2500 rpm. The haemoglobin content in the supernatant solution was estimated using a colorimeter at 560 nm. Each test was carried out in a triplicate manner with all samples (Raju et al., 2015).

Percentage membrane stabilization activity was determined by the formula,

Percentage inhibition $=A_{control}-A_{test}/A_{control}$

$A_{control}$=absorbance of control; A_{test}=absorbance of test

Heat-induced haemolysis

1mL of isotonic phosphate buffer was added to the 1 mL of different concentration (100, 200,400, 600, 800 and 1000µg/mL) samples of MEMT, MESR and combination of MEMT & MESR in a set of 6 centrifuge tubes. All the tests were done in a triplicate manner. 1mL of diclofenac (200 µg/mL) served as standard and 1mL of the vehicle taken as control. HRBC suspension was added to all tubes and mixed gently tubes were incubated in a regulated water bath at 54°C for 20 min. Another set was maintained in a freezer at -10°C for 20 min and followed by centrifuge at 3000 rpm for 3min. Supernatant haemoglobin content was determined by spectrophotometry under 540 nm (Raju et al., 2015).

The inhibition of haemolysis (%)=1-((OD2-OD1)/ (OD3-OD1)X 100

Where OD1 =absorbance of the test sample (unheated)

OD2= absorbance of the test sample (heated)

OD3= absorbance of the control sample (heated)

Hypotonicity induced haemolysis

Hypotonic solution (1 mL) of various graded dose of extracts (100,200,400,600,800 and 1000 µg/mL) were prepared and taken as in triplicate. In another set of the centrifuge tube, 1 mL of the isotonic solution containing a graded dose of extracts was also taken in triplicate manner.1 mL of vehicle (distilled water) was taken as control and 1 mL of 200 µg /mL of diclofenac for standard. HRBC suspension (1mL) was added to all test tubes and mixed gently. The mixture was incubated at room temperature for 1 hour followed by centrifuge at 3000 rpm for 3 min. Absorbance was determined spectrophotometrically at 540 nm using a supernatant containing haemoglobin (Raju et al., 2015).

The inhibition of haemolysis (%)=1-((OD2-OD1)/ (OD3-OD1) X 100

Where OD1 =absorbance of the isotonic test sample

OD2=absorbance of the hypotonic test sample

OD3= absorbance of control hypotonic sample

NBT Assay on haemocytes

The reduction of NBT to insoluble blue formazan was used as an indication for superoxide generation, although it is not entirely specific for O_2^-. A measured volume of haemocytes was taken in triplicate, using 96 well microtiter plate (Sigma M-0156) and incubated with different concentrations of MEMT, MESR in humid conditions, for 30 min at room temperature for adherence of the haemocytes. The supernatants were added with 0.3% NBT and absolute methanol. The formazan deposits were dissolved in 120 mL 2 M KOH and 140 mL DMSO. After homogenization of the contents in the wells, the absorbance was read at 620 nm in a spectrophotometer (Heroor, Beknal, & Mahurkar, 2013).

TNF-α Inhibition Assay

Inhibition of TNF-α release in lipopolysaccharide (LPS) stimulated DAL cells were evaluated by a slight modification of Prabhakar, Brooks, Lipshlitz, & Esser, 1995 This reference is not present in the reference list procedure. Different concentrations of MEMT, MESR and LPS were added to initiate inflammation in a measured volume of cell line and kept aside for 3 hours. Then the cells ($1X10^6$ cells/mL) were moved to a 96 well microplate and incubated for 18 hours (overnight) at 37°C followed by centrifuge (1800 g, 5 min, 16°C) after which the supernatant was collected. TNF-α was measured from the supernatant by the cytokine specific sandwich quantitative enzyme-linked immunosorbent assay (ELISA). The inhibition of TNF-α release by LPS-stimulated DAL cells was estimated by the ratio between the TNF-α amount secreted by treated cells (pg/mL) and the level of this cytokine (pg/mL) was observed for untreated cells stimulated with LPS (Sakthivel & Guruvayoorappan, 2013). Percentage of THF-α inhibition was reported as well as calculated from the formula of [1 – (cytokine secretion of treated cells/ cytokine secretion of cells cultivated with solvent control) 100].

RESULTS

All the extracts were subjected to a qualitative analysis of various phytoconstituents such as alkaloids, carbohydrates, proteins and amino acids, anthraquinone, glycosides, steroids, flavonoids, tannins, phenolic compounds, saponins, fats and fixed oil. Both plant extracts showed the presence of carbohydrates, alkaloids, glycosides, saponins, flavonoids, tannins, phytosterols, amino acids and proteins where the various literature showed the presence of glycosides of z-deoxy sugars, which on hydrolysis gave genins like cissogenin, tenasogenin, tenacissigenin, tenacigenoside I, tenacissoside C, tenacigenoside K, tenacigenosides G, tenacissoside H, marsdenoside B -H and11α-O-2-

Table 1. Phytochemical analysis of the Pet. ether, $CHCl_3$, methanol and aqueous extracts of MT & SR

Test	Pet.ether		$CHCl_3$		Methanol		Water	
	MT	SR	MT	SR	MT	SR	MT	SR
Carbohydrate	-	-	-	-	-	-	+	+
Alkaloid	-	-	-	-	+	+	-	-
Anthraquinone glycoside	-	-	-	-	-	-	-	-
Glycoside	-	-	-	-	+	+	-	-
Tannin	-	-	-	-	+	+	-	-
Flavonoid	-	-	+	+	+	+	-	-
Saponin	-	-	+	+	+	+	-	+
Amino acid and Protein	-	-	-	-	+	+	-	+
Phytosterol	+	+	-	-	+	+	-	-
Phenolic compound	-	-	-	-	+	+	-	-
Coumarins	-	-	-	-	-	-	-	-
Fixed oil	-	-	-	-	-	-	-	-
Mucilage	-	-	-	-	-	-	-	+

Definition of abbreviations: Pet.ether- Petroleum ether, $CHCl_3$- Chloroform, MT- *Marsdenia tenacissima*, SR- *Sansevieria roxburghiana*, + Present, - Absent

Methyl butyryl-12β-O-acetyltenacigenin B, flavonoid, alkaloid, tannins, carotenoids, saponins and anthocyanin's proteins (Obydulla, 2016). The standard procedure was followed to test the phytochemical constituent of these plant extracts. The phytochemical screening results are shown in Table 1.

Table 2 shows the effect of MEMT, MESR & Combination of MEMT & MESR on membrane protection on HRBC. Concentration dependent percentage inhibition of cell membrane protection was found in this assay, where a combination of MEMT and MESR (1000µg/mL) showed the maximum inhibition of 65.12±0.73%, MEMT (58.7±0.61%) and MESR (42.69±0.48%). Standard Diclofenac sodium at 200 µg/mL concentration showed 76.5±0.78 % inhibition. In heated solution, 1000µg/mL of combination of MEMT&MESR showed an increase in inhibition of 77.01±0.67% whereas only MEMT and MESR showed 65.97±1.71% and 44.89±0.98% respectively. In the case of hypotonic induced haemolysis, the combination of MEMT &MESR (1000µg/mL) showed the maximum protection 84.25±0.31%. Higher concentration of MEMT 1000 µg/mL showed percentage inhibition of 77.65±0.29% and 54.47±0.77% for 1000µg/mL MESR.

The effects of MEMT and MESR on NBT reduction are shown in Table 3. MEMT showed significant ($p<0.001$) inhibition of haemolysis compared with MESR which showed a less significant ($p<0.01$) effect.

Table 3. Effect of Murva extracts on NBT reduction test

Extract name	Sample concentration (µg/mL)	Mean in %±SD
MEMT	50	11.6±1.4
	100	23.5±3.4
	200	42.6±3.8
	400	68.4±2.9
	800	88.4±1.3
	1000	91.5±2.2
MESR	50	1.1±2.2
	100	08.5±3.1
	200	16.4±1.3
	400	23.5±1.1
	800	46.2±4.3
	1000	52.6±2.3

Definition of abbreviations: MEMT- Methanol extract of *Marsdenia tenacissima*, MESR- Methanol extract of *Sansevieria roxburghiana.* All the assays are performed in triplicate and results are expressed in mean %±standard deviation (SD).

Table 2. *In vitro* anti-inflammatory activity effect of MEMT, MESR & Combination on membrane protection on HRBC.

Concentration and name of extracts (µg/mL)		Percentage inhibition		
		Membrane stabilization	Heated solution	Hypotonic
MEMT	100	23.49±0.37	47.18±0.67	29.39±0.72
	200	37.32±0.49	49.46±0.48	33.08±0.48
	400	41.46±0.49	52.57±0.83	40±0.38
	600	49.35±0.61	54.92±0.48	46.99±0.58
	800	56.83±0.74	58.86±0.50	67.81±0.38
	1000	58.7±0.61	65.97±1.71	77.65±0.29
MESR	100	13.98±0.37	13.78±0.59	9.90±0.39
	200	17.88±0.37	20.82±0.58	14.48±0.39
	400	23.98±0.51	29.65±1.55	20.57±0.38
	600	26.02±0.51	33.39±0.48	38.98±0.58
	800	39.51±0.49	37.52±1.44	47.11±0.58
	1000	42.69±0.48	44.89±0.98	54.47±0.77
Combination of extracts	100	39.59±0.51	60.76±0.50	58.86±0.50
	200	40.81±0.37	64.32±1.05	70.47±0.52
	400	44.47±0.74	67.36±0.58	74.29±0.50
	600	48.29±0.88	71.24±0.57	81.08±0.61
	800	53.66±1.71	72.89±1.71	77.45±0.29
	1000	65.12±0.73	77.01±0.67	84.25±0.48
Diclofenac	200	76.50±0.78	84.12±0.31	86.29±0.58

Definition of abbreviations: MEMT- Methanol extract of *Marsdenia tenacissima*, MESR- Methanol extract of *Sansevieria roxburghiana.* HRBC- Human Red Blood Cell. All the assays are performed in triplicate and results are expressed in mean±standard deviation (SD).

Table 4. Inhibition of TNF-α release by LPS-stimulated assay on DAL cells

Extract name	Concentration of extracts (µg/mL)	Inhibition of TNF-α (%±SD)
MEMT	100	12.4±2.1
	200	59.6±1.4
	400	92.6±2.1
MESR	100	10.3±1.4
	200	48.8±1.8
	400	90.7±2.7
Control	-	12.6±2.1
Dexamethasone	10	87.5±1.3

Definition of abbreviations: TNFα- Tumour necrosis factor alpha, LPS- Lipopolysaccharide, MEMT- Methanol extract of *Marsdenia tenacissima*, MESR- Methanol extract of *Sansevieria roxburghiana*. All the assays are performed in triplicate and results are expressed in mean %±standard deviation (SD).

Effect of MEMT and MESR on inhibition of TNF-α release from DAL cells by LPS-stimulated assay results are shown in Table 4. MEMT showed significant ($p< 0.001$) inhibition of TNF-α release compared with MESR which showed less significant ($p<0.01$) effect.

DISCUSSION

There is a link between cancer and inflammation as many researchers have reported that anti-inflammatory or immunomodulatory phytoconstituents from natural sources have an anti-cancer effect. This anti-cancer effect is due to the stimulation or inhibition of particular cellular inflammatory actions and the related molecular signalling pathways (Huang, Lin, Liao, Young & Yang, 2008) Both the plants have an anti-cancer effect and are traditionally used for the treatment of cancer. MT showed an anti-cancer effect on oesophageal carcinoma (Fan et al., 2015) whereas SR showed the cytotoxic effect on EAC cell line (Haldar, Kar, Bala, Bhattacharya, & Mazumder, 2010). Inflammation is a complex biological reaction of vascular tissues to noxious stimuli.

Inflammation is connected with pain and also includes various processes like the surge of protein denaturation and vascular permeability, and membrane alteration (Raju et al., 2015). Inflammations occur due to leukocyte infiltration. The cells release lysosomal contents such as bactericidal enzymes and protease, which induce damage and inflammation. Lysosomal membrane damage triggers the release of the phospholipids into lysophospholipid and free fatty acid such as arachidonic acid which acts as the precursor for the inflammatory mediators such as prostaglandins, leukotrienes and platelet-activating factor stabilisation of lysosome membrane preventing the lysis and subsequent release of mediators (Galhena et al., 2012). The inhibition of haemolysis is considered as a marker of the anti-inflammatory activity of plant extracts. Since there is a close similarity for RBC with the lysosomal membrane, protection from RBC lysis via hypotonicity or heat might indicate the stabilization of lysosomal membranes and is used as a biochemical indication of anti-inflammatory activity. Researchers have already reported that various phytoconstituents (triterpenoids, flavonoids, and various secondary plant metabolites) can mediate analgesic and anti-inflammatory effects through its membrane-stabilizing actions (Yamada, Webber, Kirillova, Peschon, & Fausto, 1998) RBC exposed to noxious stimuli like heat, hypotonicity, or chemicals such as methyl salicylate and phenyl hydrazine that mediate the membrane damage. Membrane stabilization prevents the leakage of the inflammatory mediators (Raju et al., 2015). The results MEMT, MESR and combination of MEMT& MESR also show membrane stabilization and percentage inhibition of haemolysis in a dose-dependent manner by inhibiting both hypotonicity and heat-induced lysis of erythrocytes compared with the standard drug diclofenac sodium.

The quantification of NBT reduction proved that it was a valuable tool to measure the health condition and immunological status of a cell or organ where the haemocytes generate reactive oxygen intermediates which react with numerous biomolecules like carbohydrates, proteins nucleic acids and lipids. This interaction causes the damage of the cells and functional units which leads to a reduction of the immune response at a cellular and organismal level. NBT reduction assay is an accurate qualitative method for detecting the production of superoxide by haemocytes (Muñoz et al., 2000) The generated superoxide radicals from NBT reduction assay were scavenged by MEMT, MESR and the combination of MEMT& MESR. This significant effect is due to neutrophils present in peripheral blood, which change the yellow compound NBT into a compound with a deep blue colour (Formazone). This blue compound can be clearly detected in microscope. This reduction assay is also concentration-dependent. As a result of the host defence mechanism, upon exposure to various inflammatory stimuli such as lipopolysaccharide (LPS), macrophages initiate the release of various mediators like TNF-α and NO. In tumour progression, process inhibition of TNF expression occurs and is used to find out the possible mechanism of any anti-cancer drug. Researchers reported that a drug with the anti-inflammatory or immunomodulatory property also inhibits TNF expression in the host cell for defence purpose (Yamada et al.,1998). The present study results reveal that MEMT, MESR and a combination of MEMT& MESR possess concentration-dependent inhibition on TNF-α release.

Our results indicate that MEMT, MESR and a combination of MEMT& MESR contain various biologically active constituents which are responsible for the anti-inflammatory, immune regulatory potency and various pharmacological actions.

CONCLUSIONS

In conclusion, besides reports on various pharmacological actions of Murva, no other literature is available for the evaluation of its anti-inflammatory as well as immunomodulatory activity even though it has been used for the treatment of various diseases in Ayurveda and traditional medicine for many years. In the present research the results proved that both the

plant under the Murva category (SR and MT) and the local, safe Ayurveda drug can be used to treat cancer and cardiac disease without affecting the immune system and set the patients free from other inflammatory conditions.

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